

Targeting TFEB and TFE3 in iPSC-derived dopaminergic neurons to correct autophagic/lysosomal deficits in Parkinson's disease



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A thesis submitted for the degree of

Doctor of Philosophy

Trinity 2023

To Elle and my family

Acknowledgements

All work carried out here, and in the last 4 years was only possible due to the help and support of those around me both in and out of the lab.

To Richard Wade-Martins and Warren Hirst, thank you for giving me this opportunity work on such an interesting (and challenging) project that I have grown to be so passionate about. Thank you for giving me the opportunities and the space to develop as a scientist and become more assured in my abilities.

Thank you to my various day-to-day supervisors over the years, Phillippa Carling, Siv Vingill, Brent Ryan and Catherine Guardado for all giving me great insight when needed, teaching me so much, helping me grow in confidence and being so supportive throughout.

Thank you to my colleagues in the Wade-Martins lab over the last 4 years. It has been a pleasure to work with such intelligent and generous people. Thank you to Shikha Kataria, Maria Claudia Caiazza, Rachel Heon-Roberts, Naroa Ibarra Aizpurua, Peter Kilfeather, Charmaine Lang, Féodora Bertherat, Stewart Humble, Sandor Szunyogh, Kalina Naidoo, Ben Vallin and Daniel Schiffmann for your help in experiments or being there for a good chat (science and non-science related).

Thank you to the Hirst lab and the Bioassays and high-throughput screening lab at Biogen, for welcoming me and being such great people to meet and work with during my time in the US.

Thank you to my transfer and confirmation of status examiners Colin Goding, Sally Cowley and Katja Simon for the insightful discussions.

To my friends, thank you for giving me a life outside of work!

To Elle, my parents (Pete and Helen), my brother (Jack), my nan (Jo) and my grandad (Ian), thank you for being so supportive and being there whenever I needed. Thank you for letting me push myself (and your patience when I go off-grid) but also for making my life outside of the lab so enjoyable and giving me the occasional nudge to get out and explore if it has been a while.

Abstract

Parkinson's disease is characterised by the degeneration of dopaminergic neurons (DaNs). A key pathological hallmark within the remaining cells is the presence of proteinaceous intracellular aggregates. Clearance and recycling of damaged/aggregated proteins by the lysosome is pertinent to neuronal homeostasis and survival. Genetics, post-mortem study and disease modelling have implicated autophagic/lysosomal dysfunction as a key pathogenic mechanism contributing to PD. Mutations in PD-related genes such as *GBA*, *LRRK2* and *SNCA* have all been implicated to present deficits in lysosomal catabolic pathways.

Activation of cellular catabolism by targeting master regulators of lysosomal biogenesis, TFEB and TFE3 is an attractive therapeutic strategy in PD. Although TFEB/3 activation has been demonstrated to confer neuroprotective effects, little has been done to characterise TFEB/3 function and therapeutic viability in a human, disease-relevant model of PD.

We aimed to comprehensively characterise endogenous TFEB and TFE3 in human iPSC-derived DaNs (iPSC-DaNs), before examining how genetic and pharmacological modulation can upregulate lysosomal function and rescue deficits in patient-derived iPSC-DaNs carrying *GBA-N370S*, *LRRK2-R1441C* and *SNCA-Trp* mutations.

Strikingly, using iPSC-derived neuronal models, iPSC-derived microglia and online datasets, we found negligible TFEB expression in neuronal populations, with expression primarily associated with non-neuronal cell-types. Conversely, TFE3 was robustly expressed in neurons, making this transcription factor the prime candidate for stimulating lysosomal biogenesis in neurons to combat PD.

We developed a high-throughput qRT-PCR screen in iPSC-Ngn2 neurons to identify transcriptional regulators of *TFEB* in neurons. We identified histone deacetylases (HDACs) to robustly regulate neuronal TFEB expression, with HDAC inhibition stimulating TFEB expression and downstream lysosomal glucocerebrosidase function. Delineation of hit HDAC-targeting compounds, in-tandem with CRISPRi validation, indicated a redundant role between HDAC1, HDAC2 and HDAC3 to regulate *TFEB* expression in neurons.

Surprisingly, TFEB overexpression mainly upregulated genes associated with mitochondrial function and bioenergetics in iPSC-DaNs, demonstrating a novel, DaN-specific therapeutic function for TFEB activation in PD.

Examination of TFE3 activation in iPSC-DaNs demonstrated a major role for upregulating lysosomal biogenesis and function, more so than TFEB activation. Further, TFE3 overexpression upregulated glucocerebrosidase activity and endolysosomal function in patient-derived iPSC-DaNs that displayed perturbation in these functions. Thus, TFE3 activation is a therapeutically viable strategy for targeting lysosomal biogenesis in PD, demonstrated in a human and highly-disease relevant model.

Collectively, we demonstrate TFE3 to be the primary master regulator of lysosomal biogenesis in neurons, and activation enhanced lysosomal function in patient-derived iPSC-DaNs. We also identified HDACs as regulators of *TFEB* expression in neurons and present a novel, therapeutically viable approach for re-introduction of TFEB into neurons by HDAC inhibition. Our data shows TFE3 to have great therapeutic potential in PD but also provides a means to re-introduce TFEB in neurons to combat PD in the future.

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Abbreviations

Abbreviation	Full name
α Syn	Alpha synuclein
bHLH-LZip	Basic helix-loop-helix leucine zipper
CLEAR	Co-ordinated lysosomal expression and regulation
CMA	Chaperone-mediate autophagy
CQ	Chloroquine
CRISPR	Clustered Regularly Interspersed, Short Palindromic Repeats
CRISPRi	CRISPR-interference
CTSD	Cathepsin D
DaN	Dopaminergic neuron
dCas9	Nuclease-dead CRISPR-associated protein 9
DEGs	Differentially Expressed Genes
DQ-BSA	Dye Quenched-Bovine Serum Albumin
EM-Seq	Enzymatic Methyl-Sequencing
ER	Endoplasmic reticulum
GCase	β -glucocerebrosidase
GlcCer	Glucosylceramide
GO	Gene Ontology
GWAS	Genome wide association study
HDAC	Histone deacetylase
iPSC	Induced pluripotent stem cells
iPSC-DaNs	Induced pluripotent stem cells differentiated into dopaminergic neurons
KRAB	Krüppel associated box
LAMP1	Lysosomal associated membrane protein 1
LAMP2A	Lysosomal associated membrane protein 2A
LB	Lewy Body
LIMP2	Lysosomal integral membrane protein 2
LRRK2	Leucine-rich repeat kinase 2
LSD	Lysosomal storage disorder
mFPP	Midbrain floor plate progenitor
MiT/TFE	Microphthalmia/transcription factor E
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
PD	Parkinson's disease
PGC1 α	PPAR-g co-activator 1 α
PPAR α	Peroxisome proliferator activated receptor α
qRT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
RNA-Seq	RNA Sequencing
sgRNA	Single-guide RNA
SNc	Substantia nigra pars compacta
TFE3	Transcription Factor binding to IGHM enhancer 3
TFEB	Transcription Factor EB
TH	Tyrosine Hydroxylase
TRPML1	Transient receptor potential mucolipin 1
V-ATPase	Vacuolar ATPase

Chapter 1: Introduction

1.1 Parkinson's Disease

1.1.1 Epidemiology

Parkinson's disease (PD) is the 2nd most prevalent neurodegenerative disease behind Alzheimer's disease at a prevalence of approximately 0.3% worldwide, although this varies between subpopulations (Dexter & Jenner, 2013). Incidence increases with age, from 0.1% in 50-59-year-olds through to 1.9% in 80-89-year-olds in US populations (Van Den Eefen et al., 2002; Pringsheim et al., 2014). Incidence is expected to rise in the coming years driven by factors such as ageing populations, longevity, industrialisation and smoking rates (Dorsey et al., 2018; Marras et al., 2018). This will increase PD-associated economic burden, with predictions in the US believed to rise from \$51.9 billion to \$79 billion by 2037 (Yang et al., 2020). Sex and ethnicity also have discrepancies in PD incidence. Males have a higher risk of PD than females, and multiple studies indicate Afro-American and Asian populations have reduced PD incidence compared to other ethnicities (such as North Americans, Europeans and Australians) (Wright-Willis et al., 2010; Pringsheim et al., 2014; Gillies et al., 2014). This may be from genetic predisposition; however, China has had the largest increase in %PD between 1990-2016 associated with one of the highest %increases in GDP, suggesting socioeconomic differences could cause differing PD incidence (Dorsey et al., 2018).

1.1.2 Clinical Features of PD

Clinically, PD presents both motor and non-motor symptoms. There are three 'cardinal' motor symptoms used for diagnosis: bradykinesia (slowness of voluntary movement), resting tremor (4-6Hz in the extremities and some facial areas), and rigidity (increased muscle tone causing inflexibility and inability to relax the muscle). PD diagnosis requires clinical presentation of bradykinesia and one of the other 2 cardinal motor symptoms (Postuma et al., 2015). Postural instability also presents but is associated with more advanced disease stages. Motor symptoms increase in severity throughout

the disease correlating with degeneration of nigral dopaminergic neurons (DaNs) (Greffard et al., 2006).

Non-motor symptoms present in PD include sleep disorders (REM sleep behaviour disorder (RBD), insomnia, restless leg syndrome), neuropsychiatric symptoms (depression, anxiety, mild cognitive impairment, dementia), sensory disturbances (hyposmia, parasthesias) and autonomic dysfunction (constipation, sexual dysfunction) (DeMaagd and Phillip, 2015).

The Movement Disorders Society developed clinical criteria for PD incorporating the 3 cardinal motor symptoms and 'supportive criteria' including non-motor symptoms or response to dopaminergic therapy (Postuma et al., 2015). Clinically established PD requires bradykinesia, alongside rigidity or tremor, and the presence of 2 or more 'supportive criteria' together with the absence of 'exclusion criteria' or 'red flags' aimed to reduce misdiagnosis of similar conditions or drug-induced parkinsonism.

Non-motor symptoms vary but often appear before diagnosis in a prodromal PD phase. Hyposmia, RBD, constipation, anxiety, depression and more have been suggested to present prior to motor symptoms (Postuma et al., 2019). Indeed, approximately 50% of nigral DaNs are lost before motor symptom appear (30% when age-adjusted) (Fearnley et al., 1991; Cheng et al., 2010). Hence, study of pro-dromal biomarkers is of great interest for accurate early diagnosis and therapeutic intervention, prior to substantial DaN loss (Heinzel et al., 2019; Kuzkina et al., 2021; Bestwick et al., 2021; Mahlnecht et al., 2022).

1.1.3 Histopathological Features of PD

A major pathology in PD is preferential loss of A9 DaNs within the Substantia nigra pars compacta (SNc) that project to the dorsolateral striatum via the nigrostriatal pathway, and feeds into the basal ganglia loop for co-ordination of movement. Other midbrain DaN populations, A8 (located in the retrorubal field) and A10 (located in the ventral tegmental area) are more resilient and project to the ventral striatum, prefrontal cortex and other areas of the mesocortical and mesolimbic systems, known for emotion, reward and cognition (Bentivoglio & Morelli, 2005; Luo & Wang, 2016).

DaNs have a low frequency (2-4Hz) pace-making activity, maintaining dopamine release into the innervated region. In the striatum, this maintains steady-state regulation upon GABA-ergic medium spiny neurons (MSNs). Increased activity following stimulus elevates dopamine release facilitating movement. In PD, loss of

DaNs reduces dopamine in the ventral striatum, eliminating regulation upon MSNs that feed into the direct and indirect basal ganglia loop, dysregulating co-ordination of movement.

Several extra-nigral regions are prone to degeneration in PD linked to non-motor symptoms. Neurodegeneration between these regions vary, possibly why such a heterogenous presentation of non-motor symptoms occur. Examples of susceptible neuronal populations include: acetylcholinergic neurons of the pedunculopontine nucleus (linked to RBD and akinesia), serotonergic neurons of the anterior olfactory nucleus (hyposmia) and cholinergic neurons in the basal nucleus of Meynert (dementia) (Alexander, 2004).

Another key pathological hallmark of PD is the presence of proteinaceous aggregates called Lewy bodies (LBs) (Lewy, 1912). LBs are found not only within the SNc in PD brains, but other regions too. In fact, Lewy first described inclusions in the dorsal vagus nerve and the basal nucleus of Meynert before Tretiakoff confirmed their presence in the SNc later (Tretiakoff, 1919). LBs are primarily composed of alpha synuclein (α Syn - a small, usually disordered protein associated with the synapse), but are heterogenous structures rich in ubiquitin, lipids, microtubular fragments and organelles such as mitochondria, lysosomes and autophagic structures (Spillantini et al., 1997; Shahmoradian et al., 2019).

Braak and colleagues described an α Syn spreading pathology that begins at the enteric or olfactory systems, through to the dorsal vagus nucleus of the medulla and olfactory bulb, respectively. Once propagated in the CNS, pathology spreads up the brainstem through pontine regions (raphe nuclei, locus coeruleus) before entering the SN, proceeding out to the mesocortex and finally the neocortex (Braak et al., 2003; Hawkes et al., 2010). The spread through non-motor regions aligns with many prodromal symptoms found in PD. Supporting this prion-like spreading hypothesis, embryonic dopaminergic grafts in PD patients were contaminated by aggregates, and in mice, exogenous α Syn can propagate native α Syn aggregation (Kordower et al., 2008; Li et al., 2008). Despite this, a portion of cases do not follow Braak hypothesis. Some argue against the unilateral direction of pathology from the medulla, some cases do not present LB deposition at all (eg. *PRKN* mutations), and other neurodegenerative diseases display LB pathology in regions thought to be specific to PD (Hayashi et al., 2000; van de Warrenburg et al., 2001; Reitsdijk et al., 2017). This

underpins the heterogenous nature of PD pathology and the likelihood that multiple causalities converge to the ultimate degeneration of SNc DaNs producing PD.

Recently, identification of α Syn seeds in the CSF of patients (α Syn seeding amplification assay) has been shown as a useful diagnostic tool for PD with high sensitivity/specificity, and can even diagnose prodromal PD patients (Siderowf et al., 2023). Thus, while α Syn pathology may vary between cases, the presence of this pathology is widespread in PD and provides a good biomarker for early detection of PD.

1.1.4 PD Etiology

The etiology of PD is poorly understood. PD is a multifactorial disease, with both environmental and genetic factors underlying pathogenesis. Up to 90% of cases are sporadic (with no obvious cause) and 10% familial (rare monogenic mutation leading to disease) (Corti et al., 2011).

1.1.4.1 Environmental Factors in PD Etiology

Several environmental risks have been associated with PD. Meta-analyses and population studies identify factors likely contributing to PD risk. Environmental risk factors implicated in PD include pesticide exposure, head injury, heavy metal exposure and illicit drug use. Caffeine consumption, smoking and exercise may reduce PD risk. Studies have demonstrated pathogenic mechanisms behind some environmental risk factors such as pesticide or heavy metal exposure leading to elevated reactive oxygen species (ROS) generation (Islam et al., 2021; Pyatha et al., 2022), however the inability to demonstrate disease causality and contradictory results hamper efforts to understand environmental causes of PD (Chen & Ritz, 2018; Ball et al., 2019).

1.1.4.2 PD Genetics

1.1.4.2.1 Rare familial PD mutations

Genetics provide a useful avenue for understanding PD etiology and pathogenic processes. 15% of PD patients have documented family history and 5-10% can be attributed to monogenic mutation with Mendelian inheritance (Lesage and Brice, 2009). Despite being a fraction of cases, monogenic mutations provide crucial information regarding underlying pathological mechanisms leading to disease.

To date, there are 24 disease-causing *PARK* loci corresponding to 19 genes, although some genes are debated (eg. CHCHD2) (Table 1.1). These loci implicate pathways

such as mitochondrial, lysosomal, membrane trafficking and microtubular dysfunction in PD.

Table 1.1 - PARK loci identified (adapted from Day and Mullins, 2021; Nishioka et al., 2022)

PARK locus	Gene	Protein Function	Onset	Inheritance
<i>PARK1</i>	<i>SNCA</i>	Synaptic vesicle	Young or middle-aged	Autosomal dominant
<i>PARK2</i>	<i>PRKN</i>	Mitochondrial/ Mitophagy	Young or juvenile	Autosomal recessive
<i>PARK3</i>	<i>N/A</i>	-	Late	-
<i>PARK4</i>	<i>SNCA</i>	Synaptic vesicle	Young or middle-aged	Autosomal dominant
<i>PARK5</i>	<i>UCHL1</i>	Ubiquitin- proteasome	Young or middle-aged	Autosomal dominant
<i>PARK6</i>	<i>PINK1</i>	Mitochondrial/ Mitophagy	Young	Autosomal recessive
<i>PARK7</i>	<i>DJ-1</i>	Mitochondrial	Young	Autosomal recessive
<i>PARK8</i>	<i>LRRK2</i>	Endolysosomal, mitochondrial, microtubule	Late	Autosomal dominant
<i>PARK9</i>	<i>ATP13A2</i>	Lysosomal	Young	Autosomal recessive
<i>PARK10</i>	<i>N/A</i>	-	Late	Risk factor
<i>PARK11</i>	<i>GIGYF2</i>	-	Late	Autosomal dominant
<i>PARK12</i>	<i>N/A</i>	-	Late	Risk factor/ X-linked
<i>PARK13</i>	<i>HTRA2</i>	Mitochondrial	Young and late	Autosomal dominant
<i>PARK14</i>	<i>PLA2G6</i>	Cell membrane	Young	Autosomal recessive
<i>PARK15</i>	<i>FBXO7</i>	Mitochondrial	Young	Autosomal recessive
<i>PARK16</i>	<i>N/A</i>	-	Late	Risk locus
<i>PARK17</i>	<i>VPS35</i>	Retromer and endosomal	Late	Autosomal dominant
<i>PARK18</i>	<i>EIF4G1</i>	mRNA translation	Late	Autosomal dominant
<i>PARK19</i>	<i>DNAJC6</i>	Synaptic vesicle formation and trafficking	Young	Autosomal recessive
<i>PARK20</i>	<i>SYNJ1</i>	Synaptic vesicle formation and trafficking	Young	Autosomal recessive
<i>PARK21</i>	<i>DNAJC13</i>	Synaptic vesicle formation and trafficking	Late	Autosomal dominant
<i>PARK22</i>	<i>CHCHD2</i>	Mitochondrial	Late	Autosomal dominant
<i>PARK23</i>	<i>VPS13C</i>	Mitochondrial	Young	Autosomal recessive
<i>PARK24</i>	<i>PSAP</i>	Lysosomal	Middle or late	Autosomal dominant

1.1.4.2.2 PD Genetic Risk loci

Genetic risk variants are high frequency, low penetrance mutations or single nucleotide polymorphisms (SNPs) contributing to sporadic PD cases. Most PD risk variants have been identified by genome-wide association studies (GWAS), although candidate gene-based approaches have been used, mutations in *GBA* as an example (Sidransky et al., 2009). Recently, 90 PD-associated risk variants were identified by meta-analysis GWAS, 38 of which were novel (Nalls et al., 2019). These 90 variants account for 16-36% of PD heritability. Several genes are frequently identified by GWAS including *LRRK2*, *SNCA*, *GAK*, *HLA*, *MAPT* and *VPS13C* (Nalls et al., 2014; Nalls et al., 2019; Foo et al., 2020).

A polygenic risk score on annotated gene sets using GWAS indicate dysfunction in: protein folding/aggregation, endolysosomal function, membrane and intracellular trafficking, lipid metabolism, post-translational modification, immune response, synaptic transmission and chromatin remodelling in sporadic PD pathogenesis (Bandres-Ciga et al., 2020).

This thesis will focus on lysosomal systems heavily implicated in PD and rectifying lysosomal dysfunction in mutations associated with *GBA*, *LRRK2* and *SNCA* mutation.

1.1.4.2.3 SNCA mutation in PD

The *SNCA* gene (encoding α Syn – the primary constituent of LBs) is a small, primarily disordered, monomeric protein found in pre-synaptic terminals thought to be involved in synaptic vesicle release. α Syn structure can be divided into three sections: an N-terminal segment involved in lipid and protein binding, a hydrophobic non-A- β -amyloid component (NAC) thought prone to initiating self-aggregation, and a disordered acidic C-terminus where truncation promotes filament formation (Vasquez et al., 2020). Several rare point mutations leading to early on-set PD have been identified in the N-terminal domain of *SNCA* (such as A53T and A30P), altering lipid binding and aggregation propensity of α Syn (Meade et al., 2019). Further, multiplications of *SNCA* (duplication or triplication) cause severe early on-set PD and demonstrate a dose-dependent effect upon disease pathogenesis by α Syn (Ross et al., 2008). GWAS has also identified several variants significantly associating *SNCA* as a PD risk locus (Nalls et al., 2019).

1.1.4.2.4 *LRRK2* mutation in PD

LRRK2 encodes Leucine-rich repeat kinase 2 (*LRRK2*), a large, multifunctional protein containing: multiple protein-protein interaction domains (ARM, ANK, LRR, WD40), a catalytic core with GTPase activity via a ROC-COR domain and kinase activity via a kinase domain (Berwick et al., 2019). Mutation in *LRRK2* is associated with both familial and sporadic PD.

LRRK2 mutation comprise the most common form of familial PD, caused by several missense mutations in the ROC-COR domain (such as R1441C/G) or kinase domain (such as G2019S) leading to perturbed GTP hydrolysis and kinase hyperactivation, respectively. *LRRK2-R1441C* (a more severe mutation) and *G2019S* (the most common) are the 2 most frequent mutations responsible for up to 30% familial PD cases, and 2.5% to 10% of sporadic PD cases, respectively. The presence of both familial and sporadic PD with these mutations is likely from incomplete penetrance, increasing with age. *LRRK2-PD* closely mirrors idiopathic PD clinically and progressively, unlike many familial forms of PD. Many other *LRRK2* variants increase the risk of PD in individuals, and GWAS strongly implicates *LRRK2* as a risk locus, possibly through increased expression (Kluss et al., 2019).

1.1.4.2.5 *GBA* mutation in PD

GBA encodes the lysosomal hydrolase β -glucocerebrosidase (GCase), responsible for catabolising glucosylceramide (GlcCer) into glucose and ceramide. Homozygous mutation or compound heterozygous functional 'null' *GBA* mutations result in the rare lysosomal storage disorder Gaucher's disease (GD), whereas heterozygous mutations are associated with increased PD risk. Mutations in *GBA* constitute the most common PD risk factor, with 7-15% PD patients carrying a *GBA* variant, and GD patients having 5-fold increased PD risk (Sidransky et al., 2009). *GBA* mutation (most commonly N370S, L444P or E326K) results in perturbed GCase activity and accumulation of GlcCer or glucosylsphingosine (GlcSph). *GBA-PD* clinically presents very similar to idiopathic PD, although progression is quicker, cognitive impairment is more frequent, and the average age of onset is ~5 years earlier (Smith & Schapira, 2022).

The genetic landscape behind PD development is not fully understood, and variable gene penetrance highlights that even monogenic familial mutations may not solely be responsible for disease etiology. In this thesis we will utilise DaNs differentiated from

induced pluripotent stem cells (iPSCs) sourced from PD patients, allowing for highly relevant disease modelling inclusive of genetic variants that may contribute to pathology but are still to be uncovered. This allows for *in vitro* modelling that replicates PD patient genetic background as closely as possible.

1.1.5 Treatments in PD

To date, no disease-modifying treatment has been found for PD. Several medications are available to combat symptoms. The most common is levodopa-carbidopa. Levodopa is a brain-penetrant dopamine precursor and elevates dopamine content in the brain. Carbidopa facilitates brain bioavailability by reducing peripheral dopamine synthesis. Treatment alleviates motor symptoms but has many side effects such as dyskinesias, dystonia and hallucinations. Severe dyskinesias often result in changing medication. Similar issues regarding side effects are present with other available medications such as dopamine agonists, catechol-O-Methyltransferase inhibitors, monoamine oxidase B inhibitors, anticholinergic medication, anti-depressants and anti-psychotics (Hayes, 2019).

Many clinical trials are being undertaken to identify disease-modifying treatments (McFarthing et al., 2022). Examples of strategies include targeting inflammation, α Syn aggregation or expression, dopaminergic/non-dopaminergic symptom relief, microbiome modulation, GCase activity, LRRK2 activity, cellular therapies, mitochondrial function and more. None have been successful thus far, however.

1.2 Lysosomal Function and associated pathways in PD

1.2.1 Introduction to Lysosomal Catabolism

Lysosomes are acidic organelles, in which the luminal pH resides between 4.5-5. The proton gradient required to establish/maintain this acidity is derived by vacuolar-ATPases (v-ATPases) that hydrolyse ATP to transport protons into the lysosomal lumen, and other ion channels (such as chloride or calcium channels) (Mindell, 2012). The acidic environment is essential for catabolic hydrolases that function within lysosomes. Approximately 60 hydrolases are associated with the lysosome, contributing to degradation of many macromolecules such as: proteins (cathepsins), carbohydrates (glycosidases), lipids (phospholipases), and nucleic acids (Lükbe et al.,

2009; Settembre et al., 2013). Primarily, lysosomes function to breakdown these materials and recycle constituents for cellular use. As such, lysosomes are central to cellular catabolism with cargo delivered by two major mechanisms: endocytosis (extracellular material and recycling plasma membrane constituents) and autophagy (intracellular material).

1.2.2 Lysosomal Cargo Delivery pathways (Figure 1.1)

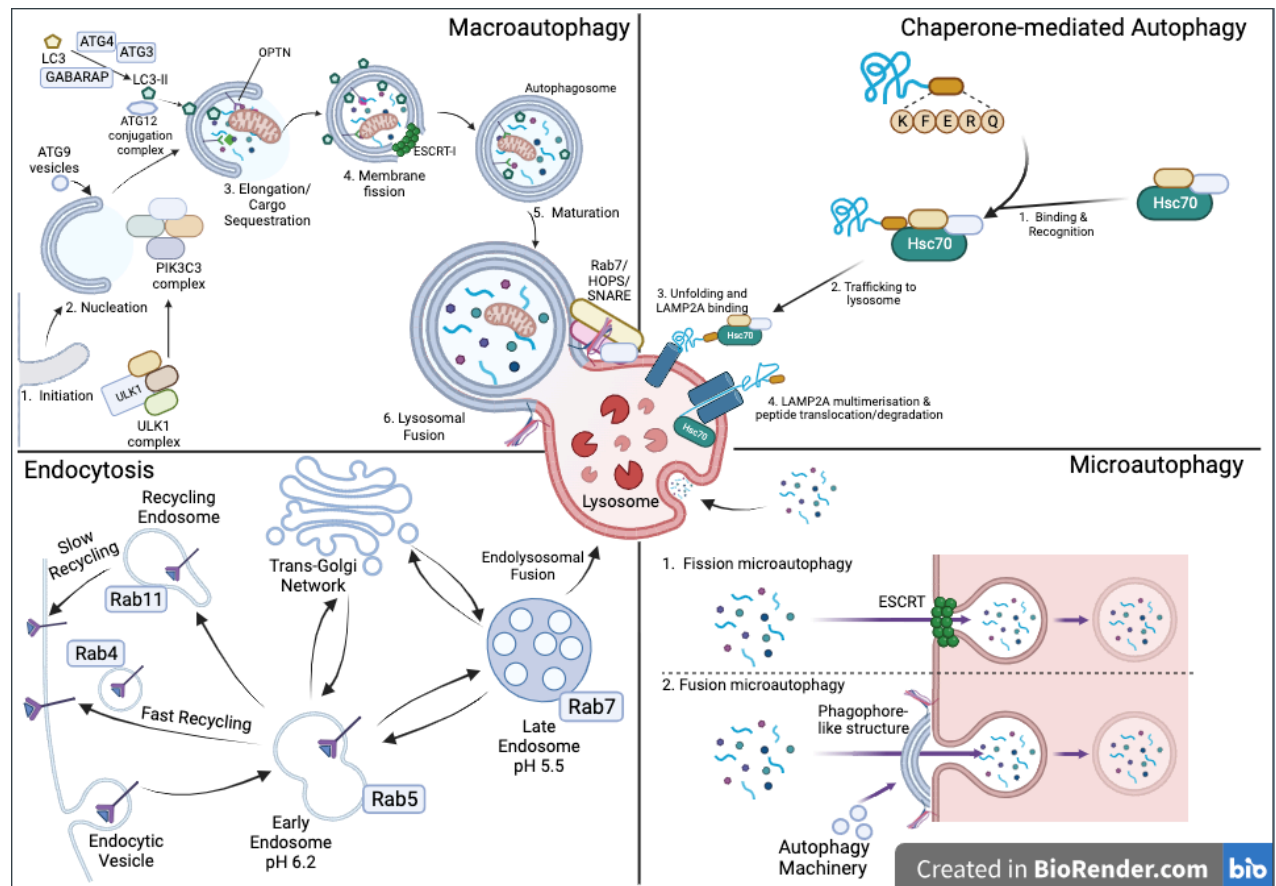


Figure 1.1 – Cargo delivery pathways to the lysosome

1.2.2.1 Autophagy

Autophagy (the process of ‘self-eating’) describes the delivery of cytoplasmic material to the lysosome. This process is important for recycling nutrients should they become scarce and rejuvenates molecular pathways by removing dysfunctional organelles and proteins. Autophagy can be split into three subcategories for cargo delivery to the lysosome: macroautophagy, chaperone-mediated autophagy and microautophagy.

1.2.2.1.1 Macroautophagy

Macroautophagy involves cargo sequestration (selectively or non-selectively) by a double membrane structure called the autophagosome, before fusion with a lysosome for degradation (reviewed in Dikic & Elazar, 2018).

Typically, macroautophagy is initiated under conditions such as stress (nutrient deprivation, oxidative stress, aggregation, ER stress and more), which triggers phagophore nucleation at the phagophore assembly site (PAS) proximal to membranous organelles enriched for the lipid, phosphatidylinositol 3-phosphate (PI3P) (such as the ER, Golgi, plasma membrane). This nucleation is driven by complexes such as Unc 51-like kinase 1 complex (ULK1) that phosphorylate PI3KC3 (PI3K Class III) complex 1, promoting elongation of the nascent phagophore membrane. Concomitantly, ATG9-containing vesicles containing lipids and proteins facilitate phagophore growth. Next, cargo and key effector proteins are recruited to the developing membrane. This is facilitated by a large complex of proteins recruited by WIPI2. A major function of the recruited complexes is activation (by ATG4) and conjugation of phosphatidylethanolamine (PE) (by ATG3) to ATG8 proteins (microtubule-associated protein light chain 3 (LC3) and gamma-aminobutyric acid receptor-associated proteins (GABARAPs)) that become embedded in the autophagosomal membrane (via the ATG12 conjugation complex). ATG8 proteins further recruit autophagic machinery, cargo and aid the elongation and closure of the autophagosomal membrane. ATG8 proteins recruit cargo for selective autophagy through interaction with cargo receptors via an LC3-interaction (LIR) domain (Dikic and Elazar, 2018). Examples of substrate-specific cargo receptors include Optineurin (OPTN) for Ubiquitin-dependent mitophagy, p62 which binds ubiquitinated substrates (including protein aggregates) and TAX1BP1 involved in pathogen sequestration. The phagophore surrounds the cargo and membrane fission completely segregates the cargo. Membrane fission is facilitated by ESCRT-I (Chang et al., 2021). Once sealed, autophagosomes undergo maturation in which ATG complexes are removed from the outer autophagosomal membrane and autophagosomes are retrogradely transported to lysosomes. During this process, autophagosomes may fuse with other vesicular compartments such as multivesicular bodies to form amphisomes prior to lysosomal fusion. Autophagosome-lysosome fusion is driven by trans-SNARE complexes and an ensemble of proteins including tethering factors, HOPS complex and Rab7 (Zhao et

al., 2021). Once fused to form an autolysosome, cargo is degraded by lysosomal hydrolases and the constituents recycled to the cell (Figure 1.1).

Macroautophagy rejuvenates many essential pathways within the cell through the selective degradation of components such as damaged organelles, aggregating proteins and even pathogens. As such, selective macroautophagy is responsible for degradation of nuclear components (nucleophagy), ER (ER-phagy), Golgi (goliphy), mitochondria (mitophagy), aggregates (aggrephagy), pathogens (xenophagy), ribosomes (ribophagy), peroxisomes (pexophagy) and even other lysosomes (lysophagy) (Aman et al., 2021; Griffey & Yamamoto, 2022). Thus, macroautophagy plays a critical role in the health and homeostasis of a variety of pathways critical to cellular function.

1.2.2.1.2 Chaperone-mediated autophagy (CMA)

Chaperone-mediated autophagy (CMA) is a highly selective mechanism for the delivery of specific proteins containing a 'bait' amino acid sequence, KFERQ (other similar sequences can be post-translationally modified to act as a bait sequence). As this amino acid sequence must be present for CMA, only a defined subset of the proteome can be degraded (interesting examples include α Syn, Tau, Huntingtin, GCase and LRRK2). The KFERQ sequence within the protein is recognised by a chaperone complex containing heat-shock cognate 71kDa protein (HSC70) and trafficked to the lysosome for degradation. At the lysosome, HSC70 and the protein substrate dock onto lysosomal associated membrane protein 2A (LAMP2A – rate-limiting protein of CMA). The substrate protein is unfolded by the chaperone complex and LAMP2A forms a translocation complex with other LAMP2A molecules. LAMP2A translocates the unfolded protein through the lysosomal membrane facilitated by lysosomal-HSC70 residing in the lysosomal lumen before degradation by cathepsins (Kauschik & Curevo, 2018) (Figure 1.1).

1.2.2.1.3 Microautophagy

Microautophagy is less understood than macroautophagy and CMA. Broadly, microautophagy involves direct engulfment of autophagic cargo by endolysosomes. Invagination of the endosomal or lysosomal compartment consumes cargo by forming a microautophagic body. Lysosomal membrane closure around the cargo occurs one of two ways: fission or fusion microautophagy. Fission microautophagy involves ESCRT machinery closing the vesicular membrane following invagination. Fusion

microautophagy requires formation of a phagophore-like structure at the site of invagination, which fuses with the lysosomal membrane and uses core-autophagic machinery and SNARE proteins similar to autophagosome-lysosome fusion. The microautophagic body is then degraded within the lysosome. Microautophagy can both non-selectively and selectively degrade cargo, with some mechanisms using autophagic receptors previously described such as LC3 or p62. Microautophagy contributes to degradation of organelles much like macroautophagy. Thus, while not greatly understood, microautophagy is a mechanism for direct delivery of numerous autophagic cargo to the lysosome itself (Schuck, 2020; Wang et al., 2023) (Figure 1.1). Herein, macroautophagy is referred to as autophagy and chaperone-mediated autophagy will be referred to as CMA.

1.2.2.2 Endosomal system

The endosomal system is a vesicular trafficking system by which extracellular material, plasma membrane and transmembrane proteins can be internalised for functions such as nutrient uptake, proteostasis, removal of pathogenic substances or cellular signalling. Further, the system is complex and bidirectional, facilitating roles such as the delivery of newly synthesised lysosomal constituents to the lysosome from the endoplasmic reticulum (ER)/Golgi.

Endocytosis begins through plasma membrane invagination (both clathrin-dependent or clathrin-independent). Membrane scission forms an intracellular vesicle separating it from the plasma membrane. This vesicle fuses to early endosomes or other vesicles to produce early endosomes near the cell periphery, directed by small GTPases such as Rab5. The endosomal system uses a pH gradient as the vesicles mature toward the lysosome, becoming more acidic.

Early endosomes are slightly acidic (~pH 6) and are the primary location for cargo sorting (Murphy et al., 1984). Cargo such as transmembrane receptors may be sorted for recycling back to the plasma membrane directly by fast-recycling involving Rab4, or slow-recycling via recycling endosomes enriched in Rab11. Remaining cargo is trafficked toward the lysosome within the maturing endosome. As the early endosome matures into a late endosome, the lumen acidifies further (pH 5.5), Rab5 is exchanged for Rab7 and cargo may be sorted into intraluminal vesicles (producing multivesicular bodies – MVBs). Late endosomes/MVBs may fuse with the plasma membrane

releasing exosomes, sort proteins/lipids toward the trans-Golgi network, or fuse with the lysosome (Naslavsky & Caplan, 2018) (Figure 1.1).

Endosomes also act as an intermediary for the delivery of newly synthesised lysosomal components in lysosomal biogenesis (discussed in Section 1.2.4.3.2).

1.2.3 Lysosomal function beyond catabolism

Despite originally being thought to only have a role in degradation, the lysosome has emerged as having multiple functions beyond this. Lysosomes may fuse with the plasma membrane in exocytosis or act as a central signalling hub for downstream cellular response for example (Figure 1.2).

1.2.3.1 Lysosomal Signalling

Lysosomes facilitates communication between themselves, regulatory complexes that scaffold onto the lysosomal membrane and even other organelles. This provides a base for nutrient sensing, responding to ROS, material exchange between organelles, membrane fusion and fission, and even propagating transcriptional changes within the cell.

1.2.3.1.1 TRPML1 in Lysosomal Ca^{2+} signalling

The lysosome is a store for several ions used for modulating the lysosomal environment, such as regulating luminal pH or membrane fusion/scission. These ions also propagate signalling cascades for downstream cellular responses.

Lysosomal Ca^{2+} is a major signalling molecule that lysosomes release. TRPML1 is the primary lysosomal channel permeable to Ca^{2+} . Lysosomal Ca^{2+} release by TRPML1 can occur following several stimuli such as starvation, lysosomal pH imbalance, ROS production or pharmacological modulation such as ML-SA1 or MK6-83 (Shen et al., 2012; Chen et al., 2014; Wang et al., 2015; Lee et al., 2015; Zhang et al., 2016). TRPML1 activation has been shown to be involved in lysosomal exocytosis, trafficking, autophagy, lysosomal biogenesis, lysosomal pH and organelle-organelle signalling (Peng et al., 2020; Wu et al., 2021). TRPML1-mediated Ca^{2+} release can modulate autophagic/lysosomal signalling cascades through modulation of nutrient-sensors such as mTORC1 or CaMMKK β /AMPK (Sun et al., 2018; Rosato et al., 2019). Interestingly, TRPML1-mediated Ca^{2+} release can transcriptionally enhance lysosomal biogenesis through stimulating protein phosphatase, calcineurin, activating master regulators of lysosomal biogenesis, Transcription Factor EB (TFEB) and

Transcription Factor binding to IGHM enhancer 3 (TFE3) (discussed in Section 1.3) (Medina et al., 2015; Martina et al., 2016). TRPML1 activation has therefore been postulated as a promising therapeutic target for combatting neurodegeneration (Tsunemi et al., 2019; Tedeschi et al., 2019; Somogyi et al., 2023).

1.2.3.1.2 Lysosome-organelle contacts

Lysosomes contact other organelles by tethering in close proximity (approximately 10 nm). Contacts facilitate material exchange. Cholesterol and lipid shuttling have been observed in mitochondrial-lysosome, ER-lysosome, and peroxisome-lysosome contact sites to maintain homeostatic lipid levels (Chu et al., 2015; Lim et al., 2019; Juhl et al., 2021). Mitochondria-lysosome contact sites can transfer Ca^{2+} and Fe^{2+} from the lysosome to mitochondria. TRPML1 facilitates Ca^{2+} transfer into mitochondria through mitochondrial channels, VDAC1 and MCU. Lysosomal contact with mitochondria may also promote mitochondrial fission (Cisneros et al., 2022). ER-lysosome contact sites have been shown to exchange cholesterol, aiding lysosomal cholesterol homeostasis and lysosomal membrane repair (Radulovic et al., 2022). Furthermore, ER-lysosome contact sites regulate lysosomal positioning within neurons and co-ordinate Ca^{2+} signalling between the two organelles (Özkan et al., 2021; Atakpa et al., 2018). Thus, extensive communication between organelles through transient contact with lysosomes, coordinate functions and maintain a homeostasis between all components involved (Figure 1.2).

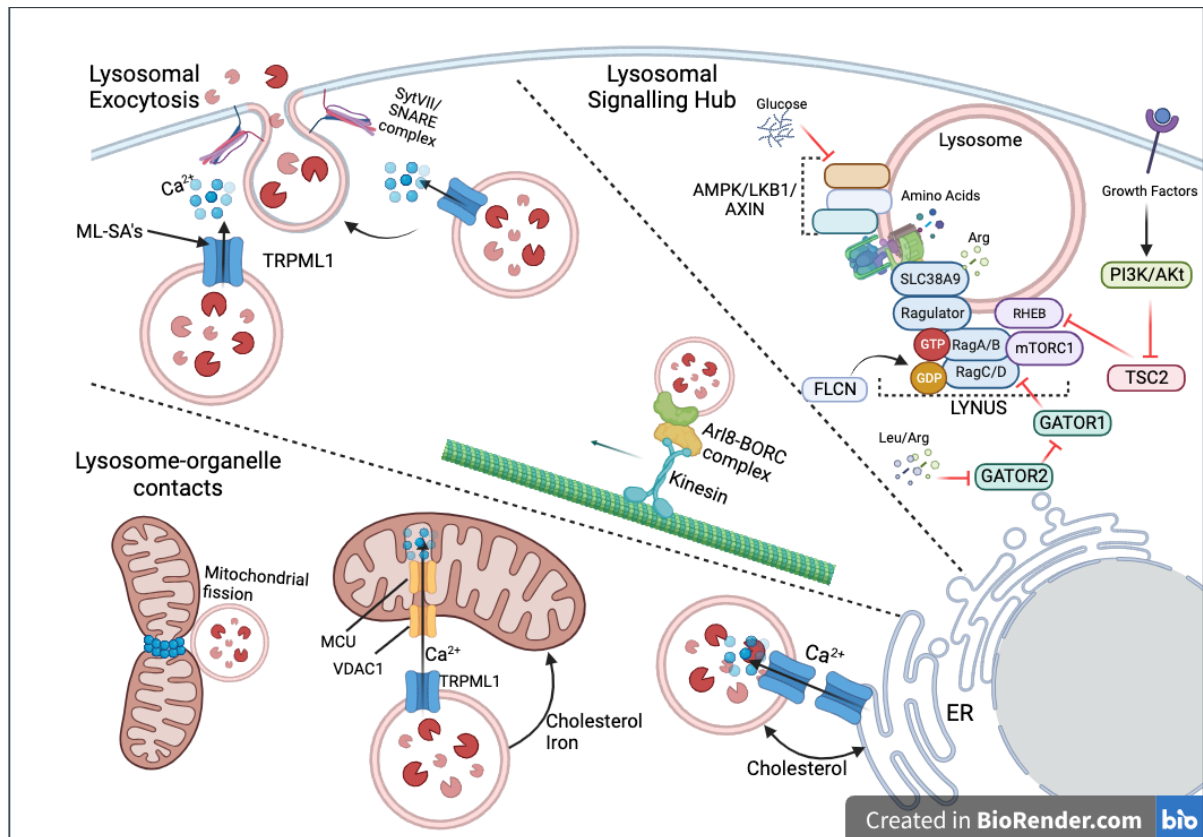


Figure 1.2 – Lysosomal signalling and function beyond catabolism

1.2.3.1.3 Lysosome as a signalling hub for nutrient sensing by mTORC1

Lysosomes provide a location for regulatory complexes to dock for nutrient sensing. The best-documented example is mTORC1.

mTORC1 is a complex including the serine-threonine kinase, mTOR, that co-ordinates cellular biosynthetic pathways by integrating information regarding cellular nutrient availability. mTORC1 activation enhances downstream anabolic pathways for cellular growth, while inhibiting catabolic pathways. Nutrient deprivation, hypoxia, energy stress and other conditions inactivate mTORC1, driving more catabolic processes such as autophagy (Melick & Jewell, 2020).

In nutrient-replete conditions, mTORC1 is activated at the lysosome, facilitated by a scaffolding complex including Rag GTPases, a Ragulator complex and Rheb. Each scaffolding protein is regulated by an array of converging pathways that integrate different nutrient presence signals from the cell and within the lysosome to modulate mTORC1 activity (reviewed in Lama-Sherpa et al., 2023) (Figure 1.2).

When mTORC1 is activated at the lysosome, mTOR is bound to substrates for regulation. mTORC1 modulates substrates to positively regulate protein synthesis (S6K and 4EBP) and negatively regulate catabolic pathways (including ULK1 inhibition

perturbing phagophore nucleation) (Saxton & Sabatini, 2017). mTORC1 activation also inhibits transcriptional regulation of catabolic processes through the inhibition of TFEB and TFE3. In the presence of nutrients, active Rag GTPases recruit TFEB and TFE3 to the lysosomal surface to be phosphorylated and inhibited by mTORC1 (Martina and Puertollano, 2013; Martina et al., 2014) (Section 1.3.2).

This is just one signalling cascade of several that use lysosomal localisation to coordinate cellular responses to different environmental stimuli within the cell. Thus, lysosomes embody an essential signalling platform within the cell to conduct cellular responses such as regulation upon catabolic processes, in a coordinated manner.

1.2.3.2 Lysosomal Exocytosis and Trafficking

While mostly perinuclear, under certain conditions, lysosomes can be anterogradely trafficked along microtubules to the periphery and fuse with the plasma membrane in a mechanism termed lysosomal exocytosis. This process exposes the luminal contents of the lysosome to the extracellular environment such that lysosomal membrane proteins can be seen on the cell surface and lysosomal hydrolases (amongst other constituents) are released into the extracellular space (Rodriguez et al., 1997; Reddy et al., 2001; Samie et al., 2013). Functionally, cells use this mechanism for several functions including membrane repair, cell-to-cell signalling, or even to pass nutrients such as lipids to neighbours (Tancini et al., 2020). Interestingly, this process can be also used to remove undegradable substrates from the lysosome. Lysosomal storage disorders (LSDs) encompass a group of diseases that carry mutations leading to loss of function in an integral lysosomal protein and accumulation of lysosomal substrates. Upregulating lysosomal exocytosis in LSDs can ameliorate storage deficits in models of LSDs (Medina et al., 2011).

Prior to exocytosis, lysosomes are trafficked by kinesin along microtubules to the periphery (Tancini et al., 2020). Once at the periphery, lysosomes are tethered to the plasma membrane and fusion is facilitated by localised Ca^{2+} release by TRPML1 (LaPlante et al., 2006; Dong et al., 2009). Lysosomal Ca^{2+} release activates Synaptotagmin VII and other fusion machinery to mediate the merging of the membranes (Zhao et al., 2008) (Figure 1.2). Again, lysosomes act here as a signalling hub for the activation of this process, stimulating fusion by releasing Ca^{2+} .

1.2.4 Lysosomal biogenesis

Following stimuli such as nutrient starvation, the lysosomal compartment must respond accordingly. Lysosomal biogenesis enhances lysosomal function when needed and maintains the lysosomal pool when prolonged high levels of catabolism need to occur. The cell may enhance lysosomal biogenesis transcriptionally through the activation of MiT/TFE transcription factors such as TFEB or TFE3; or through lysosomal reformation following the likes of chronic autophagy (Figure 1.3).

1.2.4.1 Lysosomal reformation

Lysosomal reformation may occur from endolysosomes, autolysosomes or even phagolysosomes. Mechanistically, most is known regarding autolysosomal reformation (ALR) (Yang & Wang et al., 2021). During chronic autophagy, mTOR becomes re-activated, stimulated by local release of nutrients from the lysosome following catabolism. This stimulates ALR. mTOR activation induces clathrin-dependent budding and tubulation of the lysosomal membrane. Lysosomal tubules are extended along microtubules by KIF5B, and scission by dynamin 2 produces a proto-lysosome (Nanayakkara et al., 2023) (Figure 1,3). These proto-lysosomes are non-degradative, less acidic and must mature again into lysosomes to function. Lysosomal reformation is required to maintain the lysosomal pool during prolonged periods of autophagy.

1.2.4.2 Transcriptional regulation of lysosomal biogenesis

Transcriptional regulation of lysosomal biogenesis is more energy-consuming than lysosomal reformation as it requires the biosynthesis and formation of lysosomal constituents, whereas reformation recycles existing structures. However, lysosomal biogenesis through transcription allows enhancement in volume and activity of the entire lysosomal compartment in a coordinated manner through transcription factor function. Thus, transcriptional regulation of lysosomal biogenesis enhances the catabolic capabilities of the lysosomal compartment rather than maintaining the lysosomal pool such as with reformation.

It is likely reformation and transcriptional biogenesis work in tandem, as maturation of proto-lysosomes will require the delivery of hydrolases to the organelle, and expression of these hydrolases derives from lysosomal biogenesis transcription factor function, such as from TFEB or TFE3. Section 1.3 will delve into more depth regarding TFEB/TFE3 function upon lysosomal biogenesis.

Other transcriptional regulators of lysosomal biogenesis do exist. The oncogene, STAT3 transcriptionally upregulates lysosomal hydrolases in response to lysosomal oxidative stress (Martínez-Fábregas et al., 2018). Interestingly, STAT3 interacts with TFEB in the nucleus to partially inhibit TFEB function inducing lysosome-mediated cell death following ischemic injury or lysosomal permeabilisation by RDD647, thus regulating lysosomal biogenesis dependent upon context (Li et al., 2018; Liu et al., 2023). Lysosomal biogenesis can be transcriptionally repressed by transcription factors such as ZKSCAN3, MYC, histone deacetylases (HDACs) and BRD4 (Chauhan et al., 2013; Sakamaki et al., 2017; Annunziata et al., 2019) (Figure 1.3). Many transcriptional repressors of lysosomal biogenesis work antagonistically with TFEB/TFE3 to coordinate this function (Chauhan et al., 2013; Annunziata et al., 2019).

1.2.4.3 Trafficking newly synthesised lysosomal proteins to the lysosome

Lysosomal biogenesis in both mechanisms requires the delivery of lysosomal constituents to the compartment once synthesised and processed through the ER/Golgi (Figure 1.3).

1.2.4.3.1 Transmembrane protein delivery to the lysosome

Transmembrane proteins generally follow either direct or indirect trafficking. Indirect trafficking involves initial delivery to the plasma membrane before endocytosis through the endolysosomal system terminating at the lysosome. Direct trafficking is similar to that of lysosomal hydrolases in which the transmembrane proteins are directly trafficked to the endosome from the Golgi for lysosomal delivery (reviewed by Staudt et al., 2016).

1.2.4.3.2 Hydrolase trafficking to the lysosome

The canonical method for lysosomal hydrolase trafficking involves the addition of a mannose-6-phosphate to hydrolases during Golgi processing. This process relies upon LYSET (TMEM251) to stabilise GlcNac-1-phosphotransferase, ensuring the appropriate trafficking moiety is added for correct trafficking (Pechincha et al., 2022; Richards et al., 2022). Mannose-6-phosphate receptors (M6PR) then bind to the modified hydrolase and the complex is trafficked to the endosome. Noteworthy, M6PRs can also be present at the plasma membrane to recycle exocytosed hydrolases. The acidic environment in the endosome disassociates M6PR from the

hydrolase, the receptor is recycled back to the Golgi or plasma membrane and the hydrolase is delivered to the lysosome (Staudt et al., 2016).

GCase is not trafficked by M6PRs. Lysosomal integral membrane protein-2 (LIMP2) is responsible for GCase delivery to the lysosome (Reczek et al., 2007). Once GCase has passed through the ER, LIMP2 binds and facilitates transport through the Golgi before being directly targeted to the endosomes for later fusion to the lysosome. Similar to M6PR, LIMP2 disassociates with GCase in an acidic environment releasing GCase into the lumen but remains embedded in the lysosomal membrane (Reczek et al., 2007; Zachos et al., 2012).

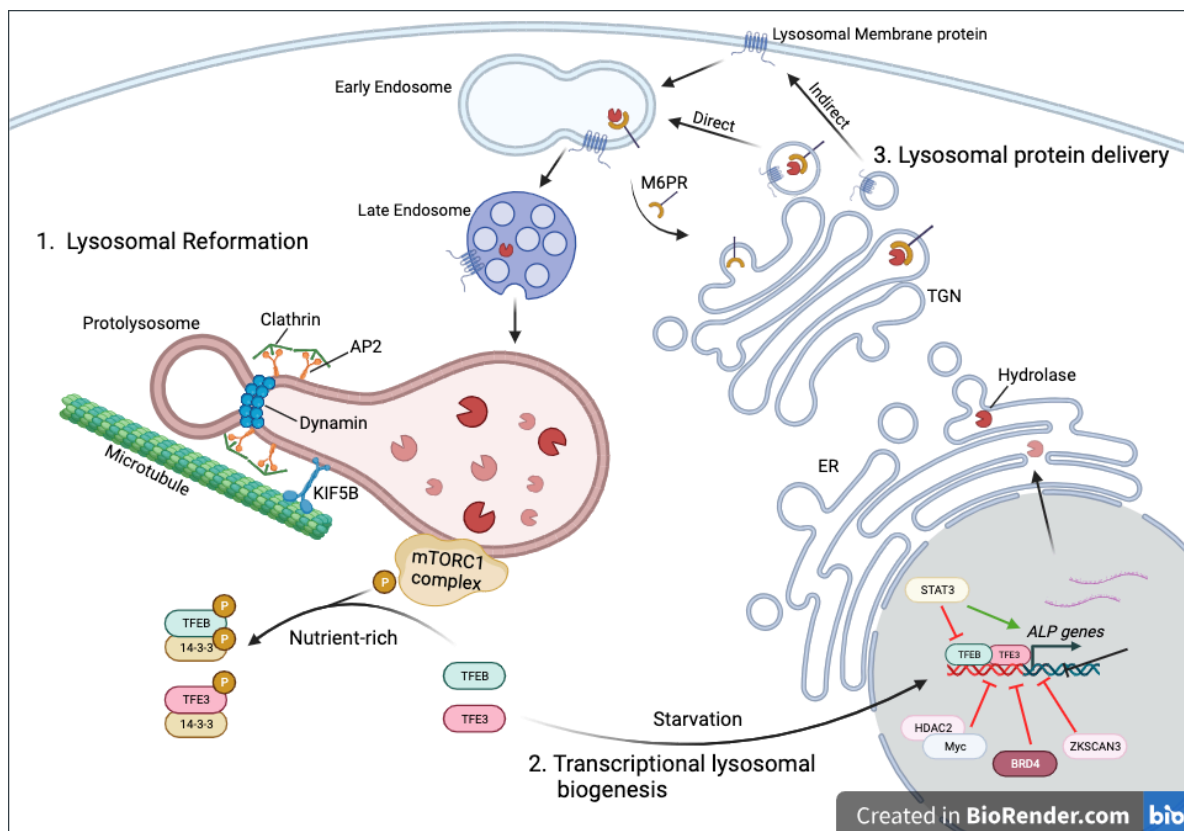


Figure 1.3 – Lysosomal Biogenesis Pathways

1.2.5 Lysosomal Dysfunction in neurodegeneration and PD

1.2.5.1 Lysosomal dysfunction in neurodegeneration and Lysosomal Storage Disorders

As neurons are post-mitotic, toxic/aggregating proteins or damaged organelles cannot disperse cytotoxic substances between progeny. Neurons must directly turnover or remove these substances. Lysosomal pathways are the primary site for removal of

these substances within neurons and lysosomal dysfunction frequently leads to neurodegeneration.

Lysosomal storage disorders (LSDs) compose a group of 70 diseases in which lysosomal dysfunction occurs from monogenic mutations in genes encoding lysosomal proteins such as hydrolases, membrane proteins, substrate transporters, and enzymatic modifiers. Mutations lead to accumulation of lysosomal substrates causing critical dysfunction of the lysosomal system. Importantly, LSDs are frequently paediatric neuropathological disorders underlining the essentiality of lysosomal function in neuronal homeostasis (Platt et al., 2018).

A common pathological feature of neurodegenerative diseases is the presence of proteinaceous aggregates (Sweeney et al., 2017). Protein aggregates are mainly degraded by selective autophagy (aggrephagy) in neurons, indicating defective degradation pathways to be a core commonality between neurodegenerative disorders (Lamark & Johansen, 2012). Indeed, post-mortem pathological study finds inclinations of autophagic-lysosomal perturbation in many neurodegenerative disorders (Mizuno et al., 2006; Dehay et al., 2010; Sasaki, 2011; Piras et al., 2016). Further, monogenic causative mutations and genetic risk variants are present in genes encoding proteins associated with lysosomal pathways for these disorders (Malik et al., 2019). As PD is the primary disorder of study in this thesis, we will discuss this further.

1.2.5.2 PD pathology overlaps with LSDs

Lysosomal dysfunction is widely implicated in PD pathology. There is a large degree of overlap between LSDs and PD, demonstrating the critical nature of lysosomal homeostasis. More than half of PD patients harbour a detrimental variant in a gene associated with LSDs, demonstrated by exome sequencing (Robak et al., 2017). Further, Parkinsonism has been described in LSDs such as Gaucher's disease (homozygous *GBA1* mutation), Fabry disease (*GLA* mutation), GM2 gangliosidosis (*HEXA* mutation), neuronal ceroid lipofuscinosis (*CLN* gene group mutations), Niemann pick disease type C1 (*NPC1* mutation) and Sanfilippo syndrome (*NAGLU* mutation) (Inzelberg et al., 1994; Nijssen et al., 2002; Goker-Alpan et al., 2004; Josephs et al., 2004; Bueckner et al., 2006; Winder-rhodes et al., 2012; Klunenmann et al., 2013; Wise et al., 2018). LSD-associated deficiencies have also demonstrated aggregation of α Syn (Saito et al., 2004; Suzuki et al., 2007; Mazzulli et al., 2011; Smith

et al., 2014). Interestingly, TFEB and TFE3 upregulate these genes, displaying the therapeutic potential of these transcription factors across these highly related diseases.

1.2.5.3 Post-mortem evidence for lysosomal dysfunction

Post-mortem pathology finds autophagic-lysosomal perturbation in PD patients by autophagosomal accumulation, and reduced levels of LAMP1 (Lysosome-associated membrane protein 1), LAMP2A and HSC70 indicative of autophagic, CMA and lysosomal disturbances (Alvarez-Erviti et al., 2010; Dehay et al., 2010). Furthermore, lipids, autophagic and lysosomal structures have been found sequestered in LBs within nigral DaNs, indicating perturbations in lysosomal catabolic homeostasis (Shahmoradian et al., 2019). Numerous studies have demonstrated reduced abundance and activity of lysosomal hydrolases in the SNc of PD patients (Gegg et al., 2012; Chiasserini et al., 2015; Moors et al., 2019; Huebecker et al., 2019). Interestingly, reduced mRNA expression of *GBA* was also observed in the SNc of PD patients, demonstrating a potential role for transcriptional enhancement of lysosomal biogenesis in PD (Moors et al., 2019).

1.2.5.4 Genetics implicating lysosomal dysfunction in PD

As mentioned in Section 1.1.4.2, several monogenic forms of PD are related to mutation in genes associated with autophagic-lysosomal pathways (Table 1.1; *ATP13A2*, *VPS35*, *LRRK2*, *PINK1*, *PRKN*). Further, PD risk loci (such as *GBA*, *CTSB*, *TMEM175*) present a strong link to lysosomal dysfunction in idiopathic PD, with an enrichment in lysosome-related pathways in GWAS hits (Nalls et al., 2019). This thesis will focus upon lysosomal perturbations associated with *SNCA*, *LRRK2* and *GBA* in PD.

1.2.5.5 SNCA and autophagic-lysosomal perturbation in PD

Mutations or multiplications in the *SNCA* gene induce pathogenic accumulation and aggregation of α Syn, indicating perturbed proteostasis in neurons. Indeed, *SNCA* mutations or multiplication can trigger lysosomal perturbation through inhibition of hydrolase trafficking and activity, such as GCase, Cathepsin B, hexosaminidase, sulfatase and β -Galactosidase (Yap et al., 2013; Mazzuli et al., 2016; Cuddy et al., 2019). Here, an aberrant interaction between pathogenic α Syn and the SNARE protein, ykt6, prevents delivery of these hydrolases to the lysosome (Cuddy et al.,

2019). Thus, substrate aggregation and reduced proteolysis have been observed in models with *SNCA* mutations (Mazzuli et al., 2016; Cuddy et al., 2019; Zambon et al., 2019).

α Syn is degraded by Cathepsin D (CTSD) in the lysosome, delivered by CMA and autophagy (it can also be degraded by the proteasome) (Webb et al., 2003; Cuervo et al., 2004; Lee et al., 2004; McGlinchey et al., 2015). As such, inhibition of CMA, autophagy or the lysosome can aggregate α Syn (Lee et al., 2004; Cuervo et al., 2004; Vogiatzi et al., 2008). Conversely, mutation, modification, aggregation or elevated expression of α Syn can perturb autophagy and CMA (as well as inhibiting hydrolase activity), demonstrating a feedback loop promoting further α Syn deposition (Cuervo et al., 2004; Martinez-Vicente et al., 2008; Decressac et al., 2013; Song et al., 2014). Other studies have suggested *SNCA* mutation to elevate autophagic flux, rather than inhibit (Oliveria et al., 2015). This was in iPSC-derived dopaminergic neurons (iPSC-DaNs) and may be compensatory resultant from inhibited hydrolase trafficking/activity caused by mutation. A recent single-cell transcriptomic analysis of iPSC-derived midbrain organoids found *SNCA-Trp* DaNs to upregulate autophagic genes corroborating a compensatory action in iPSC-DaN models (Patikas et al., 2023).

Autophagic-lysosomal perturbation, or *SNCA* mutation or multiplication can induce secretion of α Syn by neurons, likely to remove accumulating substrates from the cell (Poehler et al., 2014; Minakaki et al., 2018; Zambon et al., 2019). Lysosomal exocytosis may drive this secretion (Xie et al., 2022). As pathogenic α Syn can propagate seeding in other neurons, autophagic-lysosomal inhibition in PD may facilitate spread. Interestingly, internalisation of fibrillar α Syn may also induce lysosomal permeabilization, leading to further lysosomal dysfunction in PD (Freeman et al., 2013).

1.2.5.6 GBA and lysosomal dysfunction in PD

GCCase is a lysosomal hydrolase responsible for catabolism of GlcCer into glucose and ceramide. *GBA* mutation abrogates GCCase activity, leading to substrate accumulation within the cell (GlcCer and glucosylsphingosine (GlcSph)), although substrate accumulation is disputed in *GBA-PD* brains (Gegg et al., 2015). Mutations are thought to prevent GCCase trafficking to the lysosome, causing an accumulation of GCCase in the ER (Smith & Schapira, 2022). Indeed, *GBA-N370S* iPSC-DaNs have

hyperglycosylated GCase indicative of ER retention (Fernandes et al., 2016). GCase deficiency leads to dysfunction in other lysosomal hydrolases such as hexosaminidase, GBA2, α -galactosidase, β -galactosidase and CTSD, CTSB and CTSL (Schöndorf et al., 2014; Sanyal et al., 2020; Yang et al., 2020; Grigor-Eva et al., 2023), further perturbing lipid dysfunction and α Syn accumulation (Schöndorf et al., 2014; Fernandes et al., 2016; Magalhaes et al., 2016; Mubariz et al., 2023). Swollen lysosomes containing undegraded cargo are present in *GBA*-PD models, demonstrating lysosomal impairment (Schöndorf et al., 2014; Fernandes et al., 2016; Lang et al., 2019; Mubariz et al., 2023). Lysosomal perturbation has further been demonstrated by increased pH, impaired lysosomal calcium storage/release, reduced lysosomal reformation and impaired autophagic flux (Schöndorf et al., 2014; Kilpatrick et al., 2016; Magalhaes et al., 2016; Garcia-Sanz et al., 2017; Sanyal et al., 2020). *GBA-N370S* iPSC-DaNs have elevated α Syn release, like *SNCA* iPSC-DaNs, showing this a frequent strategy to remove accumulating cargo by DaNs (Fernandes et al., 2016). Interestingly, altered mTORC1 activity has been reported. mTOR was inhibited in *GBA*-HET mouse embryonic fibroblasts (MEFs), whereas independent studies reported increased mTOR activity in *GBA-N370S* iPSC-DaNs (Magalhaes et al., 2016; Bogetofte et al., 2023; Mubariz et al., 2023). This underlines the need to study species and disease-relevant models of PD.

Interestingly, overlap between *GBA*, *LRRK2* and *SNCA* occur. *GBA* mutation forms a pathogenic feedback loop with α Syn, whereby GCase inhibition leads to α Syn accumulation, and α Syn in turn perturbs GCase activity and trafficking, exacerbating dysfunction (Mazzuli et al., 2011; Mazzuli et al., 2016). Further, *LRRK2* kinase activity seems to inhibit GCase activity (Ysselstein et al., 2019), displaying interconnectivity in pathogenic lysosomal mechanisms in PD.

1.2.5.7 *LRRK2* and lysosomal dysfunction in PD

LRRK2 interacts and regulates many substrates functioning within the endolysosomal system, such as Rab GTPases (Jeong & Lee, 2020). As such, *LRRK2* mutations perturb endocytosis and endosomal sorting (MacLeod et al., 2013; Pan et al., 2017; Connor-Robson et al., 2019). *LRRK2* mutations have also been shown to disturb macroautophagy and CMA (Sanchez-Danes et al., 2012; Orenstein et al., 2013; Reinhardt et al., 2013; di Domenico et al., 2019; Ho et al., 2020). The effect of *LRRK2*

mutation upon macroautophagy is contradictory, as both increases and reductions in autophagic flux have been observed (Madureira et al., 2020). The direction of these changes appears dependent upon cell-type, dosage and experimental system used. At the lysosome itself, LRRK2 binds the lysosomal membrane following lysosomal inhibition, phosphorylates Rab proteins to prevent lysosomal overloading and promotes lysosomal secretion (Eguchi et al., 2018). *LRRK2* mutations have been shown to cause lysosomal swelling and increase the lysosomal localisation proximal to the nucleus (MacLeod et al., 2013; Hockey et al., 2015; Henry et al., 2015). Others have seen the opposite, with more, but smaller lysosomes, and dispersed localisation (Schapansky et al., 2018). Impaired lysosomal pH is frequently associated with *LRRK2* mutations, and aberrant regulation of v-ATPase at the lysosome have been implicated in this (Schapansky et al., 2018; Wallings et al., 2019). Lysosomal Ca^{2+} release has also been shown to be perturbed *LRRK2* mutation too (Hockey et al., 2015; Wallings et al., 2019). Interestingly, *LRRK2* is implicated in GCase activity. Reduced GCase activity has been seen in *LRRK2* iPSC-DaNs, as well as an increase in αSyn , interconnecting *LRRK2* to the pathogenic loop between αSyn and GCase deficiency (Nguyen & Krainc, 2018; Ysselstein et al., 2019). Similarly, *LRRK2* kinase inhibition increases GCase activity in both *GBA* and *LRRK2* iPSC-DaNs and can rescue lysosomal dysfunction associated with *GBA* mutation.

LRRK2 has been widely implicated in endosomal/autophagic-lysosomal function, however differing results underly the need to study models in human, disease-relevant cell types to decipher the exact role mutation has in implicated cell-types.

It is evident lysosomal dysfunction plays a large role in PD pathogenesis. The enhancement of autophagy or lysosomal biogenesis has grown as an interesting therapeutic strategy to abrogate neurodegeneration to prevent pathogenesis. The master regulators of lysosomal biogenesis, TFEB and TFE3 present a promising avenue for enhancing these pathways in neurodegeneration and PD.

1.3 TFEB and TFE3 Function and Regulation

1.3.1 MiT/TFE Transcription Factor Family

The Microphthalmia/transcription factor E (MiT/TFE) family of transcription factors are a conserved subfamily of the broader family of basic helix-loop-helix leucine zipper (bHLH-LZip) transcription factors that contain proteins such as Myc, Max and Mad

(Atchley and Fitch, 1997). There are 4 MiT/TFE transcription factors: TFEB, TFE3, MITF and TFEC (Hemesath et al., 1994). These transcription factors can homo- or heterodimerise with one another but not other transcription factors from the bHLH-LZip transcription factor family due to a 'kink' in the leucine zipper domain (Hemesath et al., 1994; Pogenberg et al., 2012). TFEB, TFE3 and MITF contain an acidic transcriptional activation domain facilitating target gene expression, whereas TFEC lacks this and appears to inhibit the transcriptional activity of other MiT/TFE transcription factors (Zhao et al., 1993). Little is known regarding TFEC function.

Like other bHLH-LZip transcription factors, MiT/TFE transcription factors bind a 6bp E-box motif: CANNTG. Bases either side of this motif or the central residues in the motif itself define transcription factor specificity (Goding and Arnheiter, 2019). MiT/TFE transcription factors also bind the M-box motif TCATGTGA unlike their bHLH-Lzip counterparts (Verastegui et al., 2000). The best documented MiT/TFE binding motif is the 10bp palindromic E-box sequenced named 'Co-ordinated lysosomal expression and regulation (CLEAR) motif: GTCACGTGAC (Sardiello et al., 2009). This motif is present near the transcriptional start site of a gene network enriched in lysosomal and autophagic genes and was initially found to be bound and regulated by TFEB, thus TFEB was deemed the 'master regulator of lysosomal biogenesis' (Sardiello et al., 2009; Palmieri et al., 2011). Since, TFE3 and MITF have been shown to bind the CLEAR motif and regulate autophagic and lysosomal biogenesis, MITF doing so primarily in melanocytes (Martina et al., 2014; Ploper et al., 2015; Möller et al., 2019). TFEB and TFE3 are thought to be ubiquitously expressed and are well established regulators of lysosomal function (Oppezzo & Rosselli, 2021). MITF is largely associated with melanosome formation and melanocyte function as well as cell survival, differentiation and proliferation. MITF expression patterns depend upon isoform, and while generally associated with melanocytes/melanoma (MITF-M), MITF is also expressed in other cell-types such as cardiomyocytes, retinal pigment epithelium, osteoclasts and mast cells (Amae et al., 1998; Tshori et al., 2006; Shahlaee et al., 2008; Lu et al., 2010). As MITF and TFEC expression is more tissue-specific in expression and less established as regulators of lysosomal biogenesis than TFEB and TFE3, we will focus on TFEB and TFE3.

1.3.2 TFEB and TFE3 regulation

TFEB and TFE3 are predominantly regulated by their spatial localisation within the cell. At basal conditions (nutrient-replete), TFEB and TFE3 are cytoplasmic. Upon nutrient starvation or cellular stress, the transcription factors translocate into the nucleus and upregulate downstream gene targets. Post-translational modifications (particularly phosphorylation) are the main mode of TFEB and TFE3 activity modulation (Table 1.2; Figure 1.4), although transcriptional regulation (Table 1.3) and translational regulation do occur.

1.3.2.1 Post-translational modifications of TFEB and TFE3

1.3.2.1.1 Phosphorylation

1.3.2.1.1.1 mTORC1 phosphorylation of TFEB and TFE3

mTORC1 regulates TFEB/TFE3 localisation, linking nutrient sensing at the lysosome to lysosomal biogenesis (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Martina et al., 2014). In nutrient abundant conditions, Folliculin activates RagC/D GTPases that subsequently bind the Rag-binding domain of TFEB or TFE3 and recruit the transcription factors to the lysosome/mTORC1 (Martina & Puertollano, 2013; Martina et al., 2014). At the lysosome, mTORC1 phosphorylates TFEB at S122, S142 and S211, or TFE3 at S321 (and perhaps S47) (Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Martina et al., 2012; Martina et al., 2014; Vega-Rubin-de-Celis et al., 2017; Nardone et al., 2023). In cell types with hyperactive mTORC1, phosphorylation may also occur at the C-terminal tail of TFEB (Peña-Llopis et al., 2011). TFEB-S211 or TFE3-S321 phosphorylation facilitates 14-3-3 chaperone binding, and is thought to promote cytoplasmic retention, sequestering TFEB and TFE3 in the cytoplasm (Roczniak-Ferguson et al., 2012; Martina et al., 2012; Martina et al., 2014). TFEB-S122 phosphorylation regulates nuclear localisation by modulating the magnitude of translocation induced when S211 is dephosphorylated and is considered a switch for TFEB translocation (Vega-Rubin-de-Celis et al., 2017). Noteworthy, S122 dephosphorylation alone cannot stimulate TFEB translocation. TFEB-S142 phosphorylation by mTORC1, but also ERK, promotes TFEB cytoplasmic localisation in nutrient rich conditions (Settembre et al., 2011).

mTORC1 phosphorylation can destabilise TFEB and TFE3. TFEB-S142 and S211 phosphorylation promotes TFEB ubiquitylation and degradation by the ubiquitin-

proteasome system (UPS), facilitated by E3-ubiquitin ligase, STUB1 (Sha et al., 2017). Recently, TFE3 was found phosphorylated at S47 (possibly by mTORC1), destabilising the protein by CUL1^{β-TrCP}-mediated ubiquitylation and degradation by the UPS (Nardone et al., 2023).

1.3.2.1.1.2 Other kinases regulating TFEB and TFE3

TFEB and TFE3 are regulated by several kinases, allowing for integration of many inputs relaying nutrient and cellular stresses to adapt and upregulate catabolic process appropriately. AKT phosphorylates TFEB(S467) and TFE3(S565) in an mTORC1-independent manner, inhibiting nuclear localisation (Palmieri et al., 2017; Pi et al., 2019). MAP4K3 phosphorylation of TFEB-S3 promotes TFEB cytoplasmic retention by promoting Rag GTPase binding and precedes TFEB-S211 phosphorylation by mTORC1 (Hsu et al., 2018). It is unknown whether similar regulation occurs on TFE3 by MAP4K3 but as TFE3 is similarly regulated by mTORC1 and has a Rag-binding domain, one may expect a similar regulatory mechanism to occur (Martina et al., 2014).

ERK1/2 phosphorylates TFE3 at S245 to promote co-activator, p300 binding and TFE3-mediated gene expression (Hershey et al., 2004). ERK1/2 also phosphorylates TFEB at the same residue as mTORC1, S142, which primes TFEB phosphorylation by GSK3 β at S138, unveiling a nuclear export signal to shuttle TFEB to the cytoplasm by CRM1 (Settembre et al., 2011; Li et al., 2018; Napolitano et al., 2018). Interestingly, GSK3 β phosphorylation of TFEB (at S134 and S138) promotes cytoplasmic localisation but does not affect TFE3, seen following GSK3 β inhibition by pharmacological PKC activation (Li et al., 2016). Cyclin-dependent kinases 4/6 (CDK4/6) also phosphorylates TFEB and TFE3 (critically at TFEB-S142 and TFE3-S246) to promote nuclear export (Yin et al., 2020). CDK1 also phosphorylates TFEB at the same position, producing the same effect during mitosis when mTORC1 is inhibited (Odle et al., 2020).

AMPK has been implicated in TFEB activation indirectly through mTORC1 inhibition and CARM1 activation (co-activator of TFEB) (Young et al., 2016; Shin et al., 2016). Interestingly, AMPK also directly phosphorylates TFEB (at residues S466, S467 and S469) and TFE3 (at residues S567, S568 and S570) promoting transcriptional activity of the transcription factors (Paquette et al., 2021).

In a cell-dependent context, PKC β has been shown to promote phosphorylation of the C-terminus of TFEB, stabilising protein expression in osteoclasts (Ferron et al., 2013).

1.3.2.1.1.3 Phosphatase regulation upon TFEB and TFE3

TFEB and TFE3 can be dephosphorylated by protein phosphatases. Calcineurin, a calcium-sensitive phosphatase activated by Ca²⁺ release from the ER in ER stress, or the lysosome by TRPML1 in conditions such as starvation, dephosphorylates TFEB and TFE3 at key residues such as TFEB-S142, TFEB-S211 and likely TFE3-S321 (Medina et al., 2015; Martina et al., 2016). Calcineurin-mediated TFEB dephosphorylation increases expression of autophagy and lysosomal proteins, through nuclear accumulation of the transcription factor. As TFEB and TFE3 upregulate *MCOLN1* (encoding TRPML1), a positive feedback loop occurs in which activation of the transcription factors increases lysosomal Ca²⁺ release capabilities (Palmieri et al., 2011; Martina et al., 2014).

Protein phosphatase 2A (PP2A) also dephosphorylates TFEB and TFE3 at regulatory residues TFEB-S211 and TFE3-S321 following oxidative stress or through PIKfyve inhibition perturbing mTORC1 signalling (Martina and Puertollano, 2018; Hasegawa et al., 2022).

1.3.2.1.2 Acetylation and Other post-translational modifications

A number of other post-translational modifications have either been predicted or found on TFEB and TFE3, such as acetylation, SUMOylation, ubiquitylation, glycosylation and cysteine oxidation (Vu et al., 2020; Franco-Juarez et al. 2022). Acetylation is one of the best documented for TFEB regulation. TFEB acetylation has been shown to be both an activating and an inhibitory moiety in different studies. HDAC inhibition or histone acetyltransferase (KAT) activity (by ACAT1, GCN5 or other KATs) acetylated TFEB residues and increased lysosomal biogenesis through nuclear localisation and TFEB transcriptional activation (Brijmohan et al., 2018; Zhang et al., 2018; Li et al., 2022). Alternatively, acetylation of different residues in TFEB (K274 and K279) reduced TFEB transcriptional activation, displaying dual roles for acetylation upon TFEB activity (Wang et al., 2020). One study found a contradictory role for acetylation of K116 on TFEB activation and function, by which deacetylation of TFEB-K116 through Sirtuin 1 activated lysosomal biogenesis, opposing results from Zhang and colleagues in 2018 (Bao et al., 2016). This was suggested to be resultant of stress conditions present. Little is known regarding TFE3 regulation by acetylation, but HDAC

inhibition stimulates translocation of the transcription factor indicating some form of conserved regulation (Li et al., 2022).

Table 1.2 - TFEB and TFE3 regulatory PTMs (adapted from Takla et al., 2023)

<i>Modification</i>	<i>Enzyme</i>	TFEB		TFE3	
		Modified residue	Effect	Modified residue	Effect
Phosphorylation	MAP4K3	S3	<i>Recruits to lysosome</i>	-	-
	mTORC1	S122	<i>Cytosolic retention</i>	S321	<i>Cytosolic retention</i>
		S142	<i>Nuclear export</i>	S47 - likely mTORC1 but unconfirmed	<i>Promotes ubiquitination and proteasomal degradation</i>
		S211	<i>Cytosolic retention</i>	-	-
	ERK2	S142	<i>Nuclear export</i>	S73 (murine Tfe3)	<i>Co-activator binding (p300)</i>
	CDK1/CDK4/6	S142	<i>Nuclear export</i>	S246	<i>Nuclear export</i>
	GSK3B	S134	<i>Cytosolic retention</i>	Not affected by GSK3b	-
		S138	<i>Nuclear export</i>	-	-
	AKT	S467	<i>Cytosolic retention</i>	S565	<i>Cytosolic retention</i>
	PKCB	S461 S462 S466 S468	<i>Stabilises TFEB</i>	-	-
		S466 S467 S469	<i>Increases transcriptional activity</i>	S567, S568, S570	<i>Increases transcriptional activity</i>
Acetylation	ACAT1	K91 K103 K116 K430	<i>Promotes Nuclear TFEB</i>	-	-
	Other KATs	K236 K237 K431	<i>Promotes Nuclear TFEB</i>	-	-
	GCN5	K274 K279	<i>Disrupts dimerization and DNA binding</i>	-	-
Deacetylation	SIRT1	K116	<i>Increases TFEB</i>	-	-

	HDACs	K91 K103 K116 K430 K236 K237 K431	transcriptional activity Cytosolic retention	-	Cytosolic retention
Ubiquitylation	STUB1	-	Proteasomal TFEB degradation	-	-
	-	K347	Proteasomal TFEB degradation	-	-
	CUL1 ^{B-} TrCP1/2	-	-	-	Proteasomal TFE3 degradation
SUMOylation	-	K347, K361	Increased transcriptional activity	K330, K460	Increases transcriptional activity
Glycosylation	SetA	S195 S196 T201 S203 T208	Promotes Nuclear TFEB	-	-
Oxidation	-	C212	Promotes Nuclear TFEB	C322	Promotes Nuclear TFE3

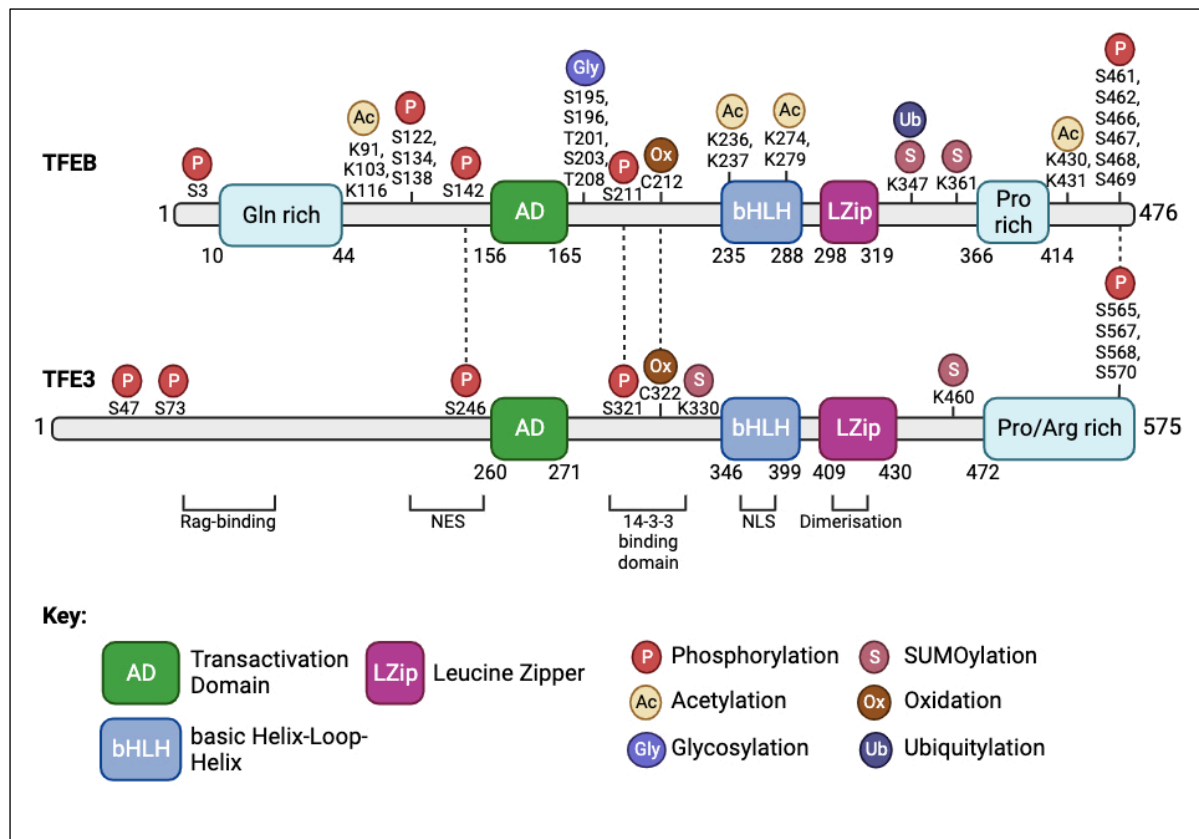


Figure 1.4 – TFEB and TFE3 post-translational modification map.

1.3.2.2 Transcriptional regulation of TFEB/TFE3

TFEB and TFE3 expression can be transcriptionally and translationally regulated. TFEB transcription is controlled by several factors and regulatory RNAs (Table 1.3). Interestingly, HDAC regulation of TFEB extends beyond post-translational modification, and HDAC2 regulates *TFEB*, *TFE3* and lysosomal gene transcription in coordination with MYC, by binding the promoters and inhibiting expression of these genes (Annunziata et al., 2019).

TFEB is a CLEAR gene and as such can upregulate its own transcription in response to stress or nutrient deprivation (Settembre et al., 2013). Similarly, nutrient deprivation induces *TFEB* expression and several autophagic genes through CREB-CRTC2 binding and promoting expression (Seok et al., 2014).

TFEB transcription is enhanced by the lipid catabolism and mitochondrial biogenesis transcription factors, peroxisome proliferator-activated receptor α (PPAR α) and PPAR- γ coactivator 1a (PGC1a), displaying metabolic regulation upon TFEB and the requirement for coordinated catabolic transcriptional responses in metabolism (Tsunemi et al., 2012; Ghosh et al., 2015). Interestingly, TFEB and TFE3 upregulate

metabolism through enhancing PGC1 α , and TFEB also enhances PPAR α and lipid catabolism genes, forming a positive feedback loop between mitochondrial biogenesis and lipid catabolism (Settembre et al., 2013; Ma et al., 2015; Salma et al., 2015).

Table 1.3 - Transcriptional regulators of TFEB (adapted from Jiao and Meng, 2022)

Name	Transcriptional Regulation	Mechanism	Cell type	Reference
CREB	Upregulation	Recruits co-activator CRTC2 in nutrient deprivation	Ms. Hepatocytes Hepa1c1c7	Soek et al., 2014
PGC1a	Upregulation	Occupies isoform with 3'-most TSS of <i>TFEB</i>	Neuro2a cells	Tsunemi et al., 2012
PPARa	Upregulation	Recruits RXR and PGC1 on <i>TFEB</i> promoter	Mouse primary brain cells BV-2 glial cells	Ghosh et al., 2015
CARM1	Upregulation	Binds <i>TFEB</i> transcriptional activation domain	MEFs	Shin et al., 2017
TFEB	Upregulation	Binds CLEAR motif in <i>TFEB</i> promoter	MEFs	Settembre et al., 2013
C-ETS2	Upregulation	Binds <i>TFEB</i> promoter	293T Cells	Ma et al., 2016
FOXO1	Upregulation	Binds <i>TFEB</i> promoter	3T3-L1 Adipocytes	Liu et al., 2016
STAT1	Upregulation	JAK2-mediated binding to <i>TFEB</i> promoter	Mouse podocytes	Alghamdi et al., 2017
KLF2	Upregulation	Upregulates TFEB-Luc activity (binds <i>TFEB</i> promoter)	HUVECs	Song et al., 2019
sXBP1	Upregulation	Binds <i>TFEB</i> promoter	Primary hepatocytes Human liver	Zhang et al., 2021

MiR-342-3p	Downregulation	Binds <i>TFEB</i> 3' UTR and inhibits transcription	HEK293T	Wang et al., 2021
MiR-128	Downregulation		Rat DaNs	Decressac et al., 2013
SMAD3	Downregulation	Binds 3' UTR of <i>TFEB</i> and inhibits transcription	HK2-cells	Yang et al., 2021
HDAC2	Downregulation	Binds <i>TFEB</i> promoter, aids c-Myc binding and inhibition	HeLa cells	Annunziata et al., 2019

Little transcriptional regulation upon the *TFE3* locus is known beyond HDAC2 and Myc regulation, although recently nuclear respiratory factor-1 (NRF-1 - regulates mitochondrial biogenesis and oxidative phosphorylation) bound and enhanced *TFE3* expression, which reduced apoptosis of a renal cell carcinoma, further underlining the intertwined function between cellular catabolism and mitochondrial metabolic homeostasis (Zhuang et al., 2021).

1.3.2.3 Translational regulation of TFEB/TFE3

TFEB translation requires EIF5A for efficient translation due to a triproline motif on TFEB (Zhang et al., 2019). In ageing mice, TFEB expression reduces in B cells concomitant with reductions in the hypusination and expression of EIF5A, as well as autophagic deficits. Spermidine restored TFEB expression and autophagy in ageing B cells by increasing EIF5A hypusination (Zhang et al., 2019). Programmed cell death 4 (PDCD4) was also shown to negatively regulate TFEB translation through inhibiting EIF4A translation upon TFEB (Chen et al., 2020). This function is associated with a TFEB-dependent perturbation of lysosomal function.

It is uncertain whether TFE3 is regulated in a similar manner from either of these studies, however EIF5A hypusination inhibitor, GC7, reduced TFE3 expression indicating regulatory overlap (Zhang et al., 2019). Recently, it was shown M2 isoform of pyruvate kinase (PKM2) facilitates translation of TFE3 to maintain Golgi integrity, suggesting a role for translational regulation in response to cellular stresses (Taguchi et al., 2023).

1.3.3 TFEB and TFE3 physiological and cellular functions (Figure 1.5)

1.3.3.1 Physiological roles for TFEB and TFE3

Early attempts to understand the roles of TFEB and TFE3 in physiology utilised knockout mouse models. TFEB knockout (KO)-mice were embryonic lethal due to deficits in placental vascularisation, whereas TFE3-KO mice were initially observed healthy (Steingrimsson et al., 1994; Steingrimsson et al., 1998; Steingrimsson et al., 2002). Tissue-specific and cell-type-specific knockout of TFEB found multiple critical roles for the transcription factor in bone resorption, lipid metabolism and obesity from KO in the liver; cytokine response in macrophages; mitochondrial, glucose and lipid homeostasis in muscle; angiogenesis in endothelial cells, and epithelial injury (Ferron et al., 2013; Settembre et al., 2013; Mansueto et al., 2017, Murano et al., 2017; Fan et al., 2018).

Although TFE3-KO mice were initially observed healthy, closer study found muscle- and liver-specific roles for TFE3 in glucose and lipid metabolism, aiding mitochondrial function, as well as a function for cytokine responses in macrophages (Pastore et al., 2016; Pastore et al., 2017). Interestingly, both studies demonstrated a cooperative role for TFEB and TFE3 by which double knockout exacerbated perturbation in these pathways.

While TFEB and TFE3 regulate highly similar gene sets, it is clear their function is not completely redundant in all contexts and is often cooperative. Function is likely to be defined by the context and tissue in question.

1.3.3.2 TFEB and TFE3 in lysosomal biogenesis and function

TFEB upregulates a large lysosomal gene set, coined the CLEAR network (Sardiello et al., 2009). These genes are enriched in many lysosome-related genes such as hydrolases, membrane proteins, v-ATPase subunits and autophagic genes (Palmieri et al., 2011). Following, TFE3 was found to bind the same motif occupied by TFEB and upregulates a highly similar set of genes, independently of TFEB activity (Martina et al., 2014). TFEB and TFE3 activation upregulates lysosomal gene expression following stimuli such as nutrient deprivation, thereby enhancing the lysosomal biogenesis and subsequent catabolism (Figure 1.5). Conversely, inhibition/depletion

of TFEB and TFE3 abrogates lysosomal gene expression/induction (Sardiello et al., 2009; Martina et al., 2014). While the activity of TFE3 upon lysosomal biogenesis is independent of TFEB, the transcription factors are partially redundant, but the presence of both is required for maximal activity in this context (Martina et al., 2014). As one may expect, TFEB and TFE3 similarly enhance other lysosomal functions. As TFEB and TFE3 upregulate TRPML1 expression, the elevated lysosomal Ca^{2+} release from TRPML1 promotes trafficking of the lysosome pool toward the periphery and subsequent lysosomal exocytosis (Medina et al., 2011; Martina et al., 2014).

1.3.3.3 TFEB and TFE3 in autophagy

TFEB and TFE3 also regulate the dedicated delivery pathways of cargo to the lysosome. Several important autophagic genes are regulated by TFEB and TFE3 (Palmieri et al., 2011; Martina et al., 2014). Consequently, TFEB and TFE3 activation enhances both bulk autophagy and cargo-selective autophagy such as mitophagy, lipophagy, ER-phagy and perhaps even lysophagy (Settembre et al., 2011; Settembre et al., 2013; Martina et al., 2014; Nezich et al., 2015; Xiong et al., 2016; Cinque et al., 2020; Nakaruma et al., 2021) (Figure 1.4).

1.3.3.4 TFEB and TFE3 in endocytosis

TFEB has also been shown to upregulate genes relating to endocytosis (Palmieri et al., 2011) (Figure 1.4). Further study of this function found TFEB to translocate to the nucleus following inhibition of endocytosis and TFEB activation increased the rate of endocytosis, a process that may aid in mTORC1 re-activation during prolonged autophagy (Nnah et al., 2019).

Thus, enhancement of lysosomal function within cells is concurrent with the elevated delivery and turnover of cellular (and extracellular) constituents, as well as retrieval of cargo from the plasma membrane to facilitate prolonged cellular response during nutrient deprivation or stress, by aiding mTORC1 reactivation.

1.3.3.5 TFEB and TFE3 as metabolic regulators

Recently, TFEB and TFE3 have emerged as metabolic regulators. As mentioned earlier, the transcription factors can upregulate PGC1 α and PPAR α , involved in lipid metabolism and mitochondrial biogenesis

Both TFEB and TFE3 regulate lipid metabolism (Figure 1.5). TFEB activation can ameliorate metabolic syndromes and obesity in mice (Settembre et al., 2013). Further,

TFE3-KO mice display perturbations in fat composition (Pastore et al., 2017). This function upon lipid metabolism seems cooperative between the transcription factors, as overexpression of each can compensate lipid accumulation and weight gain elicited by knockdown of the other, but double knockout exacerbates these issues (Pastore et al., 2017).

TFEB and TFE3 also modulate mitochondrial function beyond a role in mitophagy. TFEB and TFE3 regulate mitochondrial biogenesis and dynamics (Figure 1.5). TFEB or TFE3 KO causes mitochondrial dysfunction and impaired mitochondrial morphology in the liver or skeletal muscle (Mansueto et al., 2017; Pastore et al., 2017). Muscle-specific TFEB activation enhanced the expression of mitochondrial transcription factors, enzymes, fission proteins and complex I, II and III independently of autophagy and PGC1 α (possibly by NRF1/2 induction). A similar autophagy-independent mitochondrial function has been observed in different cell-types such as Treg cells and adipose tissue (Evans et al., 2019; Xia et al., 2022). The function in adipocytes here was dependent upon PGC1 α .

Further, TFEB and TFE3 have a role in glucose homeostasis by increasing expression of genes involved in glucose uptake, glycolytic enzymes and insulin signalling (Figure 1.5). Likewise, TFEB/TFE3 activation increased insulin sensitivity, glycogen storage and glucose metabolism, whereas knockout caused insulin intolerance and dysfunctional glucose uptake and glycogen storage perturbation (Iwasaki et al., 2012; Mansueto et al., 2017; Pastore et al., 2017; Pasquier et al., 2023).

1.3.3.6 TFEB and TFE3 in cellular stress

Both TFEB and TFE3 have displayed function in response to different cellular stresses. Both TFEB and TFE3 are activated during ER stress through a PERK and calcineurin-dependent mechanism independently of mTORC1, upregulating genes relating to the unfolded protein response (Martina et al., 2016). While thought to be cytoprotective short-term, TFEB and TFE3 promote cellular apoptosis following prolonged ER stress. Similarly, TFEB and TFE3 respond to DNA damage through a positive feedback and feedforward loop with p53 (via inhibition of mTORC1), and TFEB/TFE3 facilitate apoptosis following prolonged genotoxic stress (Brady et al., 2018). TFE3 has also been implicated in Golgi-stress response, translocating to the nucleus, binding and enhancing the expression of genes containing the Golgi apparatus stress response element (GASE) (Taniguchi et al., 2015). Interestingly, this

appears to be controlled via phosphorylation of Serine 108 on TFE3, independently of mTORC1. Further, TFE3 is translationally activated by PKM2 in the Golgi-response to aid cell survival during Golgi stress (Taguchi et al., 2023).

Thus, TFEB and TFE3 activate and facilitate the response to a variety of cellular stresses. Although regulation of cell-fate will be stress-dependent, it seems TFEB and TFE3 facilitate cytoprotection under short-term acute stress, but if stress cannot be resolved and is maintained for a prolonged period, TFEB and TFE3 will promote cellular apoptosis. (Figure 1.5).

As proteostasis, metabolism and energy balance, as well as ER stress and genotoxic stress have been associated with neuropathological mechanisms in disease, TFEB and TFE3 make attractive candidates in the treatment for neurodegeneration beyond lysosomal function alone. In this thesis however, we will primarily consider the lysosomal biogenesis function of the transcription factors in neurons.

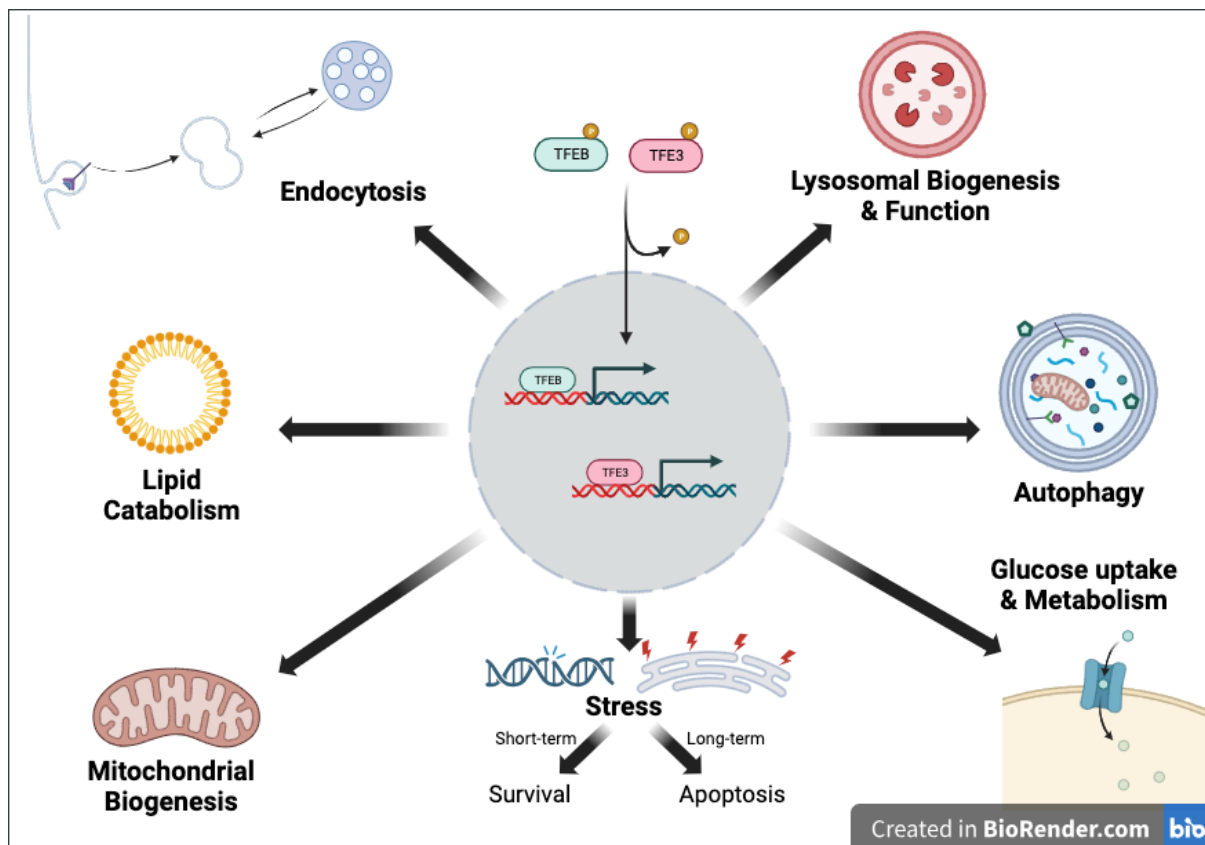


Figure 1.5 – Cellular Functions associated with TFEB/TFE3 activation

1.3.4 TFEB and TFE3 in neurodegeneration

There is a large degree of overlap between TFEB/TFE3 function and pathological mechanisms in PD. Hence, the relationship between neurodegenerative disease and TFEB/TFE3 activation have been studied.

1.3.4.1 TFEB in neurodegeneration

Mislocalisation or altered gene expression of TFEB has been demonstrated in several models of neurodegeneration, such as Huntington's disease, Alzheimer's disease, and Parkinson's disease (Tsunemi et al., 2012; Decressac et al., 2013; Tiribuzi et al., 2014; Wang et al., 2016). Conversely, an upregulation of TFEB activity has also been observed, possibly as a compensatory response to cellular stress (Coffey et al., 2014; Ugolino et al., 2016). The disparities in direction of TFEB activity likely depend upon the cell-type examined, the stress stimulus (i.e., aggregation or oxidative stress) and the progression of disease pathology present at the time of examination. For example, TFEB and downstream gene induction occurs in early disease stages showing compensation, but abrogation of function ensues as pathology develops (Decressac et al., 2013; Chen et al., 2015).

Studies have shown pharmacological or genetic activation of TFEB to ameliorate neurotoxic effects associated with neurodegenerative disease models, displaying the therapeutic potential of activating this transcription factor (Tsunemi et al., 2012; Decressac et al., 2013; Zhang et al., 2015; Li et al., 2016; Palmieri et al., 2017; Song et al., 2020).

1.3.4.2 TFE3 in neurodegeneration

TFE3 has been less extensively studied in neurodegeneration, which is surprising given TFE3 is more highly expressed in the brain than TFEB and was postulated to be associated with neuronal lysosomal function, whereas TFEB was more correlated with glial lysosomal activation (Bordi et al., 2016). Nonetheless, studies have demonstrated a function for TFE3 in neuronal response to stress in spinal cord injury, PD and Alzheimer's disease models (Bordi et al., 2016; Zhou et al., 2020; He et al., 2021).

We will discuss the relationship between TFEB/TFE3 in PD in more depth.

1.3.4.3 TFEB/TFE3 perturbation in PD

TFEB and TFE3 mislocalisation has been observed in SNc DaNs of PD patients, displaying dysregulation of lysosomal biogenesis (Decressac et al., 2013; He et al., 2021). α Syn aggregation is thought to mimic 14-3-3 proteins and inhibit TFEB nuclear localisation. Indeed, LBs co-localised with TFEB in PD brains, and increased α Syn expression in rats increased an aberrant interaction between α Syn and TFEB (Decressac et al., 2013). Similarly, an *SNCA-A53T* overexpressing rodent model displayed TFEB cytoplasmic retention by increased CRM1-mediated export and hyperactive mTORC1 signalling (facilitated by PARP1), inhibiting autophagic-lysosomal function (Mao et al., 2020). TFEB mislocalisation associated with mTORC1 hyperactivation has been observed in several PD models including *GBA-N370S* iPSC-DaNs, ATP13A2 deficient HeLa cells, 6-OHDA treated SH-SY5Y cells and iron-treated SN4741 cells, all leading to autophagic-lysosomal deficits (Bento et al., 2016; He et al., 2018; Xiao et al., 2018; Mubariz et al., 2023). mTORC1 hyperactivity has been noted in post-mortem PD brains, demonstrating multiple potential mechanisms for TFEB dysregulation (Wills et al., 2012). Interestingly, TFEB expression can be decreased to impair autophagic-lysosomal function in PD (Bento et al., 2016; He et al., 2018; Mubariz et al., 2023).

Contrary findings have been found in which increased TFEB expression or nuclear localisation occur in oxidative stress models of PD (6-OHDA or H_2O_2) and even *GBA-N370S* neurospheres (Ma et al., 2016; Morén et al., 2019; Zhuang et al., 2020). This increased TFEB activity enhances autophagic-lysosomal function and was shown to be compensatory, attenuating cytotoxicity in these models (Ma et al., 2016; Zhuang et al., 2020). It is suggested an increase in autophagic-lysosomal function precedes dysfunction to compensate for lysosomal dysfunction in early pathology. Indeed, in a *SNCA-OVX* rat model of PD, an initial increase in autophagic-lysosomal pathway (ALP) gene expression decreases with age and disease progression, demonstrating a bi-phasic axis in autophagic changes throughout PD (Decressac et al., 2013). Interestingly, TFEB transcription is also increased in the cortex and caudate nucleus of post-mortem Lewy body disease patients (Perez-Roca et al., 2019). This increase anti-correlates with *GBA* expression however, suggesting compensatory upregulation may be unable to combat pathology at certain stages of disease development.

Little is known regarding aberrant TFE3 activity in PD with the exception of mislocalisation in post-mortem SNc DaNs (He et al., 2021). Interestingly, TFE3 knockdown triggered dopaminergic neurodegeneration concurrent with a moderate decrease in autophagic-lysosomal gene expression, suggesting TFE3 has a critical role in DaN homeostasis (He et al., 2021). This is somewhat similar to TFEB, where miRNA-mediated knockdown of TFEB correlated with an exacerbation of α Syn aggregation, neurodegeneration and motor impairment in a SNCA-OVX rat model of PD (Decressac et al., 2013).

1.3.4.4 Targeting TFEB/TFE3 activation to ameliorate lysosomal dysfunction in PD

1.3.4.4.1 TFEB overexpression to combat PD

TFEB activation to ameliorate PD has predominantly been demonstrated through exogenous TFEB overexpression. TFEB overexpression increased lysosomal biogenesis, autophagosome clearance and rescued cellular death associated with an MPTP-induced cellular model of PD (Dehay et al., 2010). Since, TFEB overexpression has attenuated cellular toxicity following 6-OHDA treatment or H₂O₂ treatment *in vitro*, and TH-cell loss in *in vivo* models of PD while ameliorating motor impairments (Decressac et al., 2013; Ma et al., 2016; He et al., 2018; Arotcantera et al., 2019; Zhuang et al., 2020). TFEB overexpression frequently reduces α Syn accumulation both *in vitro* and *in vivo* (Decressac et al., 2013; Kilpatrick et al., 2015; Bento et al., 2016; Arotcantera et al., 2019). Neuroprotective benefits are generally associated with increased autophagic-lysosomal function following upregulated CLEAR gene expression. However, TFEB also conferred neuroprotection *in vivo* through neurotrophic activation of protein synthesis, via activation of MAPK1/3, AKT and mTORC1 pro-survival pathways and inhibition of apoptosis (Torra et al., 2018). Thus, TFEB activation by overexpression promotes neuroprotection and inhibits PD-associated pathology in a multi-modal manner.

1.3.4.4.2 Pharmacological TFEB activators in PD

TFEB pharmacological activators have demonstrated neuroprotective benefits. mTOR inhibitors (such as rapamycin, rapalogues (CCI-779) and Torin1) stimulate TFEB nuclear localisation and are frequently used in PD models to ameliorate pathology concomitant with autophagic-lysosomal enhancement (Decressac et al., 2013;

Siddiqui et al., 2015; Mubariz et al., 2023). Similarly, GSK3 β inhibitors (CHIR-99021 or STI-571) induce TFEB nuclear localisation and ameliorate autophagic perturbation in MPP $^{+}$ -treated *in vitro* models of PD (Ren et al., 2018).

Other interesting compounds are derivatives of Curcumin. Curcumin derivatives E4 and C1 promote TFEB translocation to stimulate autophagic-lysosomal function by AKT1-mTORC1 inhibition or direct interaction with TFEB, attenuating cellular toxicity in chemically induced models of PD (MPTP and 6-OHDA, respectively) (Song et al., 2016; Wang et al., 2020; Zhuang et al., 2020). Fascinatingly, Ambroxol, a GCase chaperone in phase II clinical trials (phase 3a also planned – Menozzi et al., 2023), has been shown to activate TFEB *in vitro*. Here, ambroxol increased TFEB expression and nuclear localisation, concomitant with enhanced CLEAR expression, lysosomal function and reduced α Syn accumulation (McNeill et al., 2014; Magalhaes et al., 2018). Conversely, ambroxol did not increase TFEB expression *in vivo*, although nuclear localisation was not assessed (Migdalska-Richards et al., 2016).

Several other compounds have demonstrated cytoprotection in models of PD, associated with TFEB nuclear translocation, such as 2-Hydroxypropyl- β -cyclodextrin, trehalose and veliparib (Table 1.2). It is noteworthy that pharmacological induction of TFEB (and TFE3) translocation can be stimulated through several cellular stressors. Thus, many compounds that activate TFEB translocation to the nucleus may act through the initiation of a cellular stress response. For example, curcumin induces ER stress in certain conditions and Trehalose damages lysosomes, inducing TFEB through calcineurin activation (Zhang et al., 2018; Rusmini et al., 2018). For TFEB activation to be therapeutically viable, induction of stress responses must be monitored so as to not exacerbate other pathogenic mechanisms in neurons. As such, we recently defined a screening strategy to identify compounds activating TFEB nuclear localisation, that were counter-screened for lysosomal inhibition (Carling et al., 2023). This principle should be considered in the future when examining TFEB/TFE3 activation.

Table 1.4 - Pharmacological TFEB activators in PD models (adapted from Jiao et al., 2023)

Compound	TFEB Activation	Model(s)	Effects
Curcumin	Nuclear TFEB by GSK3B inhibition	SHSY5Y expressing SNCA-A53T	Degradation of alpha synuclein by ALP
Curcumin C1	Bind TFEB N-terminus promoting nuclear localisation	MPTP mouse 6-OHDA/AA cells/mice iPSC-DaNs (no TFEB assessment)	Reduced cell death and by increasing ALP
Curcumin E4	Nuclear TFEB; TFEB-S142 dephosphorylation by AKT-mTORC1 inhibition	MPP-treated PC12 cells	Promote aSyn degradation and reduce cell death
Trehalose	Nuclear TFEB (AKT inhibition, lysosomal permeabilisation, PPP3CB dephosphorylation)	MPP-treated M17 cells	Reduce cell death increasing ALP
mTOR inhibitors (Torin1, rapamycin and rapalogues)	TFEB dephosphorylation by mTORC1 inhibition	AAV-SNCA-OVX rats Parkin Q311X mice GBA-N370S iPSC-DaNs	Reduce aSyn aggregation Rescue ALP/mitochondrial dysfunction Reduce DaN loss Rescue motor impairment
2-hydroxypropyl- β -cyclodextrin	Increase TFEB expression Induce nuclear TFEB	H4 cells overexpressing aSyn	Increased ALP, increased α Syn aggregate clearance
STI-571	Nuclear TFEB, by inhibiting c-Abl phosphorylation of GSK3B	MPP-treated SN4741 cells and primary midbrain neurons	Rescue ALP deficits and attenuate cell death
Veliparib	Nuclear TFEB by PARP1 inhibition = reduced SIRT1-mediated mTORC1 phosphorylation of TFEB and reduced CRM1-mediated nuclearexport	SNCA-A53T overexpressing SN4741 cells and SNCA-A53T transgenic mice	Reduced α Syn aggregation Ameliorated DaN loss Rescued motor deficits
SB203580	Nuclear TFEB by inhibiting p38-mediated TFEB phosphorylation	SNCA-A53T transgenic mice	Reduced DaN loss Rescued motor deficits Reduced aSyn accumulation Restored synaptic vesicle loss
Celecoxib	Increased TFEB expression	6-OHDA or paraquat treated SH-SY5Y	Rescued cell death

Acylated ghrelin	Increased TFEB expression and nuclear localisation	6-OHDA-treated SH-SY5Y cells and rat model	Reduced cell death/apoptosis Improved motor deficits Reduced TH-loss Rescue ALP deficits
Ambroxol (McNeill et al., 2014; Maghalaes et al., 2018)	Increased TFEB expression TFEB nuclear localization	GBA-PD derived fibroblasts SNCA-OVX SH-SY5Y GBA-HET-KO primary mouse cortical neurons	Improved lysosomal function Reduced aSyn levels ALP and mitochondrial activation Reduced aSyn aggregation
Ambroxol (Midgalska-Richards et al., 2016)	No change in TFEB expression	GBA-L444P transgenic mice hSNCA-ovx transgenic mice	Increased Gcase expression and activity Reduced aSyn aggregation

1.3.4.4.3 TFE3 activation in PD

Evidence for the therapeutic potential of TFE3 in PD is lacking. Recently however, TFE3 overexpression was shown to ameliorate neurodegeneration in an MPTP mouse model of PD, concurrent with increasing autophagic-lysosomal capacity (He et al., 2021). As TFEB and TFE3 seem similarly regulated, one may hypothesise that autophagic-lysosomal enhancement following ‘TFEB activating’ compound treatment, may be conferred by TFE3 activation. Indeed, most publications do not address TFE3 localisation in relation to pharmacological compound treatment. Thus, efforts must be made to affirm the neuroprotective effects of TFE3 activation in PD, seen by He and colleagues (2021).

Due to their wide range of functions relating to PD, in particular lysosomal biogenesis, TFEB and TFE3 are enticing therapeutic targets. Most studies examining their therapeutic potential have been in relation to TFEB, not TFE3. Further, these studies have predominantly assessed the neuroprotective effects of TFEB activation in non-human or non-disease-relevant models. Very few studies have directly assessed TFEB function in human and disease-relevant models (such as iPSC-DaNs) (Awad et al., 2015; Mubariz et al., 2023). Thus, stringent examination of endogenous TFEB and TFE3 biology in iPSC-DaNs, as well as their ability to ameliorate lysosomal dysfunction relating to PD in this model need to be assessed. Findings will provide better insight into the feasibility of therapeutic strategies combatting PD by activating TFEB/TFE3 and lysosomal biogenesis.

1.4 Modelling lysosomal dysfunction in Parkinson's disease using iPSCs

1.4.1 Somatic reprogramming to iPSCs

A major hurdle in the study of PD and other neurodegenerative diseases previously has been the absence of tractable human models that accurately replicate cell-types perturbed within the brains of patients due to the inaccessibility of this tissue. Further, the complex genetic nature of PD through incomplete penetrance of genetic permutation and many unknown disease-modifying variants, means genetically modified models of PD lack the complete genetic background that facilitate disease progression.

The finding that human somatic cells could be reprogrammed into induced pluripotent stem cells (iPSCs) provided a resolution for these issues. In 2006, Takahashi and Yamanaka discovered that retrovirally-induced expression of Oct3/4, Sox2, c-Myc and Klf4 (Yamanaka factors) in mouse fibroblasts facilitated reprogramming into pluripotent stem cells capable of differentiating into all three germ layers (Takahashi et al., 2006). This was then confirmed in human adult fibroblasts (Takahashi et al., 2007). Since, non-integrative methods of reprogramming have been used to reprogramme somatic cells into iPSCs such as Sendai virus, adenovirus, episomal plasmids, piggyBac transposons, proteins and small molecules (Claudia Caiazza et al., 2020). The most common approach for reprogramming involves Sendai virus or adenoviral delivery of reprogramming factors.

Reprogramming somatic cells into iPSCs allowed previously inaccessible human disease-affected cell-types in neurodegeneration to be modelled *in vitro*, and derived directly from patients, collected by non-invasive means such as skin biopsies.

1.4.2 iPSC-derived differentiation of dopaminergic neurons via midbrain floorplate progenitors (mFPPs)

As DaNs of the SNc are preferentially susceptible to degeneration in PD, efforts have attempted to develop efficient dopaminergic neuronal cultures expressing markers for midbrain dopaminergic neuronal lineage. As such several protocols have been developed, for example viral-mediated expression of specific transcription factors such as ASCL1, NURR1 and LMX1A (Theka et al., 2013; Mahajani et al., 2019), derivation

of DaNs from embryoid body-derived neural rosettes (Hartfield et al., 2014) or through small molecule differentiation via dual-SMAD inhibition and subsequent floor plate progenitor induction (Chambers et al., 2009; Kriks et al., 2011).

The protocol by Kriks and colleagues provided a highly efficient differentiation protocol (approximately 75% TH⁺ neurons) and derives neural precursors from the midbrain floor plate (thought to generate SNc DaNs) (2011). To summarise the Kriks protocol, mesodermal and endodermal fate is inhibited through dual-SMAD inhibition to induce anterior neural fate. Following, the neural precursors are patterned toward a midbrain floor plate fate by activation of sonic hedgehog signalling and FGF8/Wnt signalling, deriving mFPPs co-expressing the floor plate marker, *FOXA2*, and the roof-plate marker, *LMX1A*. Trophic support and factors promoting neuronal survival and growth are subsequently used to mature DaNs. By DIV50, iPSC-DaNs have been shown to be functional in culture and could be engrafted into a 6-OHDA mouse model to rescue motor impairments (Kriks et al., 2011). Further, these neurons highly resemble post-mortem DaNs, transcriptomically (Sandor et al., 2017). A recent study modified this protocol through expanding mFPPs at DIV10 to increase cellular material, allow for mFPP cryobanking and increase the efficiency of differentiation while maintaining functionality and successful engraftment into a mouse model (Fedele et al., 2017). Due to the efficient differentiation of iPSCs to DaNs using these mFPP-derived differentiation protocols and the expansion of cellular material available, the expanded Kriks protocol developed by Fedele and colleagues was undertaken in this thesis.

1.5 CRISPR interference (CRISPRi)

Clustered, regularly interspersed, short palindromic repeats (CRISPR) in combination with CRISPR-associated (Cas) proteins (CRISPR-Cas) forms a ribonuclease complex able to induce double-stranded DNA breaks in the host genome by bacteria (Jinek et al., 2012). Type II CRISPR systems have been particularly of interest as a genetic tool because the complex only requires one Cas protein (Cas9), as opposed to Type I CRISPR systems that require multiple Cas proteins (Makarova et al., 2011). With direction by a single-guide RNA (sgRNA), Cas9 can be directed to loci within the genome with high specificity and selectively induce double-stranded DNA breaks (Jinek et al., 2012; Cho et al., 2013). CRISPRn (CRISPR/Cas9 with active nuclease) was initially harnessed for genome editing. CRISPRn has proven a useful tool for

genetic engineering, however nuclease activity provides key pitfalls through off-target mutagenesis and negative selection pressures when mutating an essential gene (Qi et al., 2013; Aguirre et al., 2016).

CRISPRi is a derivative genetic tool of CRISPRn, in which a nuclease-dead Cas9 (dCas9) protein is used to dock the complex onto the genome, hindering expression of a specific gene, without damaging the DNA. Initially, CRISPRi relied upon sterically blocking transcription but knockdown was inefficient (Qi et al., 2013). Developments in CRISPRi machinery saw the addition of transcriptional repressor, KRAB (Krüppel associated box) to the complex (Figure 1.6), as well as other modifications in attempts to improve efficiency of the system (Gilbert et al., 2013; Gilbert et al., 2014; Carleton et al., 2017; Sanson et al., 2018; Alerasool et al., 2020).

CRISPR-interference

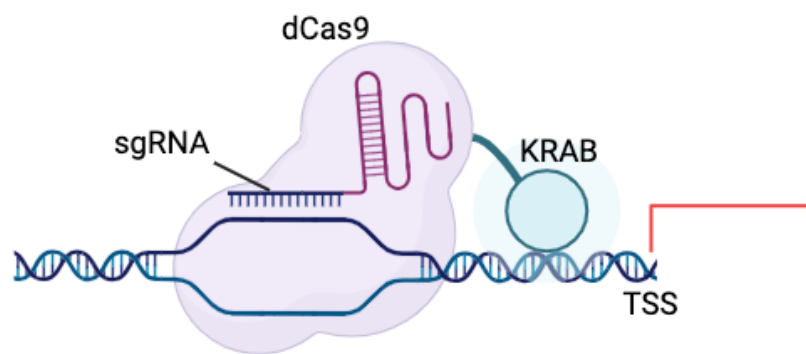


Figure 1.6 – CRISPR-interference gene knockdown by dCas9-KRAB

CRISPRi can provide highly specific and robust knockdown of target genes, for a prolonged period (through stable integration of CRISPRi machinery and vectors expressing sgRNAs), that is reproducible and effective (Larson et al., 2013; Gilbert et al., 2013; Gilbert et al., 2014). As such, CRISPRi has now been used for a multitude of different applications with success. CRISPRi is an enticing approach to screen for genetic targets relating to specific biological function and has been shown to identify hits at a similar level to that of CRISPRn screening. CRISPRi knockdown is less prone to compensation than CRISPRn, rendering the approach more suited to functional assessment of genetic perturbation (Rossi et al., 2015; Sanson et al., 2018). Genome-wide CRISPRi screening has been successfully carried out in different cell-types including iPSC-derived neurons (reviewed by Kampmann, 2018; Tian et al., 2019; Tian et al., 2021). Interestingly, a pooled genome-wide screening approach was used to

identify modulators of TFEB localisation during starvation (Kanfer et al., 2021). Beyond genetic screening, CRISPRi can also be used for disease modelling (particularly useful when assessing the effects of incomplete loss of function or correcting hyperactivity), genetic interaction mapping, as well as target validation following screening (Heman-Ackah et al., 2016). Indeed, we recently used CRISPRi in an attempt to validate hit targets identified from our TFEB/TFE3 re-localisation screen (Carling et al., 2023). Here, we demonstrated hit compounds effects upon TFEB to be independent of PRKD2 or PRKD3 knockdown.

Pitfalls for CRISPRi include stable integration of sgRNA-encoding viral vectors modifying cell genomic landscapes, not accounting for non-linear relationship between gene expression and protein function, as well as genetic knockdown in iPSC-derived models affecting differentiation. Nonetheless, CRISPRi is an incredibly useful tool to examine the modulation of expression upon function in cell-types of interest. The development of an iPSC-Ngn2 line with CRISPRi machinery stably integrated into the CLYBL safe harbour locus (i³ neurons), provides a platform to genetically examine TFEB/TFE3 regulation in a neuronal context, a tool that we will use to validate uncovered biology in this thesis (Tian et al., 2019).

1.6 Aims of this thesis

In this thesis, we will:

- Characterise endogenous TFEB and TFE3 biology in iPSC-DaNs (Chapter 3)
- Screen for transcriptional regulatory mechanisms of TFEB/TFE3 activation in iPSC-derived neurons in collaboration with Biogen (Chapter 4)
- Use CRISPRi to genetically modulate hit regulators identified from our TFEB/TFE3 activation screening in neurons (Chapter 4)
- Examine functional lysosomal effects following lentiviral overexpression, or pharmacological stimulation of TFE3 and TFEB in iPSC-DaNs (Chapter 5)
- Assess lysosomal dysfunction in patient-derived iPSC-DaNs carrying the *GBA-N370S*, *LRRK2-R1441C* or *SNCA-Trp* mutations (Chapter 6)
- Examine how activation of TFEB or TFE3 may correct lysosomal phenotypes identified in patient-derived iPSC-DaNs. (Chapter 6)

Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Thawing iPSCs

Day -1: Wells were coated with Matrigel hESC-qualified matrix for 1 hour-overnight, 37°C.

Day 0: iPSCs were removed from liquid nitrogen and placed on dry ice. Thawing media (mTESR1 medium, mTESR1 supplement, Penicillin/Streptomycin (mTESR⁺⁺), supplemented with 10µM Rho kinase inhibitor (ROCKi) was prepared, filtered through a 0.22µm filter and warmed. Spinning falcons (containing KO-DMEM) were prepared and warmed. Cryovials were thawed in a 37°C water bath. Cryovials were removed from the water bath before complete thaw (a small amount of ice remains), dried and sterilised. Cells were transferred to spinning falcons. Emptied cryovials were rinsed with KO-DMEM to collect residual cells. iPSCs were centrifuged at 350 rcf, 5 mins, room temperature. Meanwhile, Matrigel-coating was removed from prepared wells and replaced with half-volume thawing media. Following centrifugation, supernatant was aspirated, and cells resuspended in thawing media. iPSCs were then seeded into pre-coated wells and incubated overnight at 37°C, 5% CO₂

Day 1+: iPSCs were visualised on an EVOS XL Core Imaging System for correct morphology and a full media change was carried out daily with mTESR⁺⁺ until cells reached 80-90% confluence, before passaging.

2.1.2 Passaging iPSCs

iPSCs were passaged two ways, single-cell passage or colony passaging. Single-cell passaging was carried out for short-term iPSC culture. Colony passaging was carried out when expanding iPSC stocks and is less detrimental to iPSCs than single-cell.

Day -1 (all methods): Culture wells were coated with Matrigel.

2.1.2.1 *Single Cell passaging (Enzymatic)*

Day 0: Room temperature mTESR⁺⁺ with 10 μ M ROCKi and spinning falcons were prepared. Accutase/TrypLE was warmed to room temperature. Medium was aspirated from iPSCs, and cells were washed with PBS. PBS was removed and cells were incubated with TrypLE or Accutase for 5 minutes, 37°C. Cells were washed off and centrifuged at 350 rcf, 5 minutes. Meanwhile, Matrigel was removed from culture wells and replaced with half-volume mTESR⁺⁺ + Rocki. Following centrifugation, cells were resuspended with mTESR⁺⁺ + ROCKi before seeding into Matrigel-coated wells. Cells were split 1:3, but this varied dependent upon cell line proliferation and response to passaging. Cells were incubated at 37°C, 5%CO₂.

Day 1+: iPSCs were observed for correct morphology. Daily full media changes with mTESR⁺⁺ were carried out until 80-90% confluency.

2.1.2.2 *Colony passaging (non-enzymatic)*

Day 0: mTESR⁺⁺ + Rocki was prepared and replaced Matrigel in the coated-wells. Medium was aspirated from the cell culture, cells were washed with PBS, then 0.5 mM EDTA (room temperature) was added atop the cells and incubated for 2 minutes, 37°C. Cells were observed every minute until gaps began forming and a halo encircled the cells. Here, EDTA was aspirated, cells were washed off with mTESR⁺⁺ + ROCKi and seeded into the freshly coated wells. Generally, a 1:6 split was carried out, but this depended on cell line. Cells were then incubated overnight 37°C, 5% CO₂.

Day 1: iPSC colonies were visualised, and a full media change was carried out daily until 80-90% confluency. The cells were then passaged or cryobanked.

2.1.3 Cryobanking iPSCs

Day 0: Room temperature Freezing medium (90% FBS; 10% DMSO), spinning falcons and Accutase/TrypLE were prepared. Medium was aspirated, cells were washed with PBS before incubation with Accutase for 4 minutes, 37°C. Following, cells were washed off and resuspended in spinning falcons. Here, 20 μ L cell suspension was taken and added to 1.5 μ L Solution 18 live/dead stain. Spinning falcons were spun at 350 rcf, 5 minutes. During centrifugation, cells were counted on a Chemometec NucleoCounter NC-250. Following centrifugation, KO-DMEM was aspirated, cells were resuspended in Freezing medium at 4×10^6 cells/mL and aliquoted into cryovials

(1 mL/vial). Cryovials were placed in a CoolCell Freezing container (Corning, 15552771) and stored -80°C. The CoolCell freezes the cell suspension slowly at 1°C/minute. Frozen cryovials were subsequently moved to liquid nitrogen for long-term storage.

2.1.4 Differentiation of iPSCs into dopaminergic neurons through the generation of midbrain floor plate progenitors (mFPPs)

iPSCs (Information in Appendix Table S.3) were differentiated into DaNs using a midbrain floor plate progenitor expansion protocol (Fedele et al., 2017), with minor modifications. Full description and mFPP cryopreservation is described in Appendix S.1.1-S1.2.

2.1.5 i³ Neuron differentiation

Differentiation of G3dCas9 (i³ neurons) iPSCs (from Tian et al., 2019) was carried out as per Fernandopulle et al., 2018. Full description and materials are in Appendix S.1.3.

2.1.6 Materials

iPSC cultivation and differentiation materials are displayed in Appendix S.1.4.

2.2 qRT-PCR Screening (Biogen)

2.2.1 High-throughput iPSC-Ngn2 culture

Cryostored DIV3 iPSC-Ngn2 stocks were prepared by a dedicated iPSC team.

Day 0: Plating media (Table 2.1) and spinning falcons were prepared. iPSC-Ngn2 neurons were thawed at 37°C, added to spinning falcons and centrifuged at 350 r.c.f, 5 minutes. Pelleted cells were resuspended in Plating medium. 30 µL cell suspension was added to 30 µL 0.4% Trypan Blue and counted on a Nexelcom Cell Viability Counter (Nexcelcom Vision). Cells were diluted to 4x10⁵ cells/mL and seeded at 1.84x10⁵ cells/cm² into PDL BioCoat 384-well cell culture plates (Corning, 354663), using a Multidrop Combi, mounted with a Multidrop Combi accessory standard dispensing cassette (ThermoFisher Scientific, 24072670). 50 µL cell suspension was added to each well and incubated overnight at 37°C, 5% CO₂.

Day 1: 50 µL warmed, filtered Plating media was added to 384-well plates using an Integra Viaflo-384 (Integra Biosciences). Final volume: 100 µL.

Day 6 + 13: A 50% media change with Maturation media was carried out (Table 2.1).

Day 14: 50 μ L media was aspirated and treated with compound (Compound layout – Appendix S.2) for 48 hours. Compound target classes for the screening deck are displayed in Appendix S.3; Table S.8.

Table 2.1 - iPSC-Ngn2 Differentiation Media Constituents

Media (Constituents)	Concentration
Plating media	
DMEM/F12	50%
Neurobasal Medium	50%
GlutaMAX-I	0.5X
N2 supplement	1X
B27 supplement w/o Vit A	1X
Ascorbic Acid	200 μ M
Dibutyryl cAMP	1 μ M
BDNF	10 ng/mL
GDNF	10 ng/mL
Doxycycline hyclate	1 μ g/mL
CultureOne	1X
Penicillin-Streptomycin	1X
Maturation Media	
DMEM/F12	50%
Neurobasal Medium	50%
GlutaMAX-I	0.5X
N2 Supplement	1X
B27 Supplement w/o Vit A	1X
Ascorbic Acid	200 μ M
Dibutyryl cAMP	1 μ M
BDNF	20 ng/mL
GDNF	20 ng/mL
Penicillin-Streptomycin	1X

2.2.2 High-throughput qRT-PCR Screening

Sterile conditions were maintained throughout and RNaseZap (Invitrogen, AM9780) was used to prevent RNase contamination.

2.2.2.1 *Cell Lysis*

RNAasin was added to CL Lysis buffer at 1:100 dilution (Table 2.2). Media was removed from the plate by overturning onto absorbent tissue. 20 μ L Lysis Buffer(+ RNAasin) was added to cells. Assay plates were pulse-centrifuged briefly and shaken at room temperature, 5 mins, 1000 rpm on a plate shaker. Following, plates were briefly pulse-centrifuged, sealed and frozen at -80°C until further use.

Table 2.2 - CL Cell Lysis Buffer Constituents – Externally produced by Boston Bioproducts

Constituent	Concentration
-------------	---------------

Tris Base	10 mM
Tris-HCl	10 mM
NaCl	150 mM
Triton X-100	0.2% (w/v)
RNAasin Rase inhibitor	1:100

2.2.2.2 *1-step TaqMan qRT-PCR*

Lysis plates were thawed at room temperature. Meanwhile, a duplex 1-step TaqMan master mix was made (Table 2.3). 8 μ L of 1-step TaqMan Master mix was dispensed into a MicroAmp Optical 384-well Reaction plate (ThermoFisher Scientific, 4309849) using a Combi Microdrop mounted with Multidrop Combi accessory small size dispensing cassette (ThermoFisher Scientific, 24073295). 2 μ L lysate was added to the qRT-PCR mix in the corresponding reaction plate well, using an Integra Viaflo-384 system.

The lysis plate was re-sealed and stored at -80°C. The qRT-PCR plate was sealed using MicroAmp Optical Adhesive Film, briefly pulse-centrifuged before undergoing reverse transcription + inactivation in a thermal cycler (50°C – 5 mins, 95°C – 20 seconds, 24°C – Hold). All qRT-PCR plates were sequentially run using an automated robotics system, delivering the plates to QuantStudio 7 Flex Real-Time PCR system. Table 2.4 displays the reaction parameters.

Table 2.3 - 1-step qRT-PCR Reaction Constituents for 1 well

Constituent	Volume (per well)
TaqMan Fast Virus 1-step Master Mix	2.5 µL
TFEB or TFE3 FAM-MGB 60X probe	0.167 µL
HPRT1-VIC-PL 20X probe	0.5 µL
UltraPure DNase/RNase free H ₂ O	4.833 µL
Lysate	2 µL

Table 2.4 - 1-step qRT-PCR PCR reaction

Stage		Reps	Temp	Time
1	Enzyme Activation	1	95°C	20 seconds
2	PCR (cycle)	40	95°C	3 seconds
			60°C	30 seconds

2.2.2.3 Analysis

qRT-PCR data was analysed using QuantStudio software. Ct values and compound layout maps were uploaded to GeneData Screener (GDS) and *TFEB* Fold induction ($2^{-\Delta\Delta C_t}$), normalised to housekeeping gene *HPRT1*, was measured relative to DMSO. Z' values of positive compound ACY-738, relative to DMSO, were calculated for each plate. Z' > 0.3 was required for a plate to be used. Robust Z-score normalisation *within* individual plates was used to correct for plate variability.

For single concentration compound screening, compounds were screened at 10 µM in duplicate over 2 separate plates. Hits required both wells to have Z-score > 3. DMSO correction plates were run in-tandem and GDS corrected spatial bias within compound plates.

For dose-response analysis, duplicate 10-point 3-fold dilution dose-responses from 10 µM were carried out.

Fold-induction (or Z-score values) of *TFEB/TFE3* for each compound was calculated and mapped. Non-linear curve fitting was used to predict pharmacodynamic values of each compound.

2.2.3 Materials

Table 2.5 - Reagents Used for Biogen qRT-PCR Screening

Product Name	Supplier	Cat. No.
Trypan Blue (0.4%)	Invitrogen	T10282
KO-DMEM	Life Technologies	15140122
DMEM/F12	Life Technologies	11320033
Neurobasal Medium	Life Technologies	21103-049
GlutaMAX-I	Life Technologies	35050-061
N2 Supplement	Life Technologies	17502-048
B27 Supplement w/o Vit A	Life Technologies	12587010
Ascorbic Acid	Sigma	11140-050
Dibutyryl cAMP	Sigma	D0260
BDNF	Tocris	2837/10U
GDNF	R & D Systems	212-GD-050
Doxycycline hyclate	Sigma	D9891
CultureOne	Thermo Fisher Scientific	A3320201
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
RNAasin RNase inhibitor	Promega	N2515
CL Lysis Buffer	Boston Bioproducts	C-11026B
TaqMan Fast Virus 1-Step Master Mix	Thermo Fisher Scientific	4444436
<i>TFEB</i> -FAM-MGB (60X) – Hs01065086_m1	Thermo Fisher Scientific	4351368
<i>TFE3</i> -FAM-MGB (60X) – Hs00232406_m1	Thermo Fisher Scientific	4351368
<i>HPRT1</i> -VIC-MGB-PL (20X)	Thermo Fisher Scientific	4326321E
UltraPure RNase and DNase free H ₂ O	Invitrogen	10977015

2.3 Bacterial culture, transformation and plasmid purification

2.3.1 Transformation of ultracompetent *Stbl3 Escherichia coli*

LB agar plates containing antibiotic selection (Section 2.3.2) were prepared and warmed to 37°C.

Mix & Go *Stbl3 E. coli* (Production in Appendix S.4) were thawed on ice. Plasmid DNA (0.2-2 µg) was added to the competent bacteria, mixed and incubated for 5 mins on ice. Transformed bacteria were spread on the LB agar plate and incubated overnight, 37°C.

2.3.2 Bacterial culture

LB Agar (37 g LB agar powder in 1 L MilliQ water) was made and autoclaved. LB agar was melted in a microwave and poured into 10cm petri dishes. Carbenicillin (100 µg/mL) was added to cooled (but still liquid) agar to select for bacteria transformed with the plasmid of interest containing the *LacZ* antibiotic resistance gene.

Lysogeny broth (LB) medium was produced (25 g LB Broth powder in 1 L MilliQ water) and autoclaved. Ampicillin (100 µg/mL) was added to LB broth for selection of bacteria transformed with the plasmid of interest containing the *LacZ* antibiotic resistance gene (LB Selection media).

Day 1: Bacteria were streaked onto the LB agar plate with antibiotic and incubated at 37°C overnight.

Day 2: Bacterial colonies were selected using a pipette tip, transferred to a starter culture (5.5 mL LB Selection media) and incubated for 8 hours, 37°C, 250 rpm.

This culture was then used for miniprep (Section 2.4.3). If a larger culture was required (Midiprep), 55 µL starter culture was spiked into 55 mL LB Selection media in a 500 mL culture flask and incubated overnight, 37°C, 250 rpm.

Day 3: Bacterial culture was centrifuged at 3,500 r.c.f, 10 mins, 4°C and Midiprep plasmid purification was carried out (Section 2.4.3).

2.3.3 Materials

Table 2.6 - Reagents used for Bacterial work

Product Name	Supplier	Cat. No.
Stbl3 E. coli	<i>Invitrogen</i>	C737303
Ampicillin	<i>Sigma</i>	A0166-5G
Carbenicillin	<i>Gibco</i>	10177012
LB broth powder	<i>Invitrogen</i>	12780052
LB agar powder	<i>Invitrogen</i>	227000025

2.4 DNA Manipulation (Plasmid production)

2.4.1 TFEB-iRFP670 and TFE3-iRFP670 lentiviral construct production

2.4.1.1 *gBlock design*

A DNA sequence was designed using SnapGene where TFEB or TFE3 was fused to iRFP670 with a linker peptide. A Kozak sequence was included at the start of the TFEB or TFE3 sequence. Homology arms complementary to the donor EF1 α backbone were added either side of the insert.

(Upstream Homology Arm: TTCCATTTCAGGTGTCGTGAGCTAG; Downstream Homology Arm: GAATTCGTCGAGGGACCTAATAACT).

The design was assessed *in silico* for compatibility with HiFi assembly by simulating linearisation and fragment insertion using the SnapGene function 'NEBuilder HiFi DNA Assembly'.

To aid gBlock synthesis the sequence was adjusted using the Codon Optimisation Tool on IDT (<https://eu.idtdna.com/CodonOpt>). Note, the homology arms and Kozak were not codon-optimised as the DNA sequence is essential for the function of these features.

Following codon optimisation, the gBlock was ordered from IDT.

2.4.1.2 *mApple excision and linearisation of EF1 α -mApple backbone using restriction digestion*

5 μ g EF1 α -mApple construct was restriction digested using EcoRI-HF and NheI-HF in a thermal cycler (Table 2.7 and 2.8).

Table 2.7 - EF1a-mApple Restriction Digest Reagents

Reagent	Amount (10 μ L reaction)
Linearised EF1a-mApple vector	5 μ g
CutSmart Buffer (10X)	1 μ L
EcoRI-HF	1 μ L
NheI-HF	1 μ L
MilliQ H ₂ O	To 10 μ L

Table 2.8 - EF1a-mApple Restriction Digest PCR conditions

Stage		Temp	Time
1	<i>Restriction Digestion</i>	37°C	30 mins
2	<i>Enzyme Inactivation</i>	80°C	20 mins
3	<i>Hold</i>	4°C	Indefinite

2.4.1.3 Purification of linearised vector backbone by agarose gel electrophoresis

Linearised backbones were purified using agarose gel electrophoresis.

A 0.8% agarose gel (0.8 g agarose in 100 mL TBE buffer) was produced and intermittently heated in a microwave until solubilised. SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, S33102) was added to the agarose gel before it was cast. The gel was immersed in TBE in a running chamber. Loading buffer was added to the digestion reaction and loaded into the gel beside a 1 kB DNA ladder. The gel was then run at 120 V for 45 minutes, or until the loading dye ran the length of the gel.

Following, the gel was exposed to UV with a transilluminator to identify DNA fragments. The appropriate molecular weight fragment corresponding to the linearised backbone (7.115 kB) was excised and purified using a Machery-Nagel Gel and PCR Clean-up kit, according to the manufacturer's instructions.

2.4.1.4 HiFi Assembly for the ligation of TFEB-iRFP670 and TFE3-iRFP670 gBlocks into the linearised EF1 α - backbone

The gBlocks (Section 2.4.1.1) were ligated to the linearised EF1 α backbone (Section 2.4.1.2) using HiFi Assembly from NEB. A 2:1 ratio of insert:vector was used with 200 ng of linearised vector. The mass of the gBlock required was calculated using NEBioCalculator (<https://nebiocalculator.neb.com/#!/ligation>). The linearised vector and gBlock were ligated using NEBuilder Hi-Fi DNA Assembly Master Mix (NEB, E2621L) (Table 2.9). The ligation reaction was then carried out at 50°C for 5 minutes.

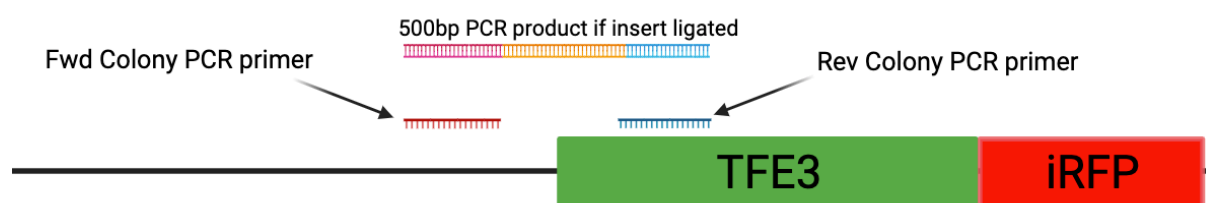
Table 2.9 - Reagents for Hi-Fi ligation of EF1a-TFE-iRFP670 constructs

Component	Amount
Linearised EF1a- vector	100 ng
TFEB-iRFP670 or TFE3-iRFP670 Insert (2:1 ratio mass)	1 μ L
2X NEBuilder Hi-Fi DNA Assembly Master Mix	5 μ L
MilliQ H ₂ O	To 10 μ L

Following ligation, the product was transformed into ultra-competent (*E. coli*) and grown on LB agar + Carbenicillin (Section 2.3.2). Colony PCR (Section 2.4.1.5) was carried out prior to sequence validation (Section 2.4.4 & Appendix S.5).

2.4.1.5 Colony PCR of bacteria transformed with EF1 α -TFEB-iRFP670 or EF1 α -TFE3-iRFP670 constructs

Colony PCR was used to screen transformed bacteria likely to contain a correct insert integration. PCR primers were designed either side of the ligation junction, such that one primer is complementary to the lentiviral backbone and the other to the gBlock. Amplification of the correct weight PCR product (~500 bp) indicates the colony likely contains a correct integration of the insert (Figure 2.1).

**Figure 2.1** - Colony PCR Schematic.

A GoTaq Master mix (Table 2.11) containing colony PCR primers specific to EF1 α -TFEB-iRFP670 or EF1 α -TFE3-iRFP670 (Table 2.10) was dispensed into PCR tubes. Another set of PCR tubes containing (LB) + Ampicillin (Section 2.3.2) were also made.

Table 2.10 - Colony PCR primers used for TFEB-iRFP670 or TFE3-iRFP670

Colony PCR Primer Name	Sequence
Fwd Colony PCR primer (EF1a backbone)	GTCCAGGCACCTCGATTAGT
Rev Colony PCR primer (TFE3-iRFP670)	CGTTCGCGTCTCTCCTGTTC
Rev Colony PCR primer (TFEB-iRFP670)	CGTCGTCGAGGCGCATTATA

Transformed bacterial colonies transformed growing on antibiotic selection LB agar were selected using a pipette tip. The bacteria on the tip were exposed to a PCR tube containing GoTaq master mix. The same tip was then placed in the corresponding PCR well containing LB + Ampicillin and incubated at 37°C to cultivate the remaining bacteria on the tip. This was repeated for 5 colonies per construct.

A PCR reaction was carried out using the PCR plate containing the GoTaq master mix (+ bacterial lysate) (Table 2.12).

Table 2.11 - Colony PCR Reaction components

Component	Amount
GoTaq Master Mix	5 μ L
100 μ M Forward Colony PCR primer	0.2 μ L
100 μ M Reverse Colony PCR primer	0.2 μ L
MilliQ H ₂ O	To 10 μ L

Table 2.12 - Colony PCR GoTaq PCR Reaction Conditions

Stage		Reps	Temp	Time
1	<i>Initial Denaturing</i>	1	95°C	2 mins
2	<i>PCR (cycle)</i>	34	95°C	45 seconds
			55°C	30 seconds
			72°C	1 min/kB
3	<i>Final Extension</i>		72°C	5 mins
4	<i>Hold</i>		12°C	Indefinite

Following, the PCR reactions were run on a 1.2% agarose gel (Section 2.4.1.3) (1.2 g agarose in 100 mL TBE). The gel was visualised using a UV transilluminator to identify PCR products at the appropriate weight (~500 bp). Successful colonies were further cultivated in a LB selection medium (Section 2.3.2) for amplification and purification of the construct (Section 2.4.3), before Sanger Sequencing (Section 2.4.4 & Appendix S.5).

2.4.2 U6-sgRNA-NLS-mTagBFP2 lentiviral construct production for CRISPRi screening

2.4.2.1 *Design and annealing of sgRNA fragment for CRISPRi lentiviral production*

2 sgRNA sequences for each HDAC target were designed using a genome-wide sgRNA library (Horlbeck et al., 2016). Sequences complementary to the designated CRISPRi lentiviral backbone (U6-sgRNA-NLS-mTagBFP2) were added allowing for sticky-end recombination following digestion.

Oligonucleotides were annealed using annealing buffer (Table 2.13) and reagents in Table 2.14. Contents were incubated at 95°C, 5 minutes before returning to room temperature.

Table 2.13 - 2X Annealing buffer components

Component	Concentration
Potassium Acetate	200 mM
HEPES-KOH pH7.4	60 mM HEPES-KOH pH 7.4
Magnesium Acetate	4 mM

Table 2.14- Annealing Reaction components

Component	Volume (50 µL)
100 µM premixed top and bottom oligonucleotide	2 µL
2X Annealing Buffer	25 µL
MilliQ H ₂ O	23 µL

2.4.2.2 *Linearisation and purification of U6-sgRNA-NLS-mTagBFP2 backbone2*

U6-sgRNA-NLS-mTagBFP2 backbone linearisation was carried out in two reactions.

1. BspI restriction digest of the backbone in NEB Buffer 2.1 overnight at 37°C (Table 2.16).
 - a. NEB Buffer was then modified to produce NEB Buffer 3.1 (Table 2.15).
2. BstXI restriction digest in NEB buffer 3.1 overnight, 37°C. (Table 2.16)

The linearised vector was purified with agarose gel electrophoresis (Section 2.4.1.3).

Table 2.15 - Buffer 2.1 to 3.1 Salt Buffer

Component	Amount
NaCl	17.5 mg
Tris-HCl	29.1 mg
NEBuffer 2.1	200 μ L
MilliQ H ₂ O	800 μ L

Table 2.16 - Reagents used for both restriction digest reactions

Reaction No.	Reagents	Amount
1	NEB Buffer 2.1	2 μ L
	U6-sgRNA-NLS-mTagBFP	10 μ g
	BspI	3 μ L
	MilliQ H ₂ O	To 20 μ L
2	Reaction 1	20 μ L
	Buffer 2.1 to 3.1 salt buffer	5 μ L
	BstXI	3 μ L
	MilliQ H ₂ O	To 30 μ L

2.4.2.3 Ligation of annealed sgRNA oligonucleotides to the linearised U6-sgRNA-NLS-mTagBFP2 backbone

Annealed oligonucleotides were diluted to 5 μ M and ligated to the linearised U6-sgRNA-NLS-mTagBFP2 backbone using the 2X quick ligase buffer (Mighty Mix; Takara, 6023) at 25°C for 30 minutes (Table 2.17). The product was transformed into ultra-competent bacteria, cultivated to increase genetic material (Sections 2.3.1-2.3.2), purified by Miniprep and Sequenced (Sections 2.4.3-2.4.4).

Table 2.17 - CRISPRi sgRNA ligation reaction components

Component	Amount
Digested U6-sgRNA-NLS-mTagBFP2 vector	20 ng
Annealed oligonucleotides	0.5 μ L
2X Mighty Mix (Quick Ligase Buffer)	2.5 μ L
MilliQ H ₂ O	To 5 μ L

2.4.3 DNA plasmid purification

Amplification of transformed bacteria was carried out in LB Selection medium (Section 2.3.2). The plasmid yield required determines the preparation kit used (Table 2.18). All preparations were carried out following the respective manufacturer's protocol.

Table 2.18 - DNA prep kit and associated bacterial culture volumes with expected DNA yield

Prep	Bacterial Culture Vol	DNA Yield expected
Miniprep (QIAGEN, 27104)	5.5 mL	<20 µg
Midiprep (Zymo, D4201)	55 mL	Up to 1.2 mg
Gigaprep (Zymo, D4204)	2.5 L	<25 mg

2.4.4 Sanger sequencing

Sanger sequencing was used to ensure correct fragment insertion (TFEB-iRFP670, TFE3-iRFP670, sgRNA oligo) into the respective backbone and absence of mutation. For sgRNA plasmid sequencing, one primer (Table 2.20: D48) prior to the sgRNA insertion site was used to screen different colonies by Sanger sequencing using Source Bioscience (Appendix S.5.2). The sequencing fragment encompasses the insertion site, the sgRNA and a segment of backbone following insertion. Thus, successful integration can be examined with one primer.

Successful colonies transformed with EF1 α -TFEB-iRFP670 or EF1 α -TFE3-iRFP670 constructs following colony PCR were further verified by BigDye Sanger sequencing (Appendix S.5.1).

Sequencing primers (Table 2.20) for each construct were designed using Primer3Plus (<https://www.primer3plus.com/index.html>).

2.4.5 Materials

Table 2.19 - Reagents used in DNA Manipulation

Reagent	Supplier	Cat. No.
EcoRI-HF	NEB	R3101L
NheI-HF	NEB	R313L
CutSmart Buffer	NEB	Provided with enzymes
Tris-Borate-EDTA (TBE)	Sigma	T4415-4L
Agarose powder	Invitrogen	16520100

SYBR Safe DNA Gel Stain	Thermo Fisher Scientific	S33102
NEBuilder Hi-Fi DNA Assembly Master Mix	NEB	E2621L
GoTaq Master Mix	Promega	M713
BlpI	NEB	R0585L
NEBuffer 2.1	NEB	B6002S
BstXI	NEB	R0113L
2X Mighty Mix (Quick Ligase Buffer)	Takara	6023

Table 2.20 - Cloning Primers used

Primer Name	Sequence	Use (location of sequence)
D48 (Fwd-Colony PCR Primer)	<i>GTCCAGGCACCTCGATTAGT</i>	Colony PCR/Sequencing (EF1a backbone)
TFE3-iRFP Rev 1	<i>CGTTGCGTCTCTCCTGTTT</i>	Colony PCR/Sequencing (TFE3)
TFE3-iRFP Fwd 1	<i>GAAGGTCCCAGGGCAGTTTT</i>	Sequencing (TFE3)
TFE3-iRFP Rev 2	<i>CACGCCCTGGGAAGAATACA</i>	Sequencing (TFE3)
TFE3-iRFP Fwd 2	<i>GTGCTCAAAGTCCAGACCCA</i>	Sequencing (TFE3)
TFE3-iRFP Rev 3	<i>GCCTGGTCTTCCTTCCTCTT</i>	Sequencing (TFE3)
TFE3-iRFP Fwd 3	<i>CCTTGTTGAAAGAGCGGCAA</i>	Sequencing (TFE3)
iRFP Rev 1	<i>CTGCGTTCTCCGTGATCCTT</i>	Sequencing (iRFP670)
TFE3-iRFP Fwd 4	<i>ACTTCCCTAGCGACCACCTC</i>	Sequencing (TFE3)
iRFP Fwd 1	<i>GATCTTCGGGTGGCGAGATG</i>	Sequencing (iRFP)
iRFP Rev 2	<i>AGCGTTCTTCAGGTACAGCA</i>	Sequencing (iRFP)
iRFP Fwd 2	<i>GGGTGTCTCAGCAAGCATGA</i>	Sequencing (iRFP)
iRFP Rev 3	<i>AGCAGCGTATCCACATAGCG</i>	Sequencing (backbone)
C13 (Rev backbone primer)	<i>AATTGTCAGTGCCCAACAGC</i>	Sequencing (backbone)
TFEB-iRFP Fwd 1	<i>AGCAGCAGCAACAACAGTTG</i>	Sequencing (TFEB)
TFEB -iRFP Rev 1	<i>CGTCGTCGAGGCGCATTATA</i>	Colony PCR/Sequencing (TFEB)
TFEB-iRFP Fwd 2	<i>TGACTTCATCCAGTTGCCCG</i>	Sequencing (TFEB)
TFEB-iRFP Rev 2	<i>TCGGCCATATTCATGCCACT</i>	Sequencing (TFEB)
TFEB-iRFP Fwd 3	<i>CTTCCCTTGCCCACCCAG</i>	Sequencing (TFEB)
C31 (sgRNA Seq Primer)	<i>GACATGATAAGATACATTGA</i>	sgRNA Sequencing (U6-sgRNA backbone)

Table 2.21 - Lentiviral vectors used for iPSC-DaN transduction in this thesis

Plasmid Name	Insert produced from this Thesis	Insert Source	Insert Type	Backbone
EF1 α -mApple	Not produced in this thesis	Courtesy of S Humble	N/A	I56-EF1 α -mApple
EF1 α -iRFP670	Not produced in this thesis	Courtesy of S Humble	N/A	I56-EF1 α -mApple
EF1 α -TFEB-iRFP670	TFEB-N1-iRFP670	Addgene: #38119 & courtesy of S Humble	gBlock	I56-EF1 α -mApple
EF1 α -TFE3-iRFP670	TFE3-2xGGGGS-iRFP670	Courtesy of BJ Ryan and S Humble	gBlock	I56-EF1 α -mApple
CMV-mTagBFP2-T2A-hSNCA	Not produced in this thesis	Courtesy of R H Roberts	N/A	pLv-CMV
FUW-FLAG-LAMP1-GCaMP7	Not produced in this thesis	Courtesy of Biogen	N/A	FUW
U6-HDAC1-1-NLS-mTagBFP2	Not produced in this thesis	Courtesy of S Szunyogh	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC1-2-NLS-mTagBFP2	Not produced in this thesis	Courtesy of S Szunyogh	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC2-1-NLS-mTagBFP2	Not produced in this thesis	Courtesy of S Szunyogh	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC2-2-NLS-mTagBFP2	Not produced in this thesis	Courtesy of S Szunyogh	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC3-1-NLS-mTagBFP2	Not produced in this thesis	Courtesy of S Szunyogh	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC3-2-NLS-mTagBFP2	Not produced in this thesis	Courtesy of S Szunyogh	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC4-1-NLS-mTagBFP2	Not produced in this thesis	Courtesy of A Nelke	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC4-2-NLS-mTagBFP2	Not produced in this thesis	Courtesy of A Nelke	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC5-1-NLS-mTagBFP2	GGGAGGAGCCGCGCAAAGA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC5-2-NLS-mTagBFP2	GGGCTCCTCCATCTTTGCGG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC6-1-NLS-mTagBFP2	GGAGGGAGAAGGCCTGAGAG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC6-2-NLS-mTagBFP2	GACCGGTCCGGCCCGATAAG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC7-1-NLS-mTagBFP2	GCTGCAGAGCGACTGTGAGA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC7-2-NLS-mTagBFP2	GTGTGAGACGGCGAGAGGAA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6-HDAC8-1-NLS-mTagBFP2	GGGACGTCCAATGGACAGGT	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2

U6- <i>HDAC8</i> -2-NLS-mTagBFP2	GCTCCTCTGATCGGCCGCAG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>HDAC9</i> -1-NLS-mTagBFP2	GATAGGAGAAGGGCACCGGC	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>HDAC9</i> -2-NLS-mTagBFP2	GTGGGTAGGTGGGAGAACCA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>HDAC10</i> -1-NLS-mTagBFP2	GCGCTGCCGCAGGCGATCCA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>HDAC10</i> -2-NLS-mTagBFP2	GCCCAGGCGCGCAGAAGGCA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>HDAC11</i> -1-NLS-mTagBFP2	GCCGCGGCACTCACATCCCG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>HDAC11</i> -2-NLS-mTagBFP2	GCCAGTGGCTGCCTCCGTGG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT1</i> -1-NLS-mTagBFP2	GCAGCGGTGGCTCCCGAGAT	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT1</i> -2-NLS-mTagBFP2	GCAGAGAGGCAGTTGGAAGA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT2</i> -1-NLS-mTagBFP2	GCGGTCTGGTGACAGCCTCAA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT2</i> -2-NLS-mTagBFP2	GGGTGACGGGACAGAGCGGT	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT3</i> -1-NLS-mTagBFP2	GCACAGCCGGAGGGCTGCCG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT3</i> -2-NLS-mTagBFP2	GAGCCAAGTGCGCGGGAAGT	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT4</i> -1-NLS-mTagBFP2	GGTGTCCGTAGAGCTGTGAG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT4</i> -2-NLS-mTagBFP2	GATCAGACGGTCCCACTGTG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT5</i> -1-NLS-mTagBFP2	GCGTGGGAGACTGTATTCGG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT5</i> -2-NLS-mTagBFP2	GGCGCTCCGGACCTGAGCCA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT6</i> -1-NLS-mTagBFP2	GTGTCGCCGTACGCGGACAA	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT6</i> -2-NLS-mTagBFP2	GAGACGCGCTCACCTCCGGG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT7</i> -1-NLS-mTagBFP2	GCGACGGGCGGTCCCGGGTG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2
U6- <i>SIRT7</i> -2-NLS-mTagBFP2	GCAGCTGCTCCCGAAACCGG	Horlbeck et al., 2016	sgRNA oligo	U6-sgRNA-NLS-mTagBFP2

2.5 Lentiviral packaging

Following sequence verification of lentiviral constructs, lentiviral packaging was undertaken using a 2nd generation lentiviral system.

Day -1: 15 cm culture dishes were coated with Poly-L-Ornithine overnight at 37°C.

Day 0: PLO was aspirated, and the culture dishes were left to dry for 1 hour. Following, low-passage Lenti-HEKs (HEK-293T) were seeded onto pre-coated 15 cm dishes at 3.1×10^5 cells/cm² in Dulbecco's modified Eagle's medium (DMEM), high glucose, GlutaMAX medium, supplemented with 10% fetal bovine serum (DMEM-GlutaMAX + 10% FBS).

Day 1: At ~90% confluency, Lenti-HEKs were transfected with psPAX2, pMD2.G, pAdvantage and the desired lentiviral construct (i.e. sgRNA or TFE-iRFP670 plasmids) using Lipofectamine 3000 (Thermo Fisher Scientific, L3000001), following the manufacturer's protocol (Table 2.22). Cells were incubated at 37°C, 5% CO₂.

Day 2: Full media change with DMEM-GlutaMAX + 10% FBS, supplemented with 1:500 ViralBoost (Alstem, VB100). Cells were incubated for 2 days.

Day 4-6: Media was collected from cultures and clarified by centrifugation at 500 r.c.f, 5 mins. Lentivirus was concentrated using Lenti-X-Concentrator (Takara, 631231), following the manufacturer's protocol and concentrated to 100X with PBS. Concentrated virus was stored at -80°C.

Table 2.22 - Lipofectamine Transfection Reagents for 1x15 cm dish

Tube	Reagent	Amount
1	OptiMEM	2.4 mL
	Lipo3000 Reagent	30 µL
2	OptiMEM	2.4 mL
	P3000	80 µL
	psPAX2	26.3 µg
	pMD.2G	9 µg
	pAdvantage	3.6 µg
	Lenti vector of interest	39 µg

2.5.1 Materials

Table 2.23 - Reagents used for Lentiviral Packaging

Reagent	Supplier	Cat. No.
Poly-L-Ornithine	Merck	P4957
DMEM, high glucose, GlutaMAX medium	Life Tehcnologies	10566016
psPAX2 vector	Addgene	12260
pMD2.G vector	Addgene	12259
pAdvantage	Promega	E1711
ViralBoost	Alstem	VB100
Lenti-X-Concentrator	Takara	631231

2.6 Lysosomal Imaging assays

2.6.1 DQ-BSA (Dye Quenched-Bovine Serum Albumin)

DQ-Red BSA (Thermo Fisher Scientific, D12051) was reconstituted to 1 mg/mL. DQ-BSA was spiked into the culture well media to a final concentration of 30 µg/mL, and incubated for 4.5 hours, 37°C, 5% CO₂

After incubation, media was replaced with a solution containing Hank's Balanced Salt Solution + CaCl₂ + MgCl₂ (HBSS++ - Gibco, 14025092), Neurofluor NeuO (1:1000 - STEMCELL Technologies, #01801) and NucBlue Live ReadyProbes Reagent (Invitrogen, R37605). HBSS++, NeuO and NucBlue was incubated on cells for 30 minutes, 37°C, 5% CO₂ before imaging at 40X using an Opera Phenix high-content imaging system at 37°C, 5% CO₂. 50 µM chloroquine was used as a negative control. Analysis of data is described in Section 2.6.3.

2.6.2 MDW-941

1 mM stock MDW-941 was spiked into cell culture wells to 5 nM final concentration and incubated at 37°C, 5% CO₂ overnight. 30 minutes prior to imaging, media(+MDW-941) was replaced with HBSS++, NeuO and NucBlue and cells were incubated for 30 minutes at 37°C, 5% CO₂. Cells were then imaged at 40X using the Opera Phenix at 37°C, 5% CO₂. Analysis of data is described in Section 2.6.3.

1 mM CBE treatment 1 hour before MDW-941 loading was used as a negative control.

2.6.3 Harmony analysis pipeline for DQ-BSA and MDW-941

The same Harmony analysis pipeline was used for DQ-BSA and MDW-941 (Figure 2.2). Live nuclei were selected by morphology and NucBlue Intensity. NeuO dye was used to identify live neurons. As neurons are polar and nuclei may not appear completely encompassed by cytoplasm, the neuronal region was expanded, and nuclei were selected within this region. The true cytoplasm was then found with these nuclei. Neurons were further selected by morphology. Following, DQ-BSA or MDW-941 spots (555-filter) were identified, and spot properties calculated normalised to per cell or per area cell to account for different cell number or size.

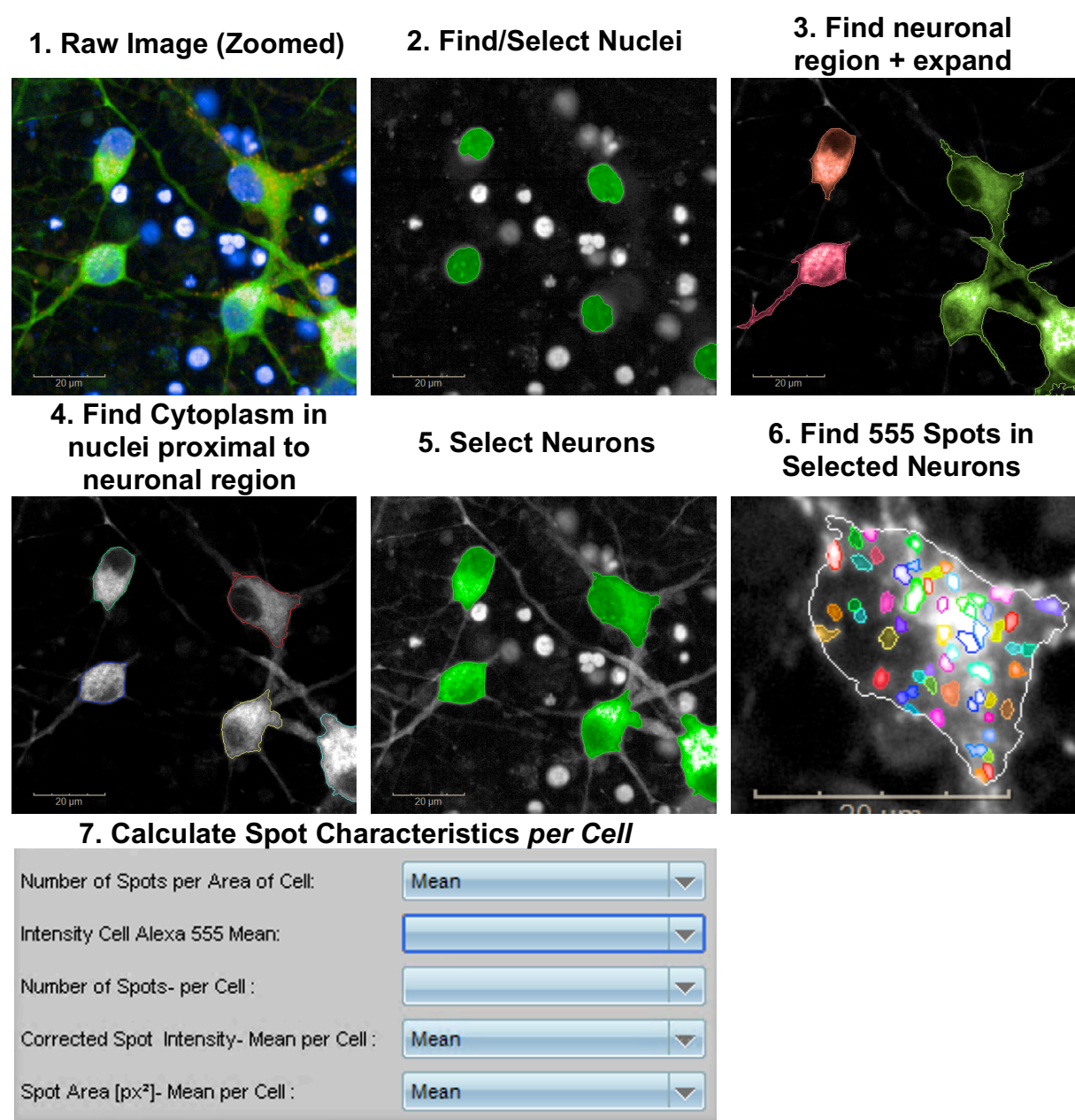


Figure 2.2 - Image analysis workflow of DQ-BSA or MDW-941 in live neurons.

2.6.4 Ratiometric lysosomal pH fluorescent dextran lysosomal pH assay

Oregon Green 488 70,000MW, Anionic Dextran and Alexafluor 555 10,000MW Anionic Dextran were reconstituted to 5 mg/mL. Dextran was administered at 25 µg/mL onto cells for 5 hours. Following, media(+dextran) was aspirated, cells were washed with HBSS and media(-dextran) was re-applied to cells, incubating overnight, 37°C, 5% CO₂. This pulse-chase was prior to any treatment of cells allowing lysosomal delivery to the lysosome. This avoids confounding pH changes resultant of impaired endolysosomal maturation.

30 minutes before imaging, NucBlue was spiked onto neurons and incubated at 37°C, 5% CO₂. Neurons were imaged at 40X on the Opera Phenix at 37°C, 5% CO₂.

Following imaging, 10 µg/mL nigericin was added to pH standards, administered to cells for 8 minutes and the plate was re-imaged.

Image analysis of lysosomal pH is displayed in Figure 2.3. Live nuclei were selected for healthy/neuronal morphology. The image was then filtered to combine and smoothen Oregon 488 and AlexaFluor 555 signal to better resemble the location of the cytoplasm. The cytoplasm was identified, and cells were further selected to remove abnormal cell morphology or cells with extreme dextran intensities. SER Spot found spots within selected neurons. Oregon 488 and AlexaFluor 555 intensity was calculated in identified spots, as was the background fluorescence of the image. A corrected 488/555 ratio was output using the formula:

$$\frac{(488 \text{ Spot intensity} - 488 \text{ background fluorescence intensity})}{(555 \text{ spot intensity} - 555 \text{ background fluorescence intensity})}$$

A pH standard curve was created with the corrected 488/555 ratio values corresponding to pH standards used (Figure 3.10 A). Lysosomal pH was then extrapolated from this curve.

50 µM CQ treatment was used as a negative control.

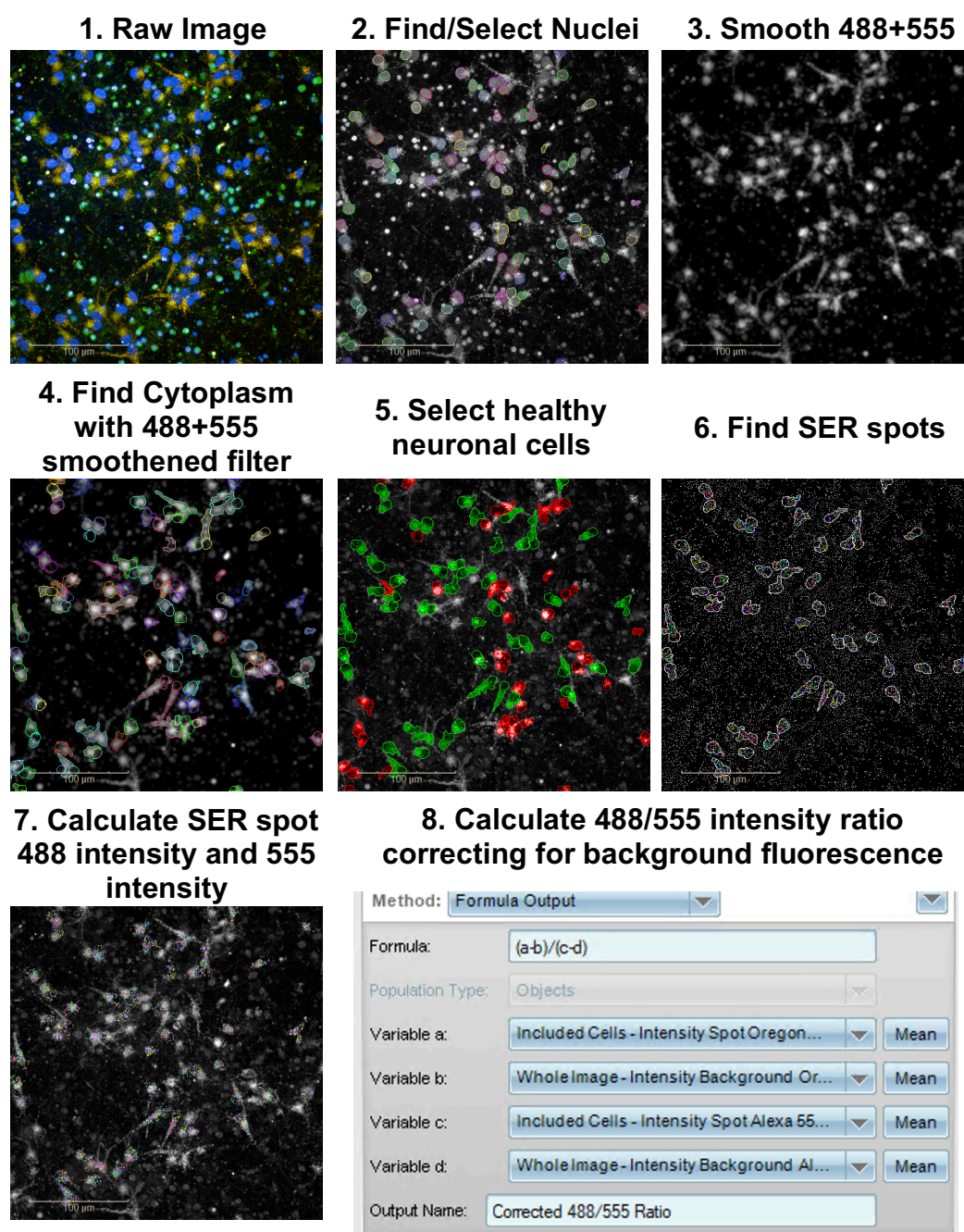


Figure 2.3 - LysoPH Image analysis workflow.

2.6.5 Lysosomal Ca^{2+} using LAMP1-GCaMP7

DIV20 iPSC-DaNs were transduced with LAMP1-GCaMP7 lentivirus and cultivated to DIV65.

3X solution of GPN (600 μM) and ML-SA5 (90 μM) was prepared in imaging media (HBSS, with Ca^{2+} and Mg^{2+} supplemented with 15 mM HEPES, 10 mM glucose, pH 7.4). Dissolution of GPN was aided with Pluronic F127 (Invitrogen, P6866) and heated to 50°C. Before imaging, media was aspirated from the iPSC-DaN culture well and replaced with 40 μL imaging media. iPSC-DaNs were imaged using the FITC channel

at 20X with an INCELL6000 imaging system for 64 seconds at 37°C. Images were captured every 4 seconds and 20 μL compounds was injected following an initial 16 seconds of imaging.

Image analysis was carried out using Columbus (Figure 2.4). Cells were identified by LAMP1-GCaMP7 signal (no nuclear mark was used) and neurons were selected by morphology. Selected cell position was tracked to assign LAMP1-GCaMP7 intensity values calculated to the same cell throughout the time-course. Thus, upon analysis, change in fluorescence can be attributed to each cell.

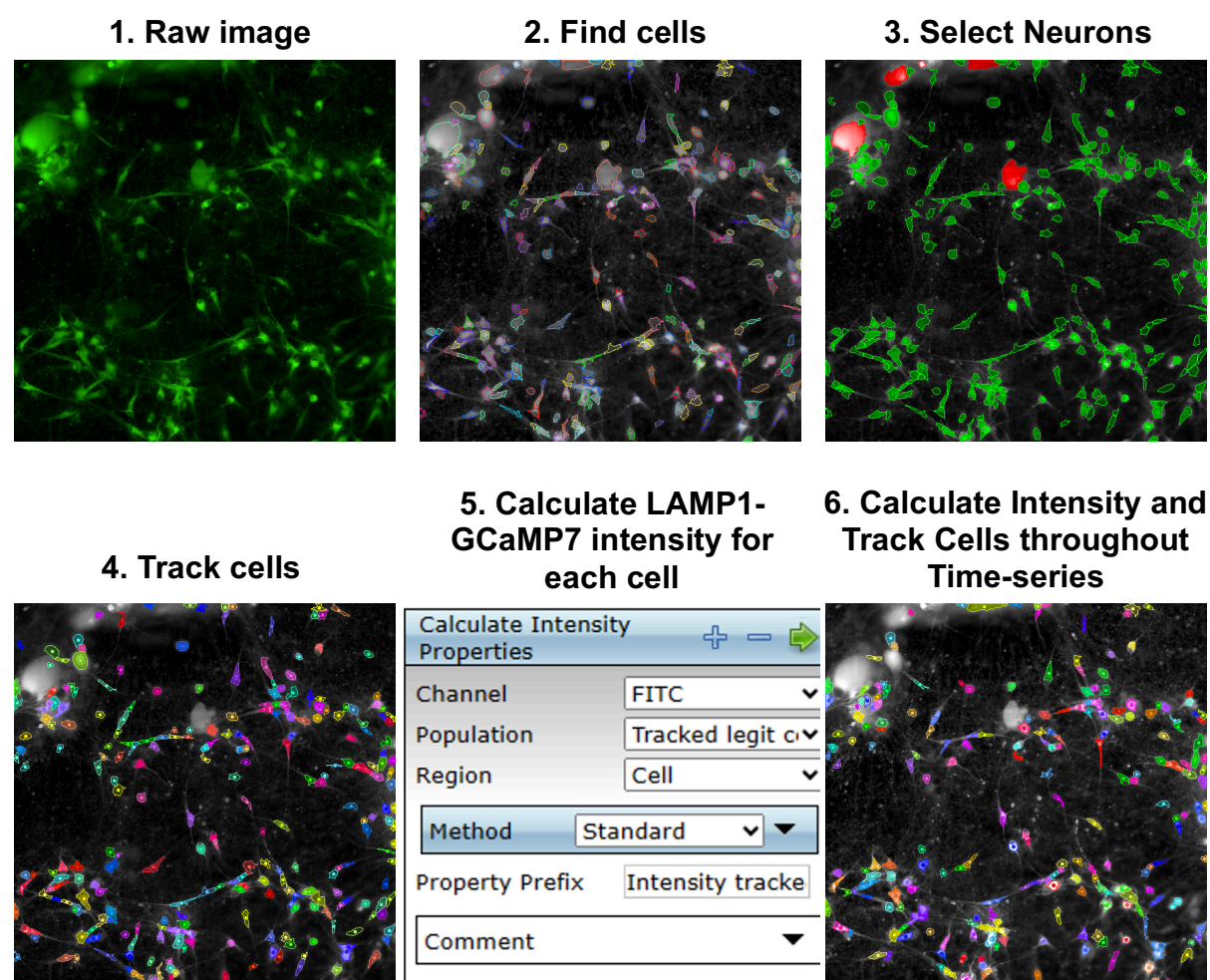


Figure 2.4 - LAMP1-GECI image analysis pipeline.

Raw fluorescence data was processed in MATLAB (script provided by Maria Claudia Caiazza). The script is designed to calculate change in LAMP1-GCaMP7 fluorescence relative to baseline fluorescence $(F - F_0)/F_0$. Area under the curve for each compound was calculated by $AUC = (\sum_{t=16}^{t=64} (F - F_0)/F_0) * 4$. Only positive values were used for AUC calculation.

2.6.6 4-MUG GCase activity (courtesy of Kalina Naidoo)

4-MUG assays were carried out in 96-well format, seeded at 100,000 cells per well. At DIV45, media was aspirated, iPSC-DaNs were washed twice with PBS and snap-frozen.

35 μ L CB⁺⁺ buffer (citrate phosphate pH 5.4, 0.25% Triton X, 0.25% (w/v) taurocholic acid) was added to each well. Plates were pulse-centrifuged and freeze-thawed on dry ice 5 times. Following, protein concentrations were quantified by BCA. 1 mM CBE was added 10 mins before 4-MUG addition to one replicate per condition. 4-MUG was added to wells at 0.86 μ M final concentration and incubated at 37°C for 1 hour. 14 μ L Glycine buffer (2 M Glycine; 2 M sodium hydroxide, pH 10.4) was then added. Fluorescence (excitation = 360, emission = 440) was assessed using a PheraSTAR FSX microplate reader.

Raw data was normalised to protein concentration and values were subtracted from CBE values to correct for non-specific signal. Data was normalised to baseline control values (Control iPSC-DaNs, DMSO or control Vector).

2.7 Omics

2.7.1 DNA methylation sequencing (Enzymatic Methyl-Seq)

DNA methylation sequencing was carried out with 3 untreated control iPSC-DaNs samples, 1 control iPSC-microglia sample (untreated), 1 control i³ neuronal sample (untreated) and 2 control iPSC-DaNs treated with DNA methyltransferase inhibitor, SCI-1027 at 50 μ M.

2.7.1.1 *DNA Extraction and quality control*

Upon collection, media was aspirated, cells were washed twice with PBS and snap-frozen with dry ice before storage at -80°C. DNA was extracted using a Gentra PureGene Cell Kit, following the manufacturer's instructions for 'DNA purification from Cultured Cells Using the Gentra Puregene Cell Kit'. DNA eluate was further purified to remove contaminants using Monarch genomic DNA purification kit, following the manufacturer's instructions.

2.7.1.2 DNA Quality Control

DNA concentration was assessed using a Qubit broad range dsDNA quantitation assay (Invitrogen, Q32850), following the manufacturer's protocol. DNA 260/280 ratios and 260/230 ratios were assessed using a nanodrop spectrophotometer.

Fragment size of extracted DNA was assessed by gel electrophoresis on a 0.7% agarose gel (Section 2.4.1.3) (0.7 g agarose + 100 mL TBE). The gel was imaged using the SYBR Safe Gel Imaging function on a ChemiDoc XRS+ imager.

Before sequencing, Oxford Genomics Centre (OGC) evaluated the DNA Integrity number (DIN) using an Agilent Tapestation.

2.7.1.3 EM-Seq Data Analysis

EM-Seq analysis was carried out following the Bismark protocol using python version 3.10: <https://felixkrueger.github.io/Bismark/>. Raw sequence quality was examined using FASTQC. Following, reads were adapter trimmed using TrimGalore (v0.6.10) by trimming 10bp from the 5' and 3' ends. The GRCh38 human reference genome was bisulfite converted and indexed using bismark_genome_preparation and bowtie2 (v2.5.0). Paired end bismark analysis called methylated cytosines in the trimmed reads, aligning to the bisulfite converted genome (Bismark v0.24.0; Samtools v1.11). Sample data was deduplicated to remove multiple alignments upon the same position. DNA methylation calls were extracted and CG, CHG and CHH methylation calls were calculated. The bismark2bedGraph function wrote a coverage file applicable to the visualisation software, Seqmonk.

SeqMonk was used to visualise methylation upon the genome by mapping bismark coverage files for each methylation context onto the GRCh38_v108 reference assembly in each sample.

Gene methylation was calculated using the 'Bisulphite methylation over features' quantitation pipeline that quantifies % methylated Cytosine residues for a methylation context within a feature (gene locus, here). Thus, % CG, CHG and CHH methylation in every gene was calculated. *TFEB* and *TFE3* methylation was extracted. CpG island methylation proximal to *TFEB* and *TFE3* was also quantified in each sample.

2.7.2 RNA-Sequencing (Online Datasets)

Raw FASTQ files and metadata from *Nichterwitz et al., 2016* (GSE76541) and *Monzon-Sandoval et al., 2020* (GSE140231) were obtained via NCBI SRA Run Selector.

Reads were pseudoaligned and quantified onto an indexed GRCh38 transcriptome assembly using kallisto (v0.46.2). In RStudio, transcript quantifications were imported using tximport and abundances were converted to Ensembl GENEID counts using a tx2gene data-frame using a GRCh38.109 assembly. DESeq2 was used for count normalisation, before counts were corrected for gene length using 'estimateSizeFactors' function so *TFEB* and *TFE3* expression could be directly compared. Gene-length-normalised *TFEB* and *TFE3* counts were plotted using ggplot2.

2.7.3 Bulk RNA-Sequencing iPSC-DaNs overexpressing TFEB and TFE3

3 control iPSC-DaNs were transduced with lentiviral mApple, TFEB-iRFP670 or TFE3-iRFP670 overexpression vectors at DIV20 and matured to DIV45 before RNA-sequencing. iPSC-DaNs were seeded in a 12-well plate (seeding density: 4.47×10^5 cells/cm²).

2.7.3.1 RNA Extraction

At DIV45, media was aspirated, cells were washed with cold PBS and snap-frozen. Upon RNA extraction, samples were thawed, and RNA was extracted using an RNEasy Mini Kit (QIAGEN, 74104) following the manufacturer's protocol. Workspaces were decontaminated with RNaseZap and sterile techniques were used.

2.7.3.2 RNA-Seq Analysis

RNA quality control was carried out by NovoGene using an Agilent 5400 before RNA-sequencing. All samples passed RNA integrity and concentration examination.

Upon completion of RNA-sequencing, sample read quality was checked using FASTQC. Following, reads were aligned to an indexed human GRCh38 transcriptome assembly and quantified using Salmon (v1.3).

Packages required were installed using the 'rnaseqGene' RStudio package. A metadata file was produced for sample details. Salmon quant.sf files were imported to R using tximport and transcript abundances were converted to Ensembl GENEID

counts in a tx2gene data-frame using the human GRCh38.109 assembly. DESeq2 was used for differential expression analysis. mApple was the reference vector for comparison. Rows (genes) with no counts were removed by pre-filtering. For principle component analysis (PCA), data was regularised logarithm (*rlog*) transformed to account for variance bias associated with high abundance values. PCA was then used (using 'plotPCA') to visualise sample separation according to the principle components that best explain variance.

Differential expression analysis for each vector relative to mApple was examined and differentially expressed genes (DEGs) were given a p-adjusted (p-adj) cut-off of 0.05. Biological pathway Gene Ontology (GO) analysis was applied to DEGs using 'enrichGO' from clusterProfiler. Upregulated DEGs ($\text{Log}_2\text{FoldChange} - \text{LFC} > 0.5$) were selected for GO analysis. A dotplot was created for the top 20 enriched upregulated GO biological pathways by TFEB or TFE3 overexpression, relative to mApple.

Volcano plots were produced by plotting LFC respective to p-adj for each gene using the 'EnhancedVolcano' package. Genes of interest were labelled on the plot. This package colours points according to LFC and p-adj value cut-offs.

- Black points: $p\text{-adj} > 0.05$; $-0.5 < \text{LFC} < 0.5$
- Green points: $p\text{-adj} > 0.05$; $\text{LFC} < -0.5$ or $\text{LFC} > 0.5$
- Blue points: $p\text{-adj} < 0.05$; $-0.5 < \text{LFC} < 0.5$
- **Red points: $p\text{-adj} < 0.05$; $\text{LFC} < -0.5$ or $\text{LFC} > 0.5$**

Heatmaps were used to identify expression patterns between samples for genes of interest. We curated 3 gene sets of 110 CLEAR genes, 115 PD-associated genes and 70 mitochondria-related genes. A heatmap using the pheatmap function was plotted for each sample using rlog transformation to normalise colour weighting for highly expressed genes.

2.8 Other assays

2.8.1 iPSC-derived model RNA extraction and protein extraction from the same sample

For collection, media was aspirated, cells were washed with cold PBS, before being removed from the well by firm rinsing using cold PBS. Cells were centrifuged at 350 r.c.f, 5 mins, supernatant was removed, and pellets were snap-frozen until use. RNA

and Protein were extracted using the AllPrep DNA/RNA/Protein mini Kit (QIAGEN, 80004), according to the manufacturer's instructions with modification. 5% SDS was used to dissolve precipitated protein for downstream BCA compatibility.

2.8.2 Immunocytochemistry

Cells cultured in 96-well or 384-well plates were used. Media was aspirated and cells were fixed using 4% Paraformaldehyde (PFA) for 7 minutes, room temperature. Fixed cells were washed twice with PBS and stored at 4°C in PBS + Azide (Santa Cruz, sc296028). Cells were permeabilised using 2% BSA, 5% Normal Donkey Serum (NDS) and 0.5% Triton X-100 for 7 minutes, room temperature, before replacement with Blocking solution (2% BSA, 5% NDS) and incubated at room temperature, 50 minutes. Primary antibodies (Table 2.28) in PBS were incubated overnight at 4°C. Following, cells were washed twice with PBS and secondary antibodies and DAPI diluted in PBS (Table 2.29) was incubated on cells for 2 hours, room temperature. Cells were then washed twice with PBS and stored at 4°C in PBS + Azide until imaging. Fluorescent images were taken on the Opera Phenix.

2.8.2.1 TFE3 Translocation Assay

DIV45 iPSC-DaNs immunostained for TFE3, TH and DAPI were imaged on the Opera Phenix at 40X. Nuclei were selected and associated TH+ cytoplasm was identified. Live DaNs were selected by nuclear/cellular morphology and thresholding TH intensity. Cytoplasmic and nuclear TFE3 intensities were calculated in TH-positive cells. A ratio of nuclear:cytoplasmic fluorescence was then calculated by:

$\frac{\text{TFE3 Intensity Nucleus}}{\text{TFE3 Intensity Cytoplasm}}$. Cells were binned into 'No Translocation', 'Mild Translocation' and 'Full Translocation' dependent upon the ratio of nuclear fluorescence. For this thesis, % Total Translocation was calculated as:

$$\frac{(\text{Number of TH+ Cells displaying Mild or Full Translocation})}{\text{Total Number of TH+ Cells}} \times 100.$$

Harmony pipeline analysis is displayed in Figure 2.5.

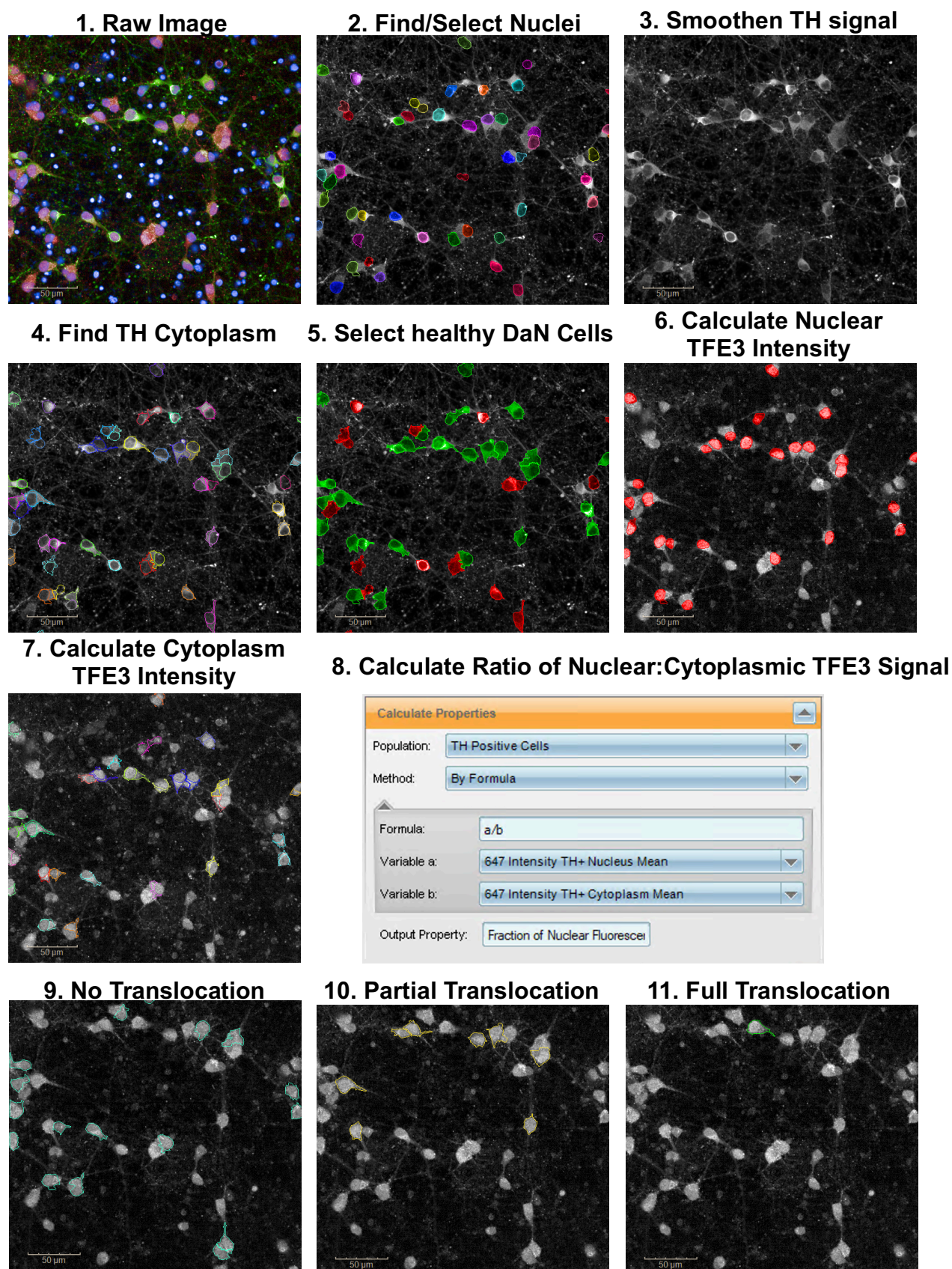


Figure 2.5 - TFE3 Translocation in iPSC-DaNs analysis pipeline.

2.8.2.2 LAMP1-positive puncta

DIV45 iPSC-DaNs immunostained for LAMP1, TH and DAPI were imaged on the Opera Phenix at 40X. Live nuclei containing a TH-positive cytoplasm were selected using nuclear/cellular morphology and TH-intensity thresholding. Find Spots was used to identify individual LAMP1-spots in the cytoplasm of DaNs. Spot properties were then calculated. In particular, the number of spots per area of cell was calculated in each dopaminergic neuron. This was averaged for the whole well before all wells in each condition in the plate was averaged. Figure 2.6 displays the Harmony analysis pipeline.

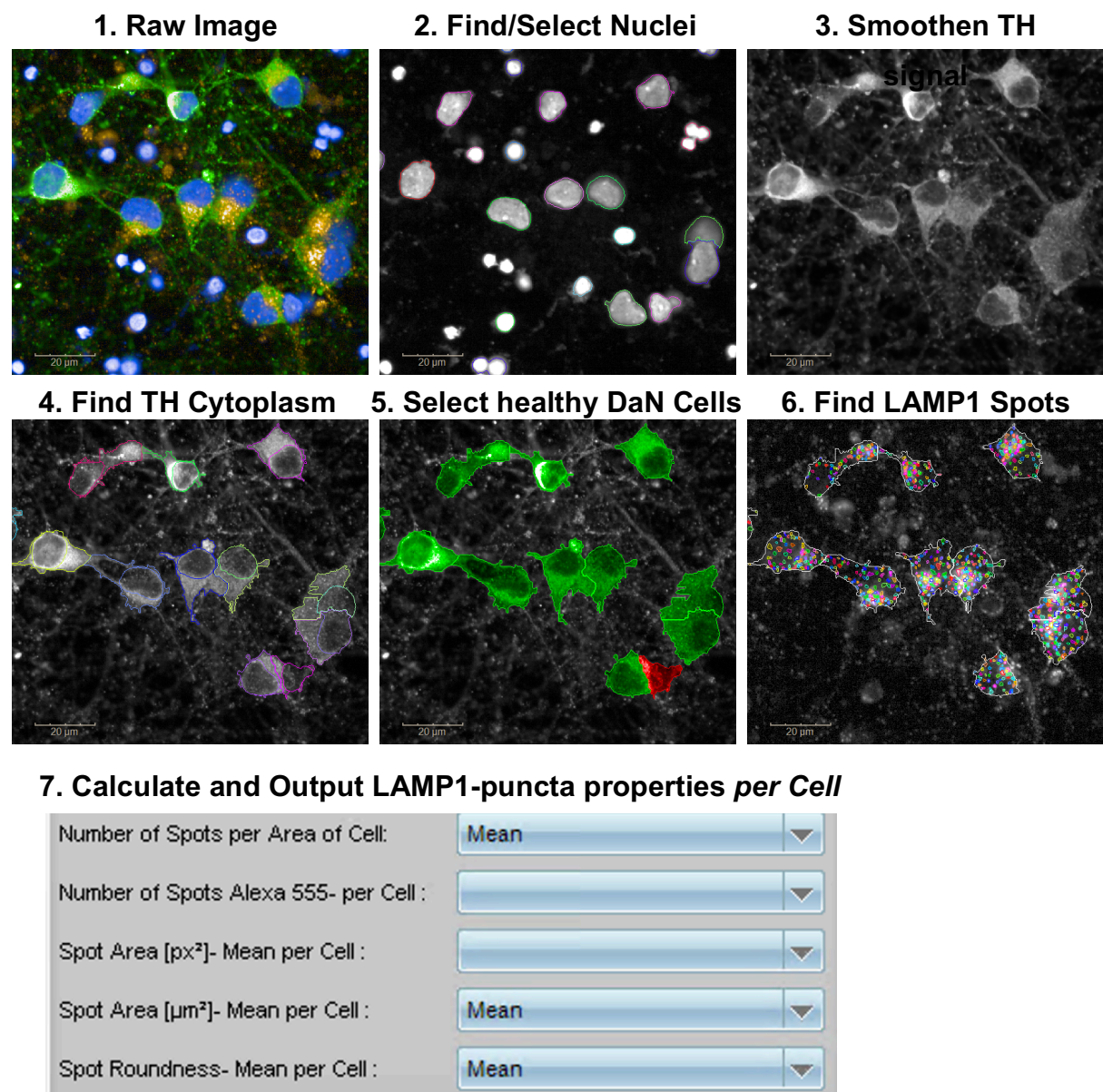


Figure 2.6 - Identification of LAMP1-positive puncta in iPSC-DaNs pipeline.

2.8.3 qRT-PCR

Following RNA extraction (Section 2.8.1), RNA was quantified using Qubit RNA High Sensitivity Kit (Invitrogen, Q32852), following the manufacturer's protocol.

cDNA was then synthesised from 400 ng RNA using SuperScript III Reverse transcriptase (Invitrogen, 18080093), following the manufacturer's instructions. cDNA was then diluted 1:5 in H₂O.

For CLEAR gene expression, qPCR was performed using FAST SYBR Green master mix (ThermoFisher Scientific, 4385610), following manufacturer's protocol. Forward and Reverse qPCR primer oligonucleotides were designed and ordered from IDT (Table 2.25). SYBR qPCR conditions are presented in Table 2.24.

Table 2.24 - SYBR qPCR Cycle Parameters

Stage		Reps	Temp	Time
1	Initial Denaturation	1	95°C	20 seconds
2	PCR (Cycle)	40	95°C	3 seconds
			60°C	30 seconds
3	Melt Curve	116	95°C	15 seconds
			60°C (+0.3/cycle)	1 min

Table 2.25 - CLEAR gene qRT-PCR Primers

Gene	Fwd Primer	Rev Primer
CTSD	GCAAAGTGGCTGGACATCGCTTG	GCCATAGTGGATGTCAAACGAGG
GBA	CTGCTGCTCTCAACATCCTTGCC	CTAGGTGCGGATGGAGAAGTCAC
MCOLN1	GACAAGTTTCGAGCCAAGGG	GATGGTGTCTCTTCCCGGA
LAMP1	CGTGTCACGAAGGCGTTTTCAG	CTGTTCTCGTCCAGCAGACACT
BECN1	CCCTGAAACTGGACACGAGT	AGTTAGTCTCTTCCTCCTGGG
ATP6V0A1	TCACCGACCTTGACTCCATCCA	GGAGTCTGGTTTGTCTGCATCC
SQSTM1	TGTGTAGCGTCTGCGAGGGAAA	ATGGTCCGTGTTTCACCTTCCG
GAPDH	CTGACTTCAACACGCACACC	ATGAGGTCCACCACCCTG

For TFEB and TFE3 expression, qPCR was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, 4444557). TaqMan Gene Expression assay probes were used (Table 2.26) and the PCR reaction was carried out as per the manufacturer's instructions. TaqMan qPCR conditions are presented in Table 2.27.

TFEB and *TFE3* probes were used with FAM reporter dye, and housekeeping gene *ACTB/GAPDH* were primer limited and used with VIC reporter dye.

Table 2.26 - TaqMan qPCR Probes

Gene	Supplier	Cat. No.
TFEB-FAM-MGB (20X) – Hs01065086_m1	Thermo Fisher Scientific	4331182
TFE3-FAM-MGB (20X) – Hs00232406_m1	Thermo Fisher Scientific	4331182
ACTB-VIC-MGB-PL (20X) – Hs01060665_g1	Thermo Fisher Scientific	4448484
GAPDH-VIC-MGB-PL (20X) – Hs02758991_g1	ThermoFisher Scientific	4448484

Table 2.27 - TaqMan qPCR Cycle Conditions

Stage	Reps	Temp	Time
1 Initial Denaturation	1	95°C	20 seconds
2 PCR (Cycle)	40	95°C	2 seconds
		60°C	30 seconds

qRT-PCR results were analysed by fold change using: $Fold\ Change = 2^{-\Delta\Delta C_t}$.

2.8.3.1 SGC qRT-PCR Screening for *TFEB* transcriptional activators

I³ neurons were seeded onto a 96-well plate at 1.84×10^5 cells/cm². At DIV14, neurons were treated with an epigenetic probe library from the Structural Genomics Consortium (SGC), previously used in our lab to target *FXN* expression in Friedrich's ataxia (Vilema-Enriquez et al., 2020). Treatment concentration and duration was used as recommended by SGC and as previously used (Table 3.3). Following treatment, media was aspirated from the wells, cells were washed once with PBS and snap-frozen before storage in -80°C. RNA was extracted in the wells using Direct-zol-96 RNA kits (Zymo Research, R2055), following the manufacturer's protocol. RNA was quantified using Quant-it RiboGreen RNA Assay Kit (Invitrogen, R11490), following the manufacturer's protocol. cDNA was then synthesised using Superscript IV Vilo cDNA synthesis kit (Invitrogen, 11754050), following the manufacturers protocol. Yield was too low to dilute RNA. Thus, the qPCR was carried out using SsoAdvanced Universal

Probes Supermix (BioRad, 1725281) following the manufacturer's protocol, as this mix has a higher tolerance to PCR inhibition. TaqMan qPCR protocol was used as above using *TFEB*-FAM TaqMan probes duplexed with *ACTB*-VIC-PL probes (Tables 2.26 - 2.27).

2.8.4 Western blotting

Protein was primarily extracted using the method described in Section 2.8.1. Otherwise, cell pellets were lysed for 30 mins on ice with RIPA buffer (50 mM Tris pH 8; 150 mM NaCl; 1% NP-40; 0.5% Sodium deoxycholate; 0.1% SDS), supplemented with PhoSTOP phosphatase inhibitor cocktail (Roche, 4906837001) and ComPLETE protease inhibitor cocktail tablets (Roche, 11836153001). Lysate was sonicated at 50 Hz intermittently for 10 second intervals over 2 minutes on ice. The lysate was then centrifuged at 1000 r.c.f for 5 mins at 4°C and the protein supernatant was extracted. Bicinchoninic assay (BCA) was used to determine the protein concentration. Pierce BCA protein assay kits were used (Thermo Fisher Scientific, 23225), following the manufacturer's protocol and fluorescence was examined using the PheraStar FSX microplate reader.

6X sample buffer (12% SDS, 30% β -mercaptoethanol, 60% glycerol, 0.012% Bromophenol blue, 375 mM Tris pH 6.8) was added to 10-20 μ g protein sample and loaded onto a 4-20% Tris-HCl SDS-PAGE gel (BioRad) alongside a PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, 26620). The gel was submerged in 1X running buffer and electrophoresis was carried out at 180 V for 35 minutes.

Separated proteins were transferred to a PVDF membrane (Biorad) by semi-wet transfer, using a TransBlot Turbo Transfer system (BioRad). The membrane was washed with Tris-Buffer Saline supplemented with 0.1% Tween 20 (TBST) before blocking with 5% milk powder in TBST for 1 hour, room temperature. Primary antibodies (Table 2.28) in 5% milk were incubated overnight at 4°C. Following, the membrane was washed 3 times with TBST for 10 minutes each before secondary antibody incubation (Table 2.29) in 5% milk for 2 hours, room temperature. Again, the blot was washed 3 times with TBST and developed using Immobilon Western Chemiluminescent HRP substrate (Millipore, WBKLS0500) or Amersham ECL Prime western blotting reagent (Merck, GERPN2232). The blot was then imaged on a ChemiDoc XRS+ system (BioRad) and relative protein amounts were quantified on ImageLab (BioRad), normalising to β -Actin.

2.8.4.1 Autophagic flux by LC3-II or p62

For measurement of autophagic activity, iPSC-DaNs were treated with a vehicle or 50 nM Bafilomycin A1 for 1 hour. Samples were then collected and blotted LC3 or p62 (Table 2.28), and normalised relative to β -Actin. Autophagic flux was examined by % induction of Bafilomycin A1 treatment relative to the vehicle treated sample.

2.8.5 Antibodies

Table 2.28 - Antibodies

Antibody Target	Concentration	Host Species (clonality)	Assay	Supplier	Cat. No.
TFEB	1:500	Rabbit (polyclonal)	ICC;WB	CellSignalling	#4240S
TFE3	1:1000	Rabbit (monoclonal)	ICC;WB	Abcam	ab179804
MAP2	1:2000	Chicken (polyclonal)	ICC	Abcam	ab92434
TH	1:1000	Chicken (polyclonal)	ICC	Abcam	ab76442
TH	1:500	Rabbit (Polyclonal)	ICC	Millipore	AB152
FOXA2	1:250	Goat (polyclonal)	ICC	R&D	AF2400
Iba1	1:1000	Goat (polyclonal)	ICC	Abcam	ab5076
LAMP1	1:500	Mouse (monoclonal)	ICC;WB	Santa Cruz	Sc-20011
GBA	1:1000	Rabbit (monoclonal)	WB	Abcam	ab128879
p62	1:1000	Mouse (monoclonal)	WB	Abcam	ab56416
CTSD	1:1000	Mouse (monoclonal)	WB	Abcam	ab6313
LC3	1:5000	Rabbit (polyclonal)	WB	Merck	L7543
Actin-HRP	1:50000	Mouse (monoclonal)	WB	Biolegend	643807

Table 2.29 - Secondary Antibodies

Species Target (Fluorophore)	Concentration	Host Species	Assay	Supplier	Cat. No.
Mouse (Alexafluor 555)	1:1000	Donkey	ICC	Life Technologies	A31570
Rabbit (Alexafluor 488)	1:1000	Donkey	ICC	Life Technologies	A21206

Rabbit (AlexaFluor 555)	1:1000	Donkey	ICC	Life Technologies	A31572
Rabbit (AlexaFluor 647)	1:1000	Donkey	ICC	Life Technologies	A32795
Chicken (AlexaFluor 488)	1:1000	Donkey	ICC	Life Technologies	A78948
Chicken (AlexaFluor 555)	1:1000	Donkey	ICC	Life Technologies	A78949
Goat (AlexaFluor 488)	1:1000	Donkey	ICC	Life Technologies	A11055
Goat (AlexaFluor 647)	1:1000	Donkey	ICC	Life Technologies	A21447
Rabbit - HRP	1:5000	Goat	WB	BioRad	1706515
Mouse - HRP	1:5000	Goat	WB	BioRad	1706516

Chapter 3: TFEB and TFE3

Expression and Transcriptional Regulation in Neurons

3.1 Introduction

Lysosomal function is essential for many converging pathways in neurons, hence lysosomal perturbation plays a central role in PD pathogenesis (Klein and Mazzulli, 2018). Enhancing autophagy and lysosomal function is considered a promising therapeutic strategy, in which TFEB has taken a central role.

As discussed in Section 1.3, TFEB and TFE3 activate a large gene set responsible for several PD-related processes within the cell including autophagy, lysosomal biogenesis, lysosomal exocytosis, lipid catabolism, mitochondrial biogenesis and ER Stress (Raben and Puertollano, 2016). Interestingly, perturbation of TFEB signalling has been associated with PD pathogenesis through reduced expression, or cytoplasmic sequestration (including in post-mortem SNc DaNs) (Decressac et al., 2013; Siddiqui et al., 2015; Mubariz et al., 2023). Conversely, TFEB activation by overexpression or nuclear re-localisation have ameliorated numerous phenotypes in several models of PD (Dehay et al., 2010; Kilpatrick et al., 2015; Torra et al., 2018; Arotcantera et al., 2019; Zhuang et al., 2020; Mubariz et al., 2023). Although TFE3 and TFEB have many overlapping functions, investigation of TFE3 in PD has been limited. Recently, TFE3 was found mislocalised to the cytoplasm in PD post-mortem DaNs (He et al., 2021). Further, TFE3 knockdown in mice caused DaN neurodegeneration, whereas TFE3 overexpression ameliorated MPTP-induced DaN loss. This suggests an essential function for TFE3 in DaN function and therefore needs be explored further as a potential therapeutic target.

Despite major interest in TFEB as a therapeutic target, little has been done to profile neuronal endogenous *TFEB* and *TFE3* expression, nor to understand the transcriptional regulation upon them in this cell-type. Many studies studying TFEB in

neurodegeneration rely upon immortalised cell lines, viral-mediated overexpression or whole-brain lysates containing many non-neuronal cell-types to study function. Those that have examined TFEB or TFE3 in a neuron-specific manner rely heavily upon antibody-based methods to detect the transcription factors. None have stringently assessed the relative abundance of TFEB or TFE3 in neurons. Interestingly, TFE3 is found more abundant than TFEB in the brain (Kuiper et al., 2004; Bordi et al., 2016). This is significant considering the suggestion that the primary effector upon lysosomal biogenesis between TFEB or TFE3 is dependent upon their relative expression (Martina et al., 2014). Thus, it is pertinent to examine TFE3 activation upon lysosomal biogenesis/function in neurons, should it be more expressed within the brain. It is also important to characterise the relative endogenous abundance of these transcription factors in neurons to decipher which is the most therapeutically viable transcription factor to target lysosomal biogenesis in neurodegeneration.

This chapter utilised human iPSC models and online datasets to characterise the expression profiles of *TFEB* and *TFE3* in neuronal and non-neuronal cell-types of the brain. We then characterise neuron-specific DNA methylation levels on the *TFEB* and *TFE3* loci using whole-genome Enzymatic Methyl-Seq on iPSC-DaNs, glutamatergic neurons, and microglia. Finally, a small epigenetic qRT-PCR screen was carried out to identify compounds activating neuronal *TFEB* transcriptional and delineate functional pathways regulating *TFEB* transcription in neurons.

Aims of this Chapter:

- Characterise TFEB and TFE3 expression in iPSC-derived neuronal and non-neuronal models
- Define expression profiles of TFEB and TFE3 in neuronal and non-neuronal populations using publicly available resources
- Identify mechanistic pathways modulating TFEB transcription in iPSC-derived neurons (DNA Methylation, Epigenetic modulator screening)
- Functionally assess compounds upregulating *TFEB* mRNA expression

3.2 TFEB and TFE3 Expression in iPSC-derived dopaminergic neurons

We first assessed endogenous TFEB and TFE3 expression in a human PD-relevant model by differentiating 3 control iPSC lines into DaNs using a modified expanded mFPP protocol (Section 2.1.4; Appendix S.1.1). At DIV45, MAP2 (neuronal marker), FOXA2 (floorplate marker) and TH (DaN marker) expression was observed (Figure 3.1 A). Differentiation efficiency, measured by the proportion of TH⁺/MAP2⁺ cells, was ~70% (Figure 3.1 B). TFEB and TFE3 expression in iPSC-DaNs was initially assessed by qRT-PCR, western blot and immunocytochemistry. Surprisingly, western blot and qRT-PCR revealed negligible TFEB expression, but robust TFE3 expression in iPSC-DaNs (Figure 3.1 C-D). Relative transcript abundance was normalised to *GAPDH* in qRT-PCR, and SH-SY5Y were positive expression controls for the western blot.

As 20-30% of the iPSC-DaN culture is non-TH⁺, and subpopulations of TH⁺ neurons exist within these cultures (Fernandez et al., 2020), we used immunocytochemistry to examine TFEB/TFE3 expression at the single-cell resolution. TFE3 expression was robust in all DaNs and even non-DaN cell-types, whereas TFEB could not be observed in any cells (Figure 3.1 E). Secondary antibody only controls (2^o only) were used to account for background fluorescence.

Despite the stark difference between TFEB and TFE3 expression in iPSC-DaNs, technical caveats exist. Different antibodies have different binding affinities and may have off-target binding. Further, qRT-PCR comparison relies upon similar primer efficiencies, and amplification kinetics to compare two genes. Thus, we analysed in-house TH-sorted RNA-Seq datasets to directly compare transcript abundance in iPSC-DaNs in an unbiased manner. Using transcripts per million (TPM) to normalise transcript abundance by gene length, RNA-Seq data confirmed minimal TFEB expression and robust TFE3 expression in iPSC-DaNs (Figure 3.1 F).

The Foundational Data Initiative for Parkinson's Disease (FOUNDIN-PD) provides multi-omic data for 95 iPSC lines differentiated into DaNs at DIV0, 25 and 65 (Bressan et al., 2023). Using bulk RNA-Seq data, we confirmed *TFE3* expression throughout differentiation, and negligible *TFEB* expression throughout all 95 iPSC-DaNs (Figure 3.1 G). Collectively, these data demonstrate TFE3 to be robustly expressed in iPSC-DaNs and an absence of TFEB expression.

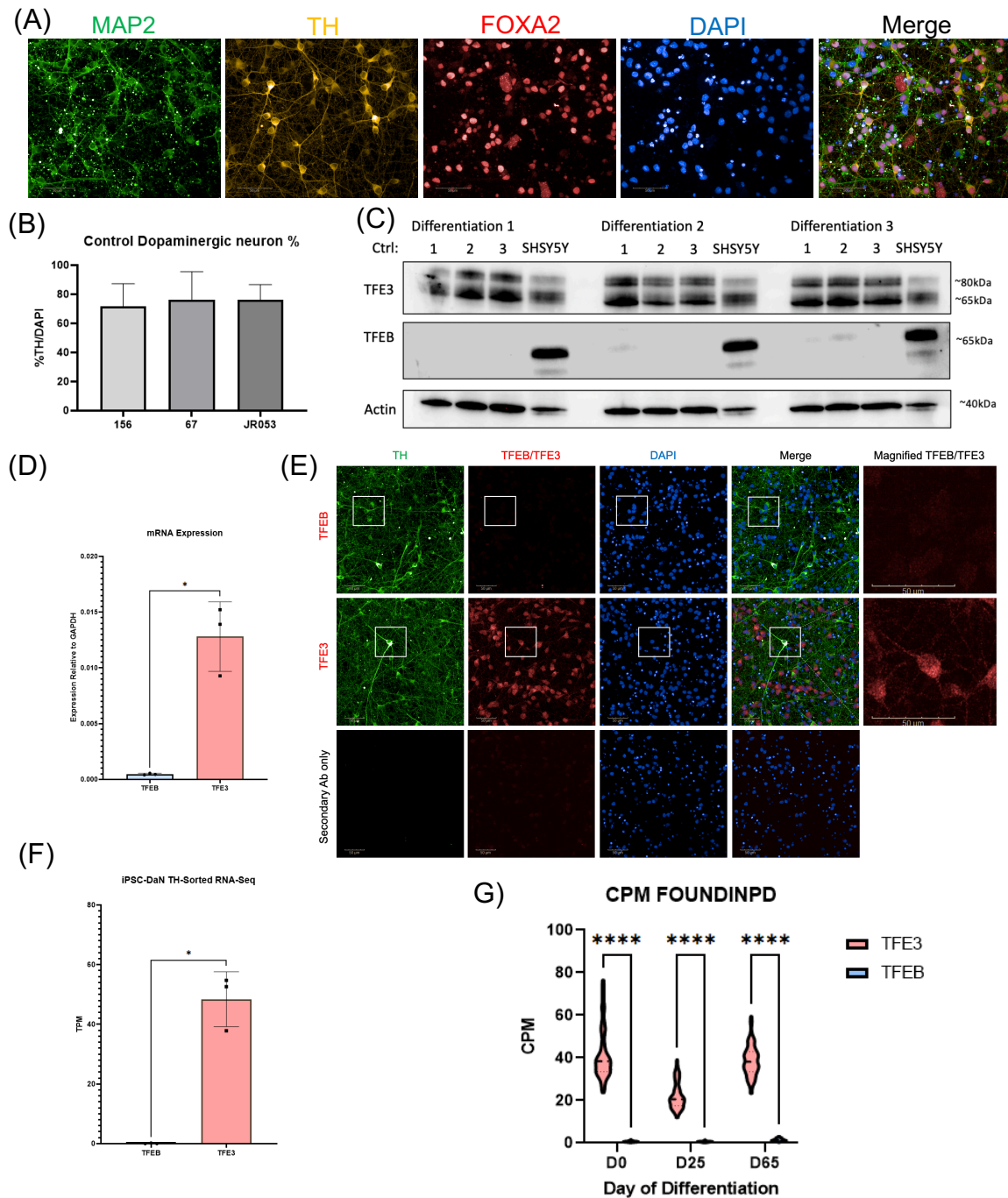


Figure 3.1 – TFEB and TFE3 expression in control iPSC-DaNs.

(A) iPSC-DaNs display expression of neuronal marker, MAP2 (green); dopaminergic marker, TH (orange); and floor plate marker, FOXA2 (red). (B) Control iPSC-DaN differentiation efficiency as a calculation of % TH+ cells/DAPI-stained nuclei. N=6 differentiations (C) Western blot of TFEB, TFE3 and loading control beta-actin, in 3 control iPSC-DaN (1: 156, 2: 067, 3: JR053) over 3 differentiations. 3 SH-SY5Y samples were used for a positive control expressing all proteins. (D) *TFEB* and *TFE3* mRNA transcript abundance in iPSC-DaNs normalised to *GAPDH* as a housekeeping gene. (E) Representative Immunocytochemical staining of 156 control iPSC-DaNs (TH stain, green) for TFEB (top row, red) and TFE3 (middle row, red). Secondary Ab only control is a negative background control. White boxes represent the region used for magnified TFEB/TFE3 staining in the far-right panels. (F) TH-sorted RNA-

Seq Transcripts per million (TPM) of TFEB and TFE3 in control iPSC-DaNs from Connor-Robson et al., 2019. (*Figure 3.1 continued...*) (G) FOUNDINPD RNA-Seq counts per million (CPM) from iPSC-DaNs of TFEB and TFE3 throughout D0, D25 and D65 of differentiation. N=3 control lines, 3 differentiations, except for (G) where N=95(D0), 94(D25) and 92(D65) according to the number of iPSC-DaN lines used. Statistical analysis carried out in (D & F) was a paired t-test. Statistical analysis for (B) was One-way ANOVA with Tukey's multiple comparisons. Two-way ANOVA with Dunnett's multiple comparisons testing was undertaken in (G) - significance between different days not displayed. * $p < 0.05$; **** $p < 0.0001$.

3.3 Using online dataset resources to define neuronal *TFEB/TFE3* expression in the brain

Despite iPSC-DaN cultures being transcriptomically similar to DaNs in the human brain (Sandor et al., 2017), we sought to further validate *TFEB/TFE3* mRNA expression profiles in DaNs from post-mortem brains. To do this, raw RNA-Seq reads from 2 laser-capture microdissected datasets of human post-mortem SNc DaNs were obtained, aligned, imported and analysed (Methods Section 2.7.2; Nichterwitz et al., 2016; Monzon-Sandoval et al., 2020). Gene-length normalised transcript abundance demonstrated *TFE3* expression to be significantly higher than *TFEB* in DaNs (Figure 3.2 A i-ii). The dataset from Monzon-Sandoval and colleagues show the presence of *TFEB* expression in DaNs, but still markedly lower than *TFE3* (Figure 3.2 A i). These results confirm *TFE3* to be the primary abundant transcription factor in DaNs, recapitulating our findings in iPSC-DaN cultures.

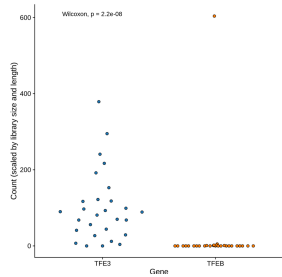
We next aimed to extrapolate this finding into *in vivo* models and other cell-types of the brain, using an online single-cell mouse RNA-Seq dataset. Ximerakis and colleagues produced a publicly accessible single-cell transcriptomic atlas of the mouse brain (2019). The database was explored using: https://portals.broadinstitute.org/single_cell/study/aging-mouse-brain. *Tfeb* and *Tfe3* expression was assessed in relation to the different cell classes identified by T-SNE (Figure 3.2 B i-iii). Purple lines indicate neuronal lineage clusters.

Dotplot comparison of expression between cell classes revealed expression levels and % cells expressing *Tfeb* to be lowest in the neuronal lineages, whereas oligodendrocytes and ependymal lineages expressed the highest levels of *Tfeb* (Figure 3.2 B iv). *Tfe3* expression was highest in immune cells, but the neuronal lineage expressed similar, if not higher expression relative to the remaining cell classes. These observations were validated by plotting logTPM of *Tfeb* and *Tfe3* for each cell in each class (Figure 3.2 B v-vi). Importantly, *Tfeb* expression was lowest in the neuronal lineage, whereas *Tfe3* displayed higher expression in this lineage. Interestingly, a small subset of neurons express *Tfeb* comparably to *Tfe3* and other lineages (Figure 3.2 B v). These neurons do not appear to cluster together by T-SNE (Figure 3.2 B iii). Averaged TPM from subcategorised neuronal classes were used to observe whether *Tfeb* is expressed in a specific population, and to compare

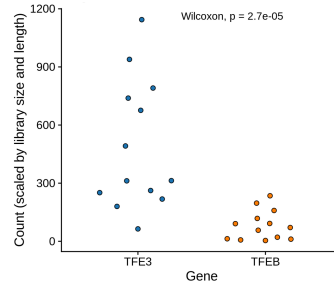
expression to *Tfe3* in cell-types. All neuronal types displayed minimal *Tfeb* expression and *Tfe3* was more highly expressed in these cells (Figure 3.2 B vii). Non-neuronal cell-types demonstrated robust *Tfeb* and *Tfe3* expression.

These data show *Tfeb* to be minimally expressed in most neurons, and *Tfe3* to be the primary neuronal transcription factor. However, non-neuronal cell-types such as oligodendrocytes and microglia express *Tfeb* within the mouse brain.

(A) (i) *Nichterwitz et al., 2016*

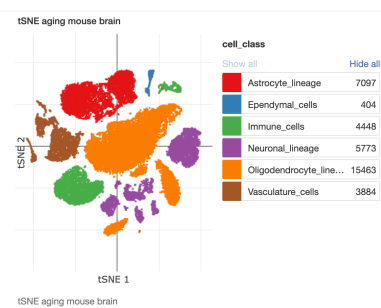


(ii) *Monzon-Sandoval et al., 2020*

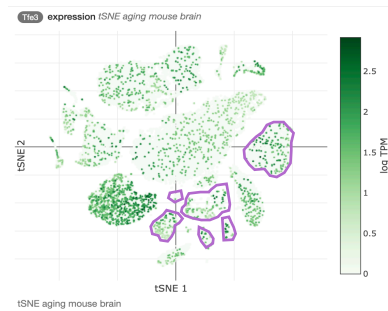


(B) *Ximerakis et al., 2019*

(i)



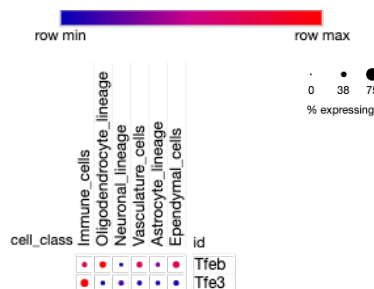
(ii) *Tfe3*



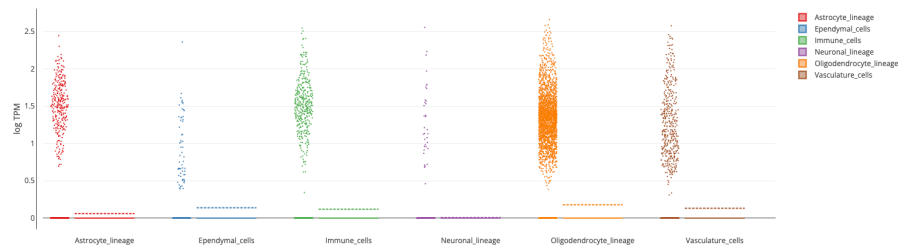
(iii) *Tfeb*



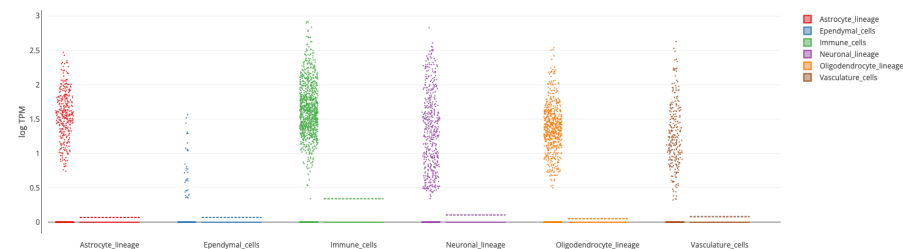
(iv)



(v) *Tfeb*



(vi) *Tfe3*



(vii)

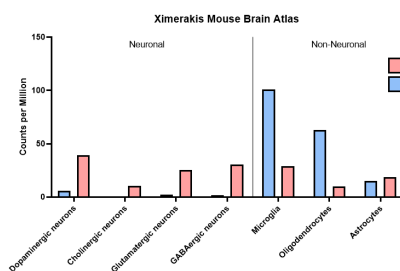


Figure 3.2 (above) – TFE3 is preferentially expressed in neuronal cell-types over *TFEB* in online RNA-Seq datasets.

(A) Laser capture microdissected human dopaminergic neuron RNA-Seq count data of *TFE3* (blue) and *TFEB* (orange) normalised to transcript size and library size, from (i) Nichterwitz et al., 2016; and (ii) Monzon-Sandoval et al., 2020. N= 13 and 28 for each dataset, respectively. A Wilcoxon signed-rank t-test was carried out on both datasets. P-value is stated on each respective graph. (B) Mouse single cell RNA-Seq data obtained from Ximerakis et al., 2019, using the website application: https://portals.broadinstitute.org/single_cell/study/aging-mouse-brain. (i) T-SNE dimensionality reduction plot coloured according to identified individual cell classes. The key displays the quantity of nuclei corresponding to each cell class. Scatter plots of logTPM shaded from white (low) to dark green (high) for each nuclei plotted for (ii) *Tfeb* and (iii) *Tfe3*. Neuronal lineage encircled by purple line. (iv) Dotplot illustrating % nuclei expressing (dot size) and logTPM (scaled from blue (low) to red (high) normalised within the respective row) of *Tfeb* (top) and *Tfe3* (bottom) in each cell class. Box plots of (v) *Tfeb* and (vi) *Tfe3* logTPM plotted with all individual nuclei in each cell class. (vii) average CPM of *Tfeb* (blue) and *Tfe3* (red) from stratified neuronal sub-types and non-neuronal cell-types of interest.

3.4 TFEB and TFE3 expression in orthogonal neuronal and non-neuronal cell types

The discovery that *Tfeb* is a non-neuronal transcription factor in mouse brains, whereas *Tfe3* is ubiquitously expressed is a novel and surprising one. The previous section displayed glutamatergic neurons to have a similar *Tfe3*-specific expression profile to DaNs, and microglia robustly expressed both transcription factors in mice. Thus, we used iPSC-derived glutamatergic neurons (iPSC-Ngn2), and iPSC-derived microglia (iPSC-microglia, courtesy of J.Wanting and S.Cowley) to confirm this observation in human models and confirm the ability of iPSC-models to replicate this phenotype.

iPSC-microglia expressed both TFEB and TFE3 by immunocytochemistry (Figure 3.3 A). TFE3 expression was also evident in iPSC-Ngn2 neurons here, however weak TFEB cytoplasmic signal was also apparent. qRT-PCR revealed increased *TFEB* and *TFE3* expression in iPSC-microglia compared to iPSC-Ngn2 and iPSC-DaNs, and both neuronal cultures expressed similar *TFE3* levels and negligible *TFEB* levels (Figure 3.3 B). By western blotting, TFE3 expression was evident in all cell types and significantly higher in iPSC-Ngn2 compared to iPSC-DaNs, whereas TFEB was only observed in iPSC-microglia (Figures 3.3 C i-ii).

These data confirm TFEB expression in non-neuronal cell types (such as microglia) and minimal expression in neurons, whereas TFE3 is expressed throughout multiple cell-types in the CNS.

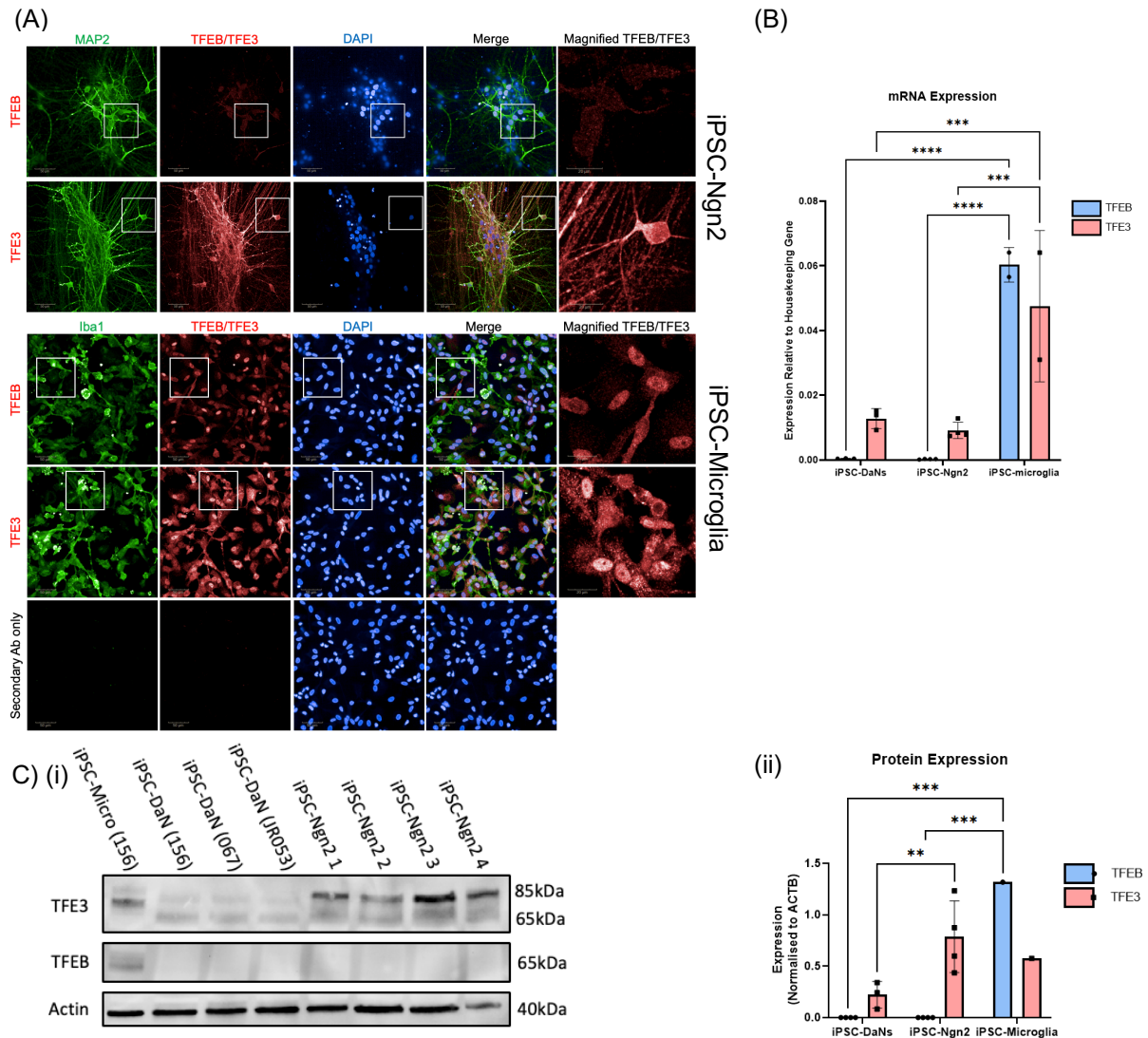


Figure 3.3 – iPSC-derived neurons and microglia replicate *TFEB* and *TFE3* expression profile.

(A) Immunocytochemical staining of iPSC-derived Ngn2 neurons (top), and iPSC-derived microglia (bottom) identified by cell-type markers MAP2 (neuronal marker – green) and Iba1 (microglial marker – green), stained for *TFEB* (red, top row of respective panels) and *TFE3* (red, second row of respective panels). Secondary antibody only used as indication of background fluorescence. White boxes represent the region used for magnified *TFEB/TFE3* staining in the far-right panels. (B) qRT-PCR mRNA expression of *TFEB* (blue) and *TFE3* (red) in iPSC-DaNs, iPSC-Ngn2, and iPSC-microglia, normalised to housekeeping gene *GAPDH*. (C) (i) Western blot of iPSC-microglia, iPSC-DaNs and iPSC-Ngn2 (numbered 1-4 for differentiation) displaying *TFE3* (top), *TFEB* (middle) and loading control beta-actin (bottom) protein. (ii) Quantification of western blot protein levels of *TFEB* (blue) and *TFE3* (red) normalised to beta-actin loading control. N= 3 control iPSC-DaNs, 1 differentiation; 1 line iPSC-Ngn2, 4 differentiations; and 2 control iPSC-microglia lines, 1 differentiation. 2-way ANOVA with Sidak's multiple comparison test of *TFEB* or *TFE3* between cell-types was carried out. ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

3.5 DNA Methylation in the mouse brain anti-correlates with *Tfeb* and *Tfe3* expression

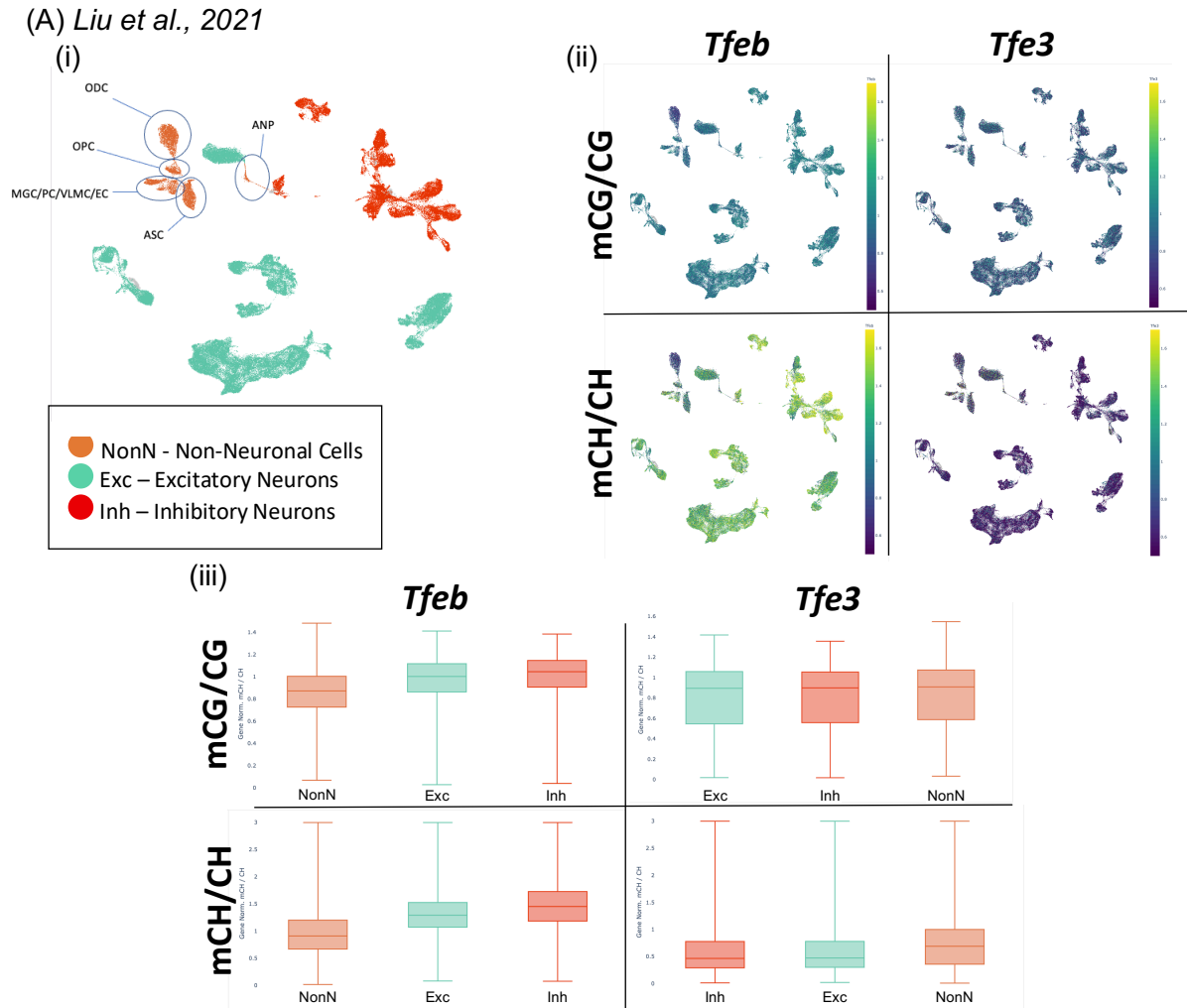
The mechanisms underpinning transcriptional repression of *TFEB* in neurons were next investigated. DNA methylation is a common base modification deposited on cytosine residues in the genome. Methylated cytosines are primarily present upon CG dinucleotides in mammals. The effects of DNA methylation upon transcription are dependent on cell-type and location relative to the locus (Moore et al., 2013). Non-CG methylation (mCN - N= A, C or T) occurs less frequently but is most common in neurons and stem cells (He and Ecker, 2015). Neuronal non-CG methylation is associated with transcriptional repression (Guo et al., 2014).

We used publicly available mouse brain DNA methylation datasets to examine whether DNA methylation on *Tfeb* may explain the neuron-specific repression observed. We initially used a mouse brain single-nucleus DNA methylation-Seq dataset using the website: <http://neomorph.salk.edu/omb/home> (Liu et al., 2021).

Figure 3.4 A i displays UMAP separation of populations into non-neuronal (orange - NonN), excitatory neurons (teal - Exc) and inhibitory neurons (red - Inh). *Tfeb* and *Tfe3* scatter plots of CG and non-CG methylation levels relating to each nucleus colour-scaled as 'per-cell normalised mCG or mCH fraction of the gene body' were used. *Tfe3* displayed consistently low methylation levels throughout all clusters for both CG and CH (non-CG) methylation (Figure 3.4 A ii-iii). *Tfeb* DNA methylation appeared higher in neurons than non-neuronal clusters, particularly for non-CG methylation (Figure 3.4 A ii-iii).

Interestingly, oligodendrocytes (ODC) that displayed high *TFEB* mRNA levels (Figure 3.2 C), had amongst the lowest *Tfeb* non-CG DNA methylation, suggesting a correlation between DNA methylation and gene expression. Quantitative analysis demonstrated both CG and non-CG DNA methylation on the *Tfeb* locus elevated in neuronal classes compared to non-neuronal classes (Figure 3.2 A iii). *Tfe3* DNA methylation was comparable throughout all cell classes. Further, non-CG methylation was higher on *Tfeb* than *Tfe3* in neurons. Thus, a correlation between *Tfeb* DNA methylation (particularly non-CG) and gene repression is present in neurons when comparing to other cell-types and to *Tfe3* DNA methylation patterns.

We also used another publicly available mouse neuronal DNA methylation dataset to confirm this finding through accessing: http://neomorph.salk.edu/Striatum_Inhibitory_Neuron.php (Lavery et al., 2020). Qualitative assessment of non-CG DNA methylation tracks from mouse neurons displayed much greater non-CG methylation on the *Tfeb* locus compared to *Tfe3* (Figure 3.4 B ii). Interestingly, knockout of *Dnmt3a*, a *de novo* DNA methyltransferase, ablated non-CG methylation found on the *Tfeb* locus (Figure 3.4 B iii). Together, 2 publicly accessible datasets were used to display increased non-CG methylation on the *Tfeb* locus in neurons, correlating to transcriptional repression observed in previous sections.



(B) *Lavery et al., 2020*

(i) *Tfeb* – Wild-type – mCH tracks



(ii) *Tfe3* – Wild-type – mCH tracks



(iii) *Tfeb* – *Dnmt3a* KO – mCH tracks

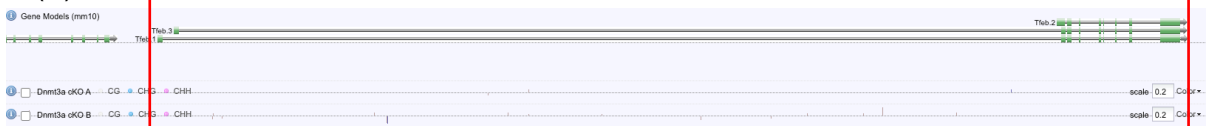


Figure 3.4 – Online mouse brain methylation datasets show elevated DNA methylation on the *Tfeb* locus in neurons.

(A) Single-cell mouse brain DNA methylation atlas from Liu et al., 2021 using the website application: <http://neomorph.salk.edu/omb/home>. (i) UMAP dimensionality reduction plot segregating individual nuclei colour-stratified into non-neuronal cells (NonN – orange), excitatory neurons (Exc – green) and inhibitory neurons (Inh – red). Non-neuronal cell-types

(Figure 3.4 continued...) circled and labelled as ODC (oligodendrocytes), OPC (oligodendrocyte progenitor cells), MGC (microglia), PC (perivascular cells), VLMC (vascular leptomeningeal cells), EC (endothelial cells), ASC (astrocytes), and ANP (Adult neuron precursor). (ii) Normalised DNA methylation levels for in mCG (top row) and mCH (bottom row) on *Tfeb* (left column) and *Tfe3* (right column) loci in individual nuclei, colour-scaled from 0.5 (lower methylation - blue) to 1.7 (higher methylation - yellow). (iii) Boxplot quantification of normalised DNA methylation levels in CG (top row) or CH (bottom row) context for *Tfeb* (left column) or *Tfe3* (right column) in each defined population (NonN – orange, Exc – green, Inh – red). Quantification provided by the website application. (B) Non-CpG DNA methylation tracks of sorted and sequenced murine neurons retrieved from Lavery et al., 2020 using the website application: http://neomorph.salk.edu/Striatum_Inhibitory_Neuron.php. Non-CpG methylation tracks from Wild-type mice on (i) *Tfeb* and (ii) *Tfe3* loci displayed. (iii) Non-CpG methylation tracks from *Dnmt3a* knockout mice on the *Tfeb* locus. Red boxes encompass the gene locus of interest.

3.6 *TFEB* transcriptional modulation using DNA Methyltransferase inhibitor SCI-1027 in iPSC-DaNs

The previous section established a correlation between DNA methylation at the *Tfeb* locus and its suppression in neurons. To address the causality of DNA methylation upon *TFEB* expression, we treated iPSC-DaNs with a pan-methyltransferase inhibitor, SCI-1027 and examined expression changes.

Strikingly, SCI-1027 treatment for 24 hours robustly increased TFEB intensity by immunocytochemistry in iPSC-DaNs (Figure 3.5 A). This increase was dose-dependent and also increased TFE3 intensity, suggesting broad de-repression (Figure 3.5 B). qRT-PCR displayed a 4-fold increase in *TFEB* mRNA (Figure 3.5 C i). Upon inspection of raw Ct values, both *TFEB* and *ACTB* increased (Figure 3.5 C ii). *TFEB* qRT-PCR primer efficiency is reasonable at 105% to Ct34, after which efficiency is greatly reduced (Figure 3.5 C iii). Thus, the increased *TFEB* fold-change may be resultant from TFEB Ct values falling below the range of primer efficiency.

Western blotting saw no increase in TFEB expression following SCI-1027 treatment, compared to DMSO (Figure 3.5 D). iPSC-DaNs treated with SCI-1027 had abnormal cell morphology (Figure 3.4 A). This and the reduced *ACTB/TFEB* Ct values indicate cytotoxicity was induced by the compound. Live nuclear count assessed using Hoescht 33342 was significantly reduced following SCI-1027 treatment (Figure 3.5 E). These data are inconclusive regarding modulation of *TFEB* expression by DNA methyltransferases, largely due to compound-induced cytotoxicity confounding observations.

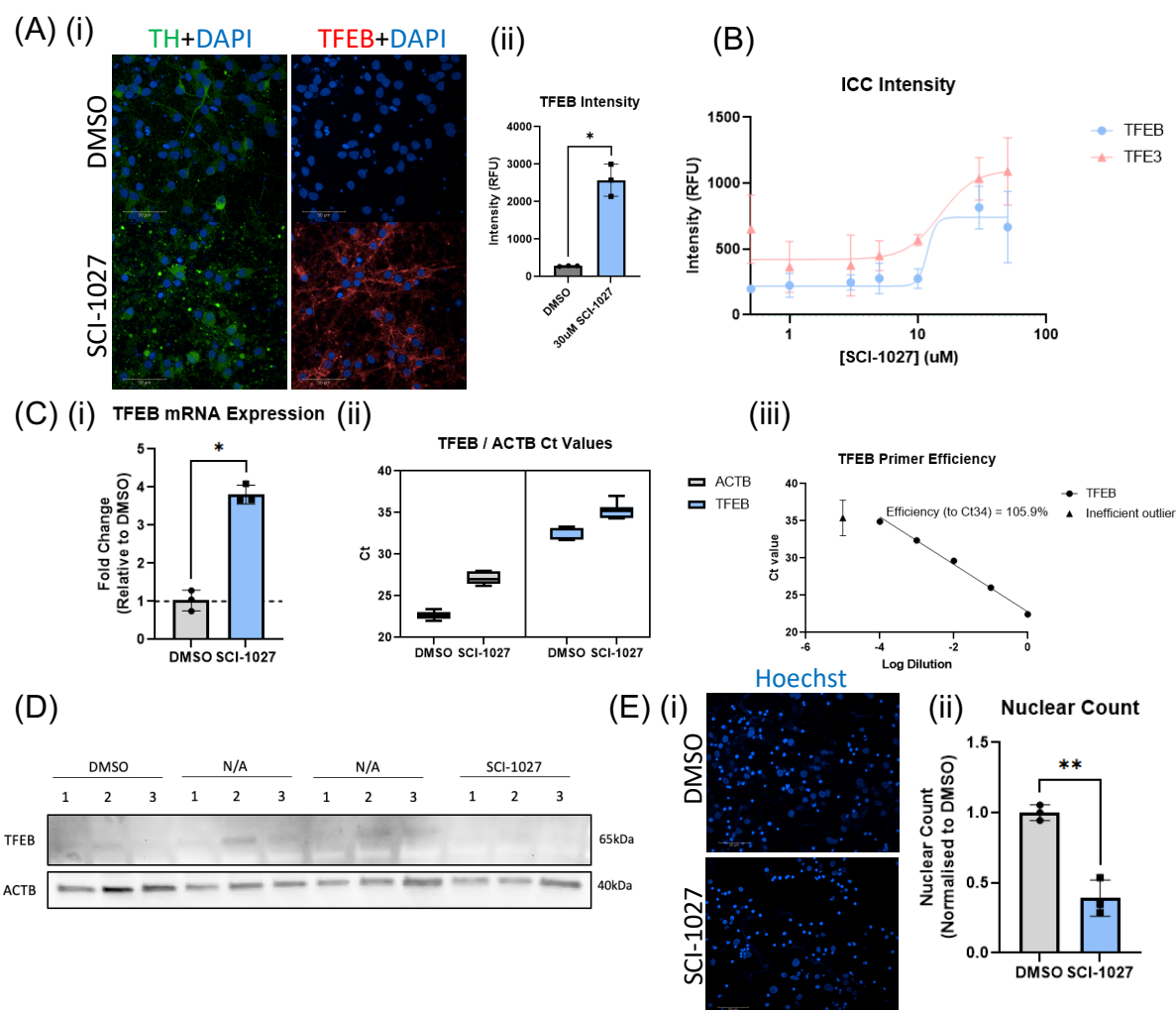


Figure 3.5 – DNA methyltransferase inhibition by SCI-1027 treatment in control iPSC-DaNs.

(A) (i) Representative immunocytochemical staining of control iPSC-DaNs identified by TH (green), treated with DMSO (top row) or SCI-1027 (bottom row) stained for the presence of TFEB (red). (ii) Quantification of SCI-1027 treatment on TFEB intensity from immunocytochemistry in iPSC-DaNs. N=3 control lines, 2 differentiations. (B) SCI-1027 dose-response quantification of TFEB (blue) and TFE3 (red) intensity by immunocytochemistry. N=3 control lines, 2 differentiations. (C) (i) qRT-PCR *TFEB* mRNA fold change responding to SCI-1027 treatment. N = 3 control lines, 2 differentiations. (ii) Raw *ACTB* and *TFEB* qRT-PCR Ct values following treatment of SCI-1027 in iPSC-DaNs. (iii) *TFEB* primer efficiency curve in 10-fold dilutions using iPSC-DaN overexpressing TFEB-GFP. Primer efficiency displayed on the graph. Dilution beyond efficiency plotted as inefficient outlier. N=3 replicates, 1 sample. (D) Western blot of TFEB and beta-actin in control iPSC-DaNs (1 – 156, 2 – 067, 3 – JR053), treated with DMSO, and SCI-1027. N/A indicates alternative treatment not relevant for this section. N=3 control lines, 1 differentiation. (E) (i) Representative image and (ii) quantification of nuclear count in iPSC-DaNs treated with SCI-1027, stained with Hoescht. N=3 control lines, 3 differentiations. Statistical analysis undertaken was paired t-test. * $p < 0.05$, ** $p < 0.01$.

3.7 DNA Methylation Sequencing by Enzymatic Methyl-Seq in iPSC-derived models

Thus far, DNA methylation has anti-correlated with TFEB expression in neurons and other brain cell-types, but methyltransferase inhibition in iPSC-DaNs was inconclusive in modulating expression. We decided to assess whether DNA Methylation upon the *TFEB* locus replicated in human iPSC-derived models.

Enzymatic Methyl-Seq (EM-Seq) is a single-base resolution DNA methylation sequencing technique alternative to bisulfite sequencing (BS-Seq).

EM-Seq uses enzymatic conversion of methylated cytosines to 5-carboxycytosines by TET2 and non-modified cytosines to uracil by APOBEC3A. BS-Seq directly deaminates non-modified cytosines in less optimal reaction conditions for DNA (Figure 3.6 A). Consequently, EM-Seq reduces DNA damage, GC content bias, improves mapping efficiency and allows for reduced input material compared to BS-Seq (Vaisvila et al., 2021).

3 control iPSC-DaNs, 1 iPSC-Ngn2 line and 1 iPSC-microglia line were differentiated. 2 control iPSC-DaNs were also treated with 50 μ M SCI-1027 to observe if DNA methylation was ablated following treatment. DNA was extracted, concentration was quantified, and quality was assessed prior to sequencing (Figure 3.6 B; Table 3.1). 2 samples fell below the required 260/280 ratio of 1.8 (Ngn2_neuron and 156_DaN). DNA was run on a 0.7% agarose gel. All samples bar Ngn2_neuron had high molecular weight and minimal shearing (Figure 3.6 C). Before sequencing, DNA integrity was examined using an Agilent TapeStation. The Ngn2_neuron sample had a low DIN (DNA integrity number) of 3.8 (Figure 3.6 D; Table 3.1). Overall, DNA concentration and quality was appropriate for the assay. Ngn2_neuronal sample had poor integrity, but EM-Seq has been shown to perform well with poor integrity samples from FFPE DNA inputs (Vaisvila et al., 2021). Based on this information and the quality of the remaining samples, sequencing was undertaken.

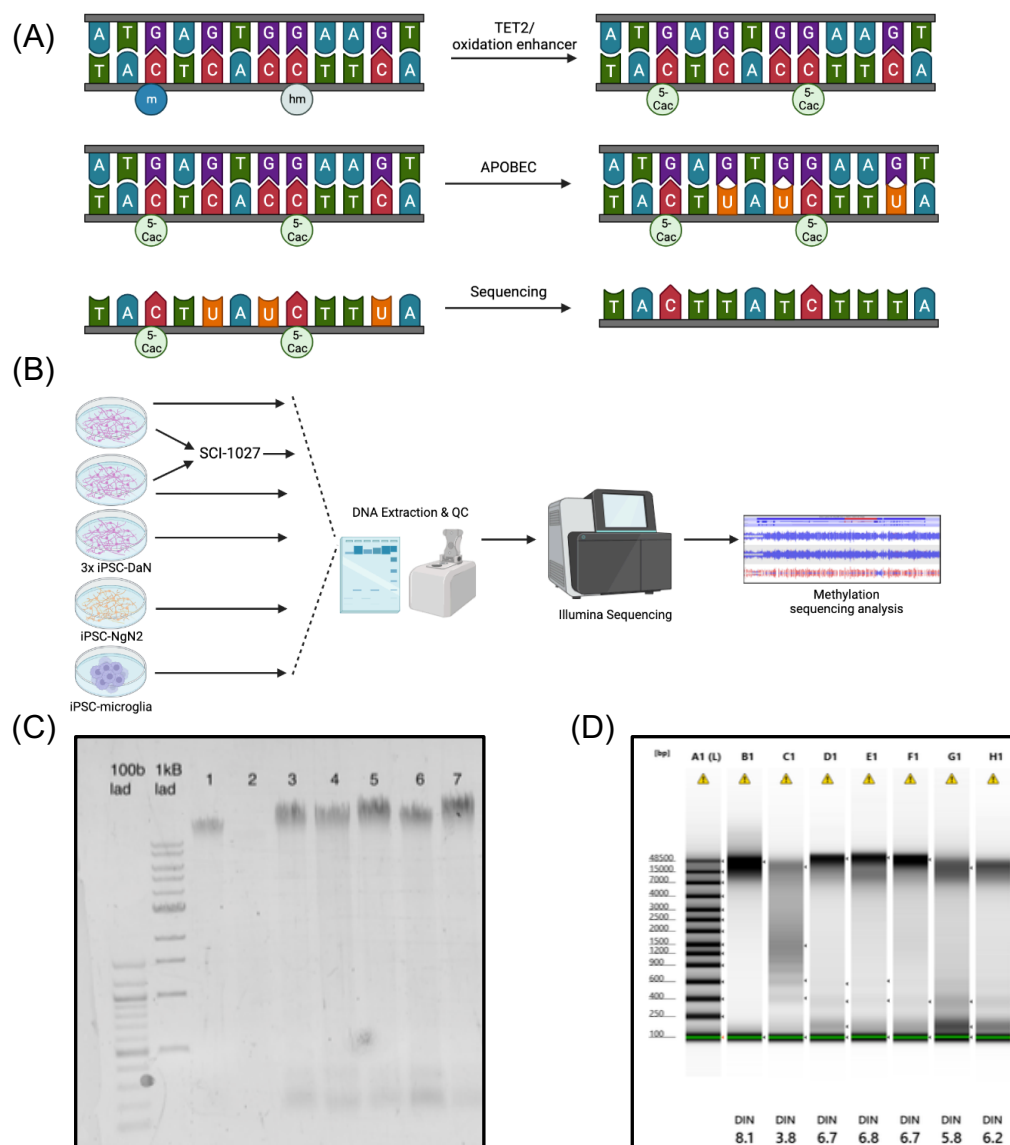


Figure 3.6 – DNA methylation sequencing by EM-Seq in iPSC-DaNs, iPSC-Ngn2 and iPSC-microglia.

(A) Schematic of EM-Seq theory. Briefly, methylated or hydroxymethylated cytosines are first enzymatically converted to 5-carboxycytosines by TET2 and an oxidation enhancer (protecting these cytosines from downstream deamination). APOBEC is then used to enzymatically deaminate non-modified cytosines to uracils. The uracil is then sequenced as a thymine in the genome and modified cytosines will be sequenced as cytosines. Methylated cytosines can be compared and identified upon alignment and comparison to a bisulfite-converted reference genome. Figure adapted from NEB. (B) EM-Seq study design. 3 control iPSC-DaN lines, 1 iPSC-Ngn2 line and 1 control iPSC-microglial line were differentiated. DNA was extracted, cleaned-up and quality checked for DNA concentration, integrity and purity. Samples were then sent to OGC for further QC and sequencing. Raw reads received were then processed computationally, described in Section 2.7.1.3 (C) DNA samples run on a 0.7% agarose gel beside a 100bp and 1kB ladder. Sample names corresponding to lane number in Table 3.1. (D) OGC DNA integrity analysis using an Agilent TapeStation. A1(L) corresponds to the ladder control. Sample name with corresponding DNA integrity number (DIN) in Table 3.1.

Table 3.1 - DNA Sample Quality Control

Lane No.	Sample Name	[DNA] (ng/μL)	260/230	260/280	DIN
1	156_Microglia	28.8	2.11	2.01	8.1
2	Ngn2_neuron	23.2	1.11	1.72	3.8
3	156_DaN	23.2	1.87	1.48	6.7
4	067_DaN	20.4	1.77	1.8	6.8
5	JR053_DaN	23.6	1.77	1.89	6.7
6	067 + SCI-1027	18.6	2.09	1.91	5.8
7	JR053 + SCI-1027	23.2	1.43	1.83	6.2

Raw reads were trimmed, and quality was assessed by FASTQC. Bismark was used to call methylated cytosines mapped onto a bisulfite-converted GRCh38 reference genome. Good mapping efficiency (60-70%) was observed (Table 3.2). Global %CG methylation was ~75% in neurons and higher in microglia (80.4%) (Table 3.2). Surprisingly, only minor reductions in %CG methylation were observed following SCI-1027 treatment (-5.2% and -3.1%), whereas %non-CG minimally increased (0.1% and 0.2%) (Table 3.2). Methylation tracks for CG, CHG and CHH contexts were visualised using SeqMonk (Figure 3.7 A). Section 2.7.1.3 discusses the analysis in more detail.

Table 3.2 - Bismark sequencing reads, mapping efficiency and global % methylation levels

Sample Name	No. Reads	% Mapping Efficiency	%mCG	%mCHG	%mCHH	%mCN or %mCHN
156_Microglia	305,478,372	61.3	80.4	0.3	0.3	1.7
Ngn2_neuron	298,539,351	63.8	75.1	0.9	0.7	2.0
156_DaN	399,599,331	68.7	75.9	0.6	0.6	2.2
067_DaN	399,599,331	65.6	77.5	0.4	0.5	2.1
JR053_DaN	351,106,055	68	76.9	0.5	0.5	2.1
067 + SCI-1027	364,674,074	63.6	72.3	0.5	0.5	3.1
JR053 + SCI-1027	317,148,837	67.5	73.8	0.6	0.6	2.5

Gene Methylation was quantified using the 'Bisulphite methylation over features' pipeline on SeqMonk. No significant differences were observed between *TFEB* and *TFE3* loci in CG or non-CG methylation (Figure 3.7 B). No differences in CG methylation were observed between cell-types for each locus, however iPSC-microglia displayed reduced non-CG methylation on both *TFEB* and *TFE3* loci, and iPSC-Ngn2 displayed elevated non-CG methylation on the *TFEB* locus, compared to *TFE3* (Figure 3.7 B). These cell-types only had one sample however, so statistical differences cannot be examined. SCI-1027 treatment did not robustly alter DNA methylation on either locus in iPSC-DaNs, although a near significant decrease was observed in CG methylation of *TFE3* (Figure 3.7 C).

Inspection of CpG islands on the *TFEB* and *TFE3* locus revealed two CpG islands either side of the *TFEB* locus (one 5' side, and one 3' side), whereas *TFE3* had only one 5' CpG island (Figure 3.7 D). No significant differences between *TFEB* and *TFE3* on the 5' CpG islands were found, however, methylation on the 3' CpG island of *TFEB* was lower in microglia than neuronal samples (Figure 3.7 E).

Overall, our data shows there to be no evidence for differential DNA methylation on the *TFEB* locus in neurons compared to *TFE3* or other cell-types that express *TFEB*.

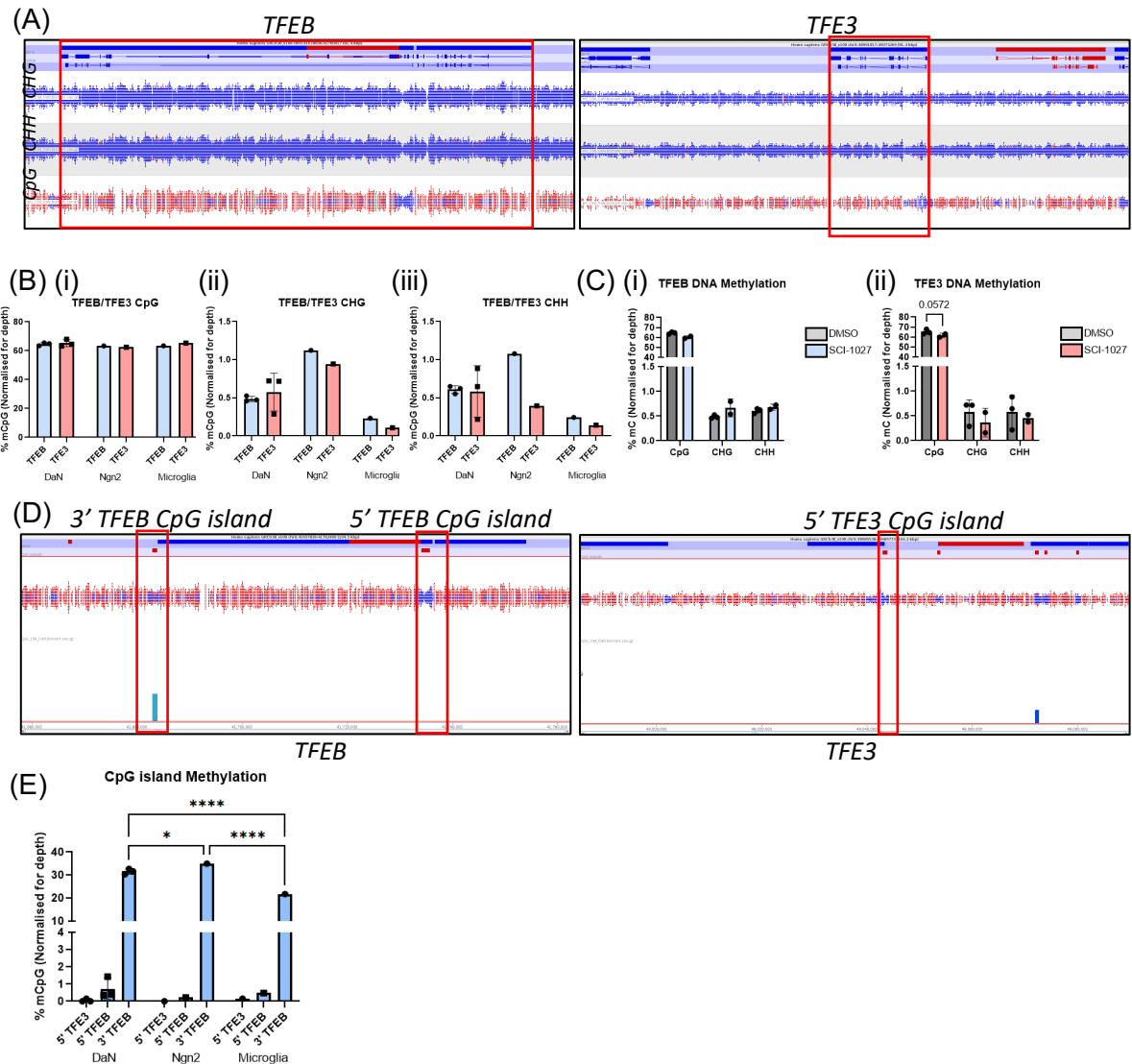


Figure 3.7 - Whole genome EM-Seq in iPSC-derived neurons and microglia.

(A) Representative methylation tracks for *TFEB* (left) and *TFE3* (right) in control iPSC-DaN line (156). CHG (top), CHH (middle) and CpG (bottom) tracks display the frequency of methylated (red) and non-methylated (blue) cytosines in each context. Red box outlines the gene of interest. (B) Quantitation of % methylation in (i) CpG, (ii) CHG and (iii) CHH context within the gene body of *TFEB* (blue) and *TFE3* (red). (C) Quantitation of % methylation of CpG, CHG, and CHH methylation in control iPSC-DaNs treated with DMSO or SCI-1027, in (i) *TFEB*, or (ii) *TFE3*. (D) Representative CpG methylation tracks and quantitation of % CpG methylation (bottom) in CpG islands proximal to *TFEB* (left) or *TFE3* (right). Red boxes indicate the proximal CpG islands of the genes. (E) Quantitation of % methylation in the proximal CpG islands of *TFEB* (blue) and *TFE3* (red) in iPSC-DaNs (DaN), iPSC-Ngn2 (Ngn2) and iPSC-microglia (Microglia). N= 3 iPSC-DaNs; 1 iPSC-Ngn2; 1 iPSC-microglia. Two-way ANOVA, with Sidaks multiple comparison was undertaken for comparison. *, $p < 0.05$; **** $p < 0.0001$.

3.8 A small epigenetic qRT-PCR screen for activators of neuronal *TFEB* mRNA expression

As there was no compelling data showing differential regulation of *TFEB* by DNA methylation in neurons, we decided to carry out a small epigenetic screen in iPSC-Ngn2 neurons to identify transcriptional regulators of *TFEB*. We used a small, well-characterised epigenetic probe library from the Structural Genomics Consortium (SGC), consisting of 38 compounds (Ackloo et al., 2017; Table 3.3). This library has been used successfully in-house previously to target Frataxin expression in Friedrich's ataxia (Vilema-Enriquez et al., 2020).

Briefly, induced iPSC-Ngn2 neurons were seeded into 96-well plate format, and at DIV14 were treated with SGC compounds in triplicate wells for 48 hours or 6 days (dependent on recommended treatment time). RNA was extracted directly from the well and cDNA was synthesised, before normalised cDNA volumes were run in a duplex *TFEB/ACTB* qRT-PCR reaction (Figure 3.8 A, Methods Section 2.8.3.1). Prior to screening, *TFEB* and *ACTB* primer efficiency was confirmed in duplex (Figures 3.8 B-C). To examine the effect of confounding lysosomotropic compounds inducing *TFEB* mRNA expression, iPSC-Ngn2 were treated with chloroquine (CQ). CQ treatment caused a non-significant 1.8-fold increase in *TFEB* expression (Figure 3.8 D). Hit compounds were defined as increasing *TFEB* fold-change above 3 Standard deviations (S.D) of DMSO (~2.2-fold-change in this screen) (Figure 3.8 E). Thus, lysosomotropic compounds were unlikely to be identified as hits.

Screening identified 3 hit compounds (7: BI-9564, 12: GSK484, 21: LAQ-824) (Figure 3.8 E). LAQ-824, a pan-HDAC inhibitor (HDACi), substantially increased *TFEB* by 15-fold (Figure 3.8 E). This effect was confirmed by multiple follow-up qRT-PCR experiments in iPSC-Ngn2 neurons, and induction was found vastly greater than in the initial screen (~55 fold-change) (Figure 3.8 F). Thus, pan-HDACi, LAQ-824 robustly induces *TFEB* expression in iPSC-Ngn2 neurons.

Table 3.3 - Structural Genomics Consortium Compounds, Targets, Concentrations and treatment times

Compound No	Compound Name	Target	Concentration [μM]	Treatment Time
1	(+)-JQ1	BRD2,3,4, BRDT(BET)	1	48h
2	(R)-PFI-2	SETD7	4	48h
3	A-196	SUV420H1/H2	4	6d
4	A-366	G9a, GLP	4	6d
5	BAY-598	SMYD2	4	48h
6	BAZ2-ICR	BAZ2A, BAZ2B	4	48h
7	BI-9564	BRD9, BRD7	4	48h
8	Bromosporine	Pan-bromodomain	5	48h
9	CI-994	Class I HDAC inhibitor	-	Not screened
10	GSK2801	BAZ2A, BAZ2B	4	48h
11	GSK343	EZH1/2	4	6d
12	GSK484	PAD-4	10	48h
13	GSK591	PRMT5	4	6d
14	GSK864	Mutant isocitrate dehydrogenase 1	1	48h
15	GSK-J1	JMJD3, UTX, JARID1B	5	48h
16	GSK-LSD1	LSD1	1	48h
17	I-BRD9	BRD9	4	48h
18	I-CBP112	CREBBP, EP300	4	48h
19	IOX1	2-oxoglutarate oxygenases	-	Not screened
20	IOX2	HIF prolyl hydroxylases	-	Not screened
21	LAQ824	Pan-HDAC inhibitor	6	48h
22	LP99	BRD9, BRD7	4	48h
23	MS023 (HCl Salt)	Type I PRMTs	4	6d
24	MS049 (HCl Salt)	PRMT4, PRMT6	4	6d
25	NI-57	BRPF1, BRPF2, BRPF3	4	48h
26	NVS-CECR2-1	CECR2	1	48h
27	OF-1	BRPF1, BRPF2, BRPF3	4	48h
28	OICR-9429	WDR5	4	48h
29	Olapirib	PARP1, PARP2	1	48h
30	PFI-1	BRD2,3,4, BRDT (BET)	4	48h
31	PFI-3	SMARCA, PB1	4	48h

32	<i>PFI-4</i>	<i>BRPF1B</i>	4	48h
33	<i>SGC0946</i>	<i>DOT1L</i>	4	6d
34	<i>SGC707</i>	<i>PRMT3</i>	4	48h
35	<i>SGC-CBP30</i>	<i>CREBBP, E3P00</i>	3	48h
36	<i>UNC0638</i>	<i>G9a, GLP</i>	4	6d
37	<i>UNC0642</i>	<i>G9a, GLP</i>	4	6d
38	<i>UNC1215</i>	<i>L3MBTL3</i>	4	6d
39	<i>UNC1999</i>	<i>EZH1/2</i>	4	6d

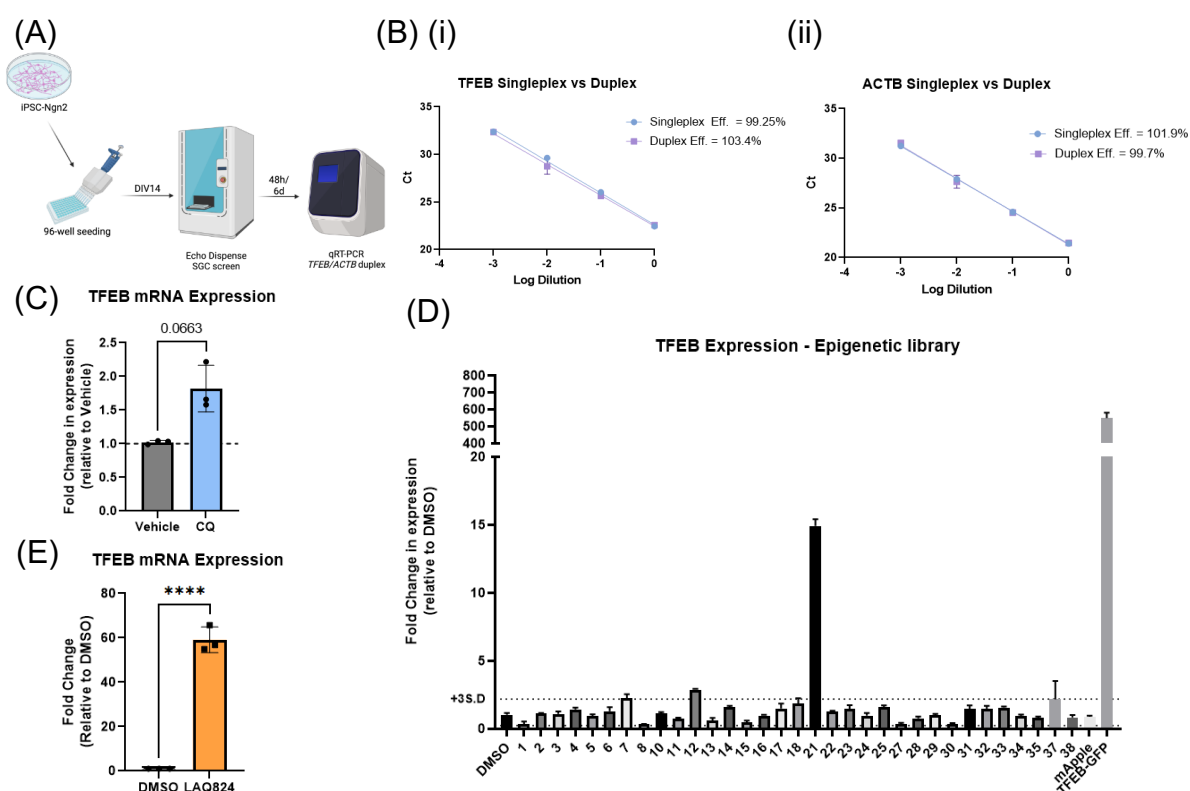


Figure 3.8 – qRT-PCR screening Structural Genomics Consortium epigenetic probe library for neuronal *TFEB* mRNA inducers.

(A) Schematic workflow of qRT-PCR screening. Briefly, iPSC-Ngn2 were cultivated to DIV14 in a 96-well format. Neurons were treated in triplicate using a Labcyte Echo 555 acoustic dispenser. Compounds names, treatment times and concentrations can be found in Table 3.3. Following treatment, RNA was extracted directly from the wells and quantified. cDNA was synthesised and a duplex qRT-PCR reaction containing *TFEB* and *ACTB* was carried out. (B) Primer efficiencies for (i) *TFEB* and (ii) *TFE3* were examined for comparison between singleplex and duplex in 10-fold dilutions using a *TFEB*-overexpressing iPSC-DaN sample. Singleplex (blue) and duplex (purple) efficiency is stated on the respective graphs. (C) *TFEB* mRNA induction following treatment with chloroquine (CQ) was assessed in iPSC-Ngn2 neurons. N=3 differentiations. P-value is displayed above the bars. (D) Screening results displaying *TFEB* mRNA fold-change for examined compounds 1-38. *TFEB*-GFP was used as a positive control. mApple was used as a viral-negative control, DMSO a vehicle control. Dotted line indicates the 3 S.D. threshold above DMSO. N=3 replicates, 1 experiment. (E) qRT-PCR follow-up with LAQ824 (compound 21) treatment in iPSC-Ngn2 neurons. N=3 differentiations. Statistical analysis carried out was paired t-test. **** p<0.0001.

3.9 Effects of pan-HDAC inhibition, using LAQ824, on *TFEB*, *TFE3* and downstream lysosomal gene expression in iPSC-DaNs

Next, we assessed the effects of LAQ-824 in iPSC-DaNs. LAQ-824 treatment on control iPSC-DaNs showed robust induction of *TFEB* mRNA and not *TFE3* mRNA (Figure 3.9 A). Further, treatment mildly increased TFEB protein expression, whereas TFE3 was unchanged following western blot (Figure 3.9 B).

Significant and robust increases in the mRNA expression of CLEAR genes *CTSD*, *MCOLN1*, *GBA*, *LAMP1* and *BECN1* were observed by qRT-PCR, and non-significant but trending increases were found in *ATP6V0A1* and *SQSTM1* (Figure 3.9 C). These data suggest HDAC inhibition elevates both TFEB expression and downstream lysosomal mRNA expression, independent of TFE3 expression.

Western blotting revealed LAQ-824 significantly increased GCase protein expression and CTSD maturation in iPSC-DaNs (Figure 3.9 D-E). Near significant increases in LAMP1 and mature-CTSD were also observed (Figure 3.9 D). Immunocytochemical staining of the endolysosomal compartment by LAMP1 revealed no change following treatment in iPSC-DaNs (Figure 3.9 F). In summary, LAQ-824 preferentially upregulated *TFEB* expression in neurons over *TFE3* and upregulated downstream lysosomal gene expression and hydrolase maturation.

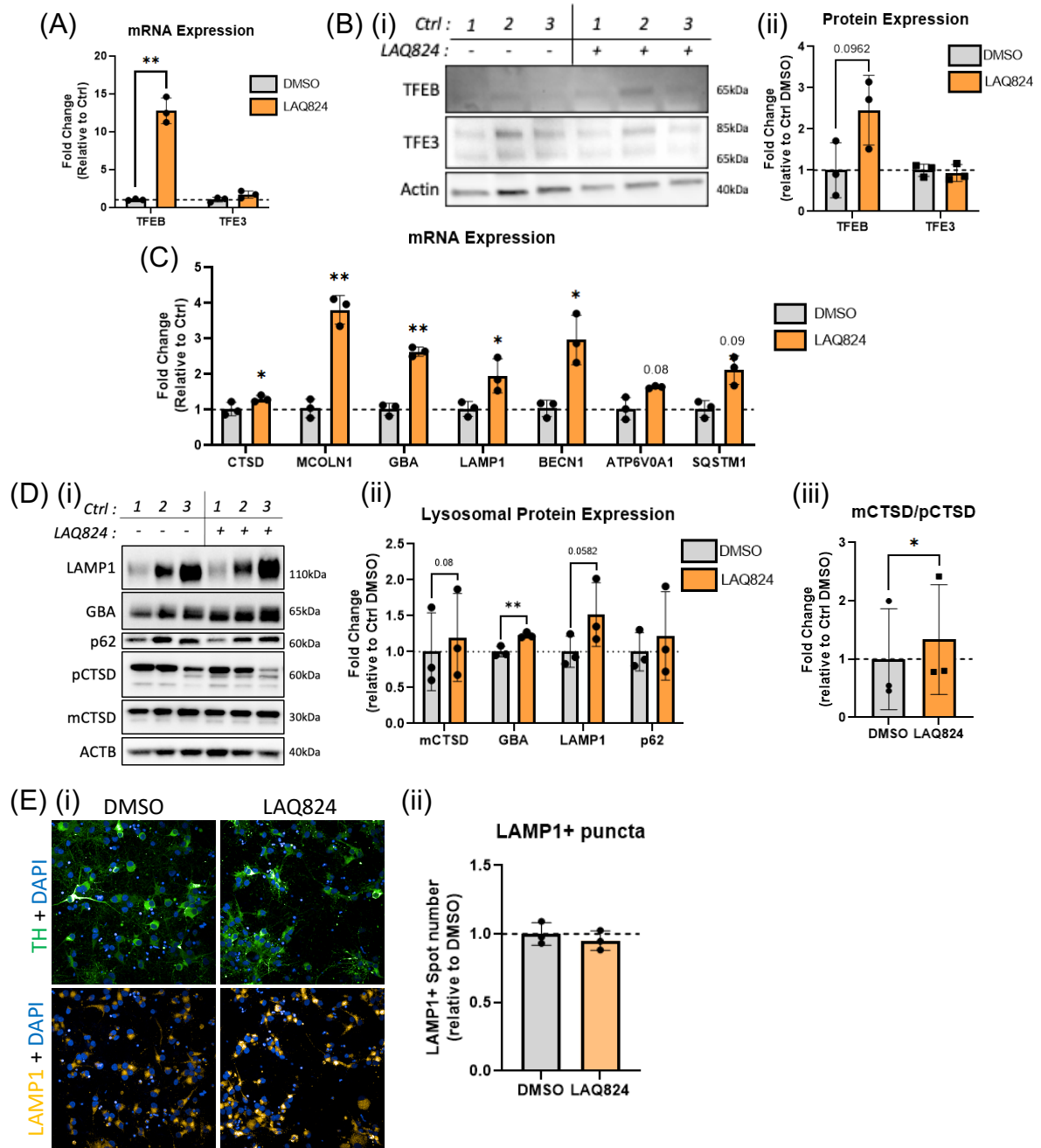


Figure 3.9 – pan-HDAC inhibitor, LAQ824, upregulates TFEB and downstream lysosomal gene expression in control iPSC-DaNs.

(A) qRT-PCR *TFEB* and *TFE3* fold change in mRNA expression in iPSC-DaNs following treatment of LAQ824. (B) (i) Representative image and (ii) quantification of TFEB and TFE3 fold change protein levels using western blots from control iPSC-DaNs (1 – 156, 2 – 067, 3 – JR053) following treatment with LAQ824. (C) CLEAR mRNA Fold change responding to LAQ824 treatment in control iPSC-DaNs using qRT-PCR. (D) (i) Representative images and (ii) quantification of lysosomal protein changes following treatment of LAQ824 in control iPSC-DaNs by western blot. (iii) Ratio of mature CTSD (mCTSD):immature CTSD (pCTSD) isoforms with LAQ824 treatment in control iPSC-DaNs. (E) (i) Representative images and (ii) quantification of immunocytochemical staining of LAMP1 puncta (yellow) in iPSC-DaNs (stained for TH, green) treated with LAQ824. N=3 control lines, 3 differentiations. Paired t-test was undertaken. * $p < 0.05$, ** $p < 0.01$, numeric p-value displayed above bars if $p < 0.1$.

3.10 Development and introduction to live-imaging-based lysosomal assays in iPSC-DaNs

Live-cell, fluorescence-based lysosomal assays can provide high-content information and high-throughput assaying for functional lysosomal characterisation following treatment or in disease-associated models. Here, imaging assays for lysosomal pH, endolysosomal proteolysis and lysosomal GCase have been utilised.

Lysosomal pH was assessed through the endolysosomal co-delivery of high molecular weight dextrans tagged with pH-sensitive 488 fluorophore or a pH-insensitive fluorophore (Alexafluor 555). Following pulse-chase delivery of dextrans, the fluorescence ratio of 488:555 in lysosomes can be assessed and back-calculated with a standard curve to define the lysosomal pH within a cell (Figure 3.10 A-B; Section 2.6.4). CQ is known to alkalinise the lysosomal compartment. Indeed, 24-hour CQ treatment for iPSC-DaNs raised the pH of the lysosomal compartment using this assay (Figure 3.10 C). A control for lysosomal acidification in iPSC-DaNs has not been tested/identified yet.

DQ-BSA (Dye Quenched Bovine Serum Albumin) is an endolysosomal proteolysis assay. DQ-BSA is BSA heavily loaded with BODIPY TR-X dye allowing self-quenching. Following endolysosomal delivery, DQ-BSA is cleaved, and the self-quenched dye bridges are broken, producing fluorescent puncta indicative of active endolysosomal proteolysis (Marwaha and Sharma, 2017). Spot number and spot intensity indicate the number of active endolysosomes and proteolytic activity within the endolysosomes, respectively. In iPSC-DaNs, CQ can ablate DQ-BSA signal (Figure 3.10 D).

MDW-941 is a BODIPY-tagged cyclophellitol that binds proximal to the active site of GCase, thereby staining the active hydrolase. MDW-941 stains lysosomal GCase preferentially to non-lysosomal GCase and has previously been used as an activity-based probe (Witte et al., 2010). Spot number and spot intensity of MDW-941 indicates the number of GCase containing lysosomes, and the abundance of GCase within the lysosome, respectively. In iPSC-DaNs, conduritol B epoxide (CBE) pre-treatment (a covalent GCase inhibitor) abolished MDW-941 signal, and CQ caused trending reductions in both spot number and intensity (Figure 3.10 E). ML-SA5, a TRPML1 agonist, increased both spot number and spot intensity in control iPSC-DaN (Figure 3.10 F).

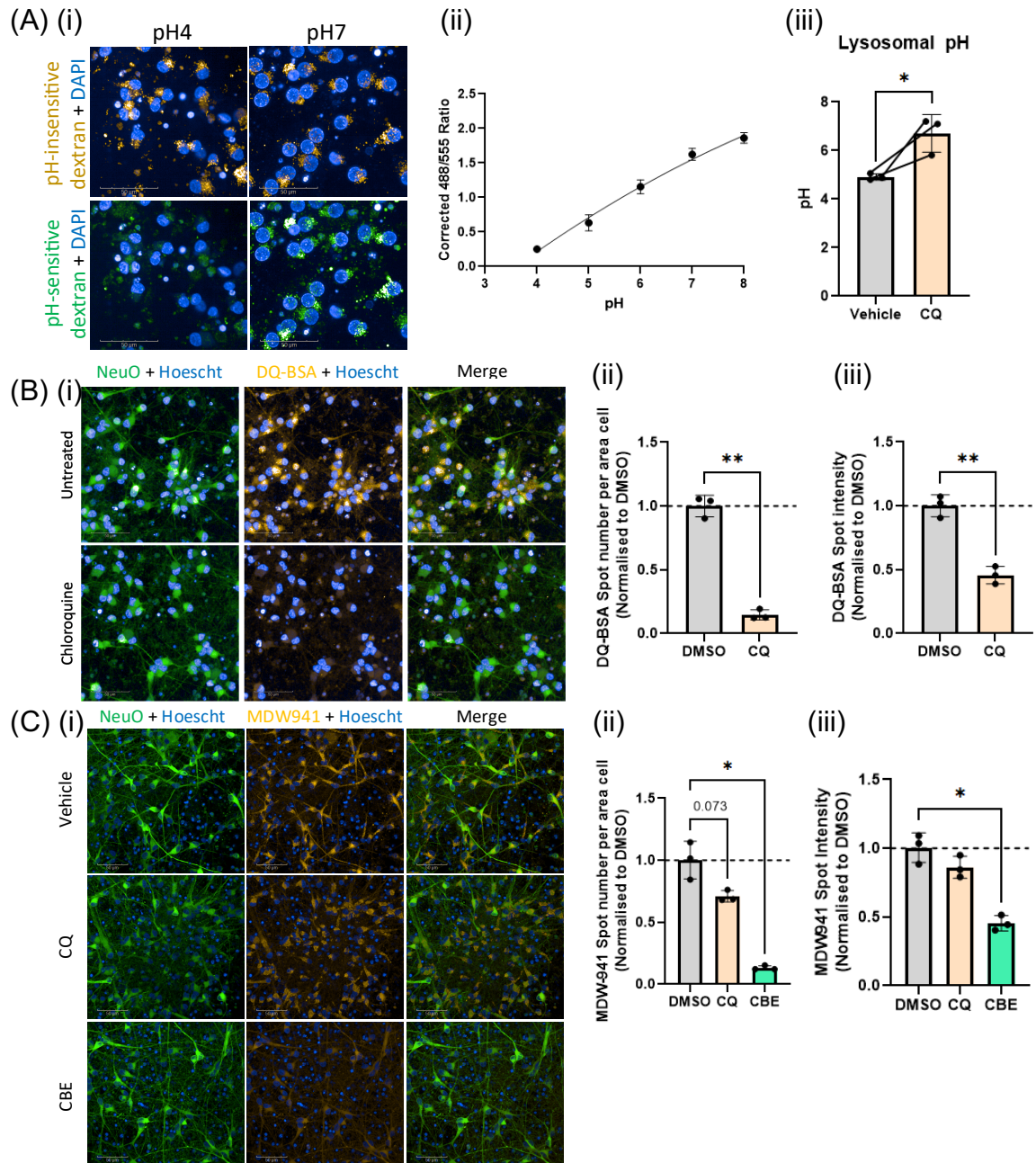


Figure 3.10 – Live-cell fluorescence-based lysosomal functional imaging assays in iPSC-DaNs and tool compounds.

(A) Ratiometric lysosomal pH dextran assay. (i) Representative image of iPSC-DaNs co-loaded with a pH-sensitive 488 fluorescent dextran (green) and a pH-insensitive 555 fluorescent dextran (orange) at pH 4 (left column) and pH 7 (right column). (ii) Representative pH curve with corresponding dextran intensity ratios in iPSC-DaNs. (iii) Quantification of lysosomal pH in iPSC-DaNs following CQ treatment. Paired t-test. (B) DQ-BSA (endolysosomal proteolysis) (i) Representative images of DQ-BSA stained (orange – middle column) live control neurons (stained with NeuO neurofluor - green) treated with vehicle (top) or CQ (bottom). (ii) Quantification of DQ-BSA puncta (ii) number and (iii) intensity following treatment of CQ. Paired t-test. (C) MDW-941 (lysosomal glucocerebrosidase probe). (i) Representative images of MDW-941 (orange – middle column) in live-control iPSC-DaNs (stained with NeuO neurofluor – green and Hoescht - blue) treated with Vehicle (top), CQ (middle) or CBE (bottom). Quantification of MDW-941 puncta (ii) number and (iii) intensity following treatment. RM One-way ANOVA with Dunnett's multiple comparison test. N=3 control lines, 3 differentiations. * $p < 0.05$, ** $p < 0.01$ p-value above bars: $p > 0.1$.

3.11 Assessing LAQ-824 effects upon lysosomal function in iPSC-DaNs using live-cell-based fluorescent imaging assays

LAQ-824 treatment increased TFEB and lysosomal gene expression in iPSC-DaNs, suggesting elevated lysosomal biogenesis. Live-cell based imaging assays were used to assess lysosomal pH, proteolysis and GCase following treatment. Surprisingly, LAQ-824 treatment significantly reduced DQ-BSA spot number but not spot intensity (Figure 3.11 A). Treatment also mildly increased lysosomal pH (Figure 3.11 B). MDW-941 spot number significantly decreased, however a significant increase in spot intensity was observed (Figure 3.11 C). These results indicate perturbed lysosomal function following HDAC inhibition, but treatment increased the abundance of GCase present in lysosomes.

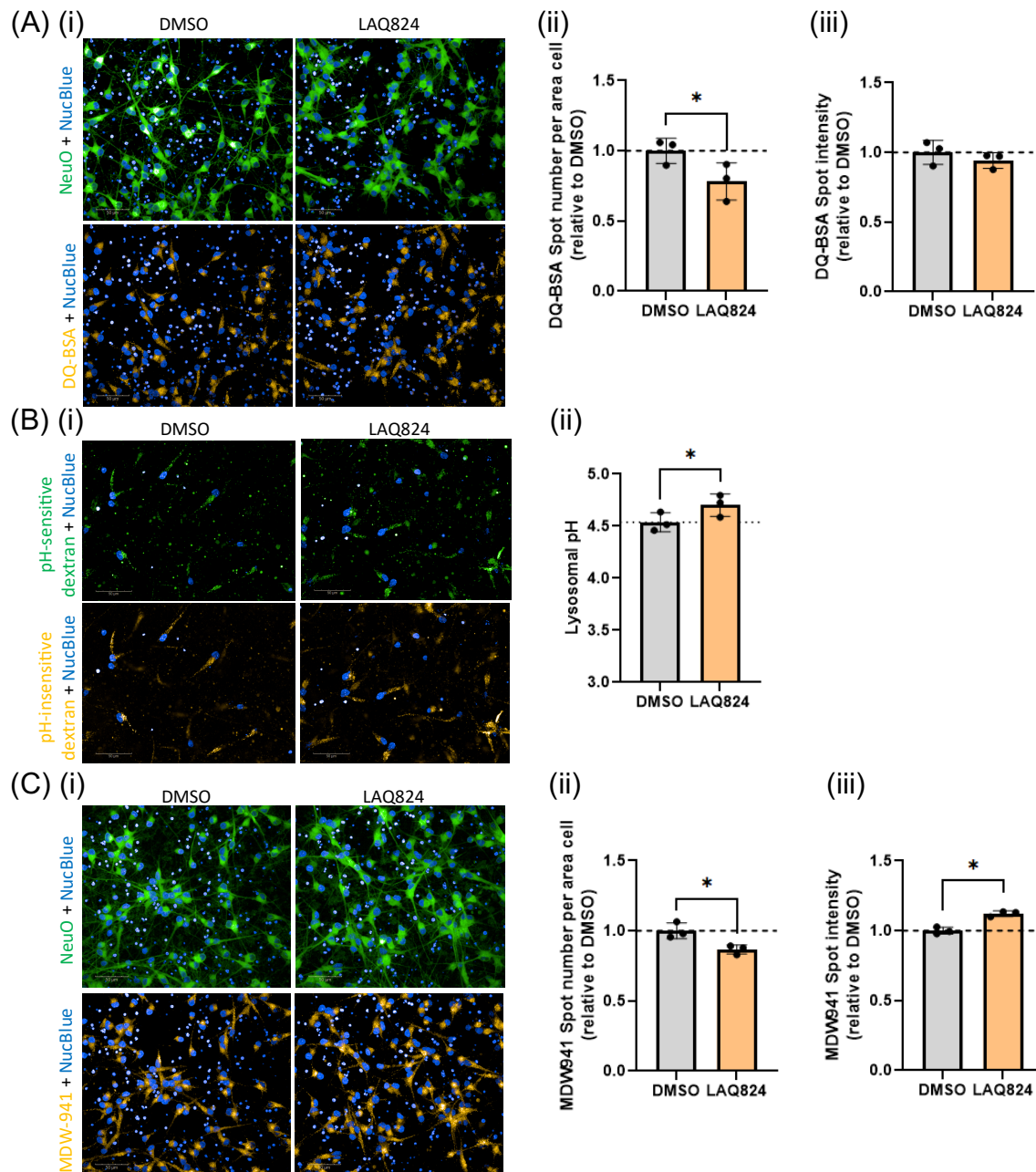


Figure 3.11 – Effects of pan-HDAC inhibition using LAQ824 on lysosomal function in live-DaNs using live-cell fluorescence-based imaging assays.

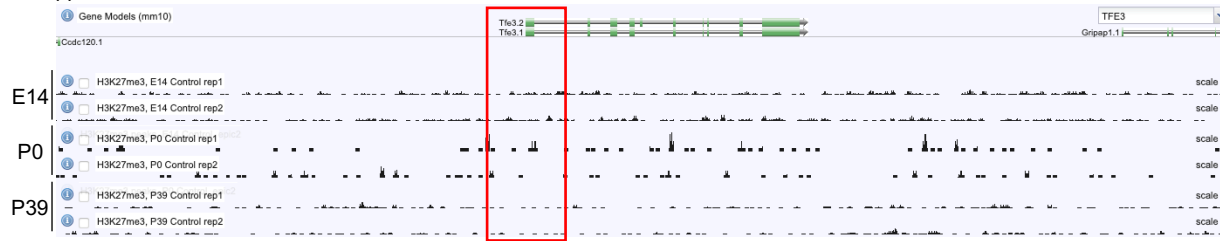
(A) (i) Representative images of DQ-BSA (orange) loaded live iPSC-DaNs (stained with NeuO – green) treated with LAQ824 (right column). Quantification of LAQ824 treatment on DQ-BSA puncta (ii) number and (iii) intensity in iPSC-DaNs. (B) LysoPH assay (i) Representative images of live iPSC-DaNs loaded with pH-sensitive (green) and pH-insensitive (orange) dextrans, treated with LAQ824. (ii) Quantification of lysosomal pH in response to LAQ824 treatment in iPSC-DaNs. (C) (i) Representative images of MDW-941 (orange)-loaded live iPSC-DaNs (stained with NeuO – green and Hoescht - blue) treated with LAQ824. Quantification of MDW-941 puncta (ii) number and (iii) intensity in iPSC-DaNs following LAQ824 treatment. N=3 control lines, 3 differentiations. Paired t-test. * $p < 0.05$.

3.12 Online Mouse ChIP-Seq dataset displays differential H3K27me3 histone modification proximal to the transcriptional start site of *Tfeb*

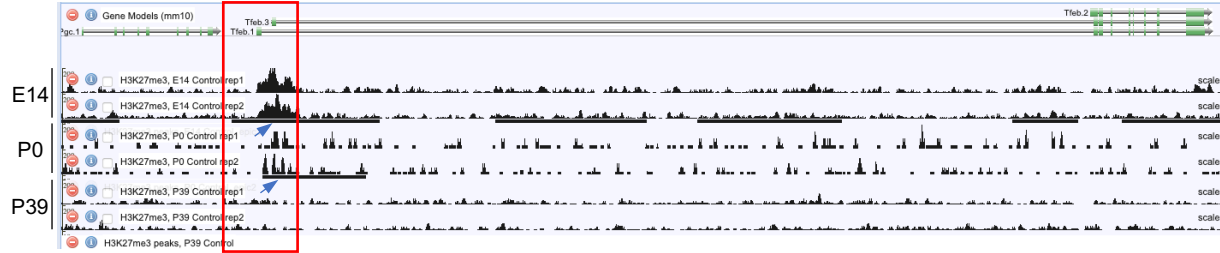
Recently, a publicly accessible online multi-omic dataset of murine neurons was published containing ChIP-Seq tracks for multiple histone modifications (Lie et al., 2022). Qualitative inspection of *Tfeb* and *Tfe3* loci revealed higher levels of H3K27me3 (a transcriptionally repressive histone modification) proximal to the *Tfeb* transcriptional start site (TSS) than the *Tfe3* TSS, and numerous H3K27me3 peaks were present throughout the *Tfeb* locus (Figure 3.12 A – horizontal black lines indicate modification peaks). This mark dissipated in aged mice but was still more abundant in the *Tfeb* locus. Interestingly, H3K27Ac, a mark of transcriptional activation, displayed a peak near the *Tfe3* TSS but not the *Tfeb* TSS in P39 mice (Figure 3.12 B – horizontal black line beneath the tracks indicate modification peaks). The *Tfeb* locus had this histone modification throughout the locus but had less prominent peaks near the TSS compared to *Tfe3*. These data and our results suggest repressive histone modifications of H3K27 upon the *Tfe3* locus may play a role in the transcriptional repression of *TFEB* in neurons.

(A) H3K27me3 – repressive epigenetic mark

(i) *Tfe3*



(ii) *Tfeb*



(B) H3K27Ac – activating epigenetic mark

(i) *Tfe3*



(ii) *Tfeb*

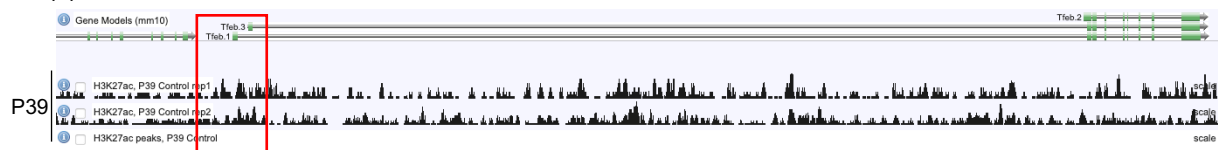


Figure 3.12 – Histone 3 Lysine 27 modification of *Tfeb* and *Tfe3* loci in mouse neurons from online ChIP-Seq dataset.

ChIP-Seq histone modification tracks from Lie et al., 2022, using the website application: https://brainome.ucsd.edu/annoj/mm_dnmt3a_ko/. (A) H3K27me3 tracks of (i) *Tfe3* and (ii) *Tfeb* loci from E14, P0 and P39 control mice. (B) H3K27me3 tracks of (i) *Tfe3* and (ii) *Tfeb* loci from P39 control mice. Black horizontal bars underneath ChIP-Seq enrichment values are quantified peak regions of corresponding histone modification. Red boxes indicate region of gene locus around transcriptional start site. Blue arrows show epigenetic peak of interest present within region proximal to the transcriptional start site (peaks displayed by horizontal black bars).

3.13 Discussion

In this chapter, we comprehensively demonstrated *TFE3* to be the primary expressed transcription factor between *TFEB* and *TFE3* in neurons. *TFEB* expression was more associated with non-neuronal cell-types. This finding replicated across a number of datasets from multiple models and multiple species, including iPSC-DaNs (both in-house and online), iPSC-Ngn2 neurons, iPSC-microglia, human post-mortem DaNs, and mouse single cell brain datasets.

TFE3 has previously been described as the primary transcription factor associated with autophagy in neurons, and *TFEB* activation is related to glia (Bordi et al., 2016). Our expression data supports this finding. However, studies have also observed *TFEB* presence in some neuronal models, frequently using antibody-based biochemical approaches (some at very high concentrations) (Awad et al., 2015; Liu et al., 2019; Mubariz et al., 2023). Assessment of *TFEB* transcript levels has been regarding fold change mRNA induction following treatment/perturbation, rather than considering total abundance (Awad et al., 2015; Mubariz et al., 2023).

From the single-cell mouse brain atlas data analysed here, sub-populations of neurons may express *TFEB*, and publications where *TFEB* has been observed may mirror these sub-populations. Online ChIP-Seq data displayed an age-dependent reduction in the repressive histone modification H3K27me3, suggesting *TFEB* neuronal transcriptional repression is a dynamic process, rationalising how subpopulations may express *TFEB*. It is clear however, that *TFEB* is minimally expressed in most neurons, and *TFE3* is the primary neuronal transcription factor of the two.

Recently, neuronal knockdown of *Tfe3* in mice caused neurodegeneration of DaNs (He et al., 2021). Our data suggest *TFEB* to be absent at physiologically relevant levels in DaNs, which may explain why *TFE3* knockdown triggered neurodegeneration, as *TFEB* cannot compensate for the loss of *TFE3*.

Two avenues of research were considered from these observations:

- 1) Understand neuron-specific transcriptional regulation of the *TFEB* locus and identify mechanisms to pharmacologically re-introduce expression.
- 2) Examine the therapeutic benefits of *TFE3* activation in neurons given its robust expression in the brain.

This chapter began to understand the former. Pharmacologically upregulating *TFEB* expression may enhance lysosomal function in neurons in co-operation with *TFE3*.

Indeed, previous publication showed that expression of both transcription factors is required for maximal response of lysosomal function (Martina et al., 2014). This raises the question as to why TFEB is repressed by neurons at all. This is likely during differentiation. TFEB promotes and maintains pluripotency in progenitor cells, and knockdown of TFEB/TFE3 enhances neuronal differentiation (Yuizumi et al., 2021). During differentiation, TFEB silencing may be required for appropriate lineage specification. We postulate that following terminal differentiation, therapeutic upregulation of TFEB expression could prove beneficial.

DNA methylation negatively correlated with *Tfeb* expression in mouse brain datasets. DNA methylation regulates neuronal differentiation, making it an attractive mechanism for *TFEB* modulation (Wang et al., 2016). We initially attempted to modulate DNA methylation through DNA methyltransferase inhibitor, SCI-1027.

Effects of DNA methyltransferase inhibition with SCI-2017 upon TFEB expression in neurons were inconclusive, impeded by toxicity. Global DNA methylation was not robustly altered by SCI-1027 treatment, perhaps because the treatment time was not sufficient to incur methylation changes (24 hours). This seems unlikely as DNA methylation half-life is in hours rather than days (Maegher, 2014). Basally, iPSC-models found no differences in methylation between *TFEB* and *TFE3* in any methylation context.

Interestingly, a 3' CpG island present on the *TFEB* locus and absent in *TFE3* was more highly methylated in neurons than microglia. Little is known about the function of 3' CpG islands, especially those that are intergenic. One publication suggests methylation of 3' CpG islands promotes transcription of genes (Yu et al., 2013). Thus, DNA methylation does not functionally correlate to the regulation of human neuronal *TFEB*.

A small epigenetic qRT-PCR screen found a pan-HDACi, LAQ824, to robustly induce *TFEB* expression in iPSC-Ngn2 neurons. This was replicated in iPSC-DaNs. LAQ824 also increased expression of downstream lysosomal gene expression and even enhanced the maturation of CTSD in iPSC-DaNs, although this seems paradoxical as an increase in lysosomal pH was later observed. HDAC inhibition selectively induced *TFEB* expression and not *TFE3*. Online ChIP-Seq datasets also saw H3K27Ac peaks present in the *TFE3* locus but not the *TFEB* locus, strengthening the hypothesis that differential histone acetylation at each locus may selectively regulate expression in neurons. Previous publications identified HDAC2 to transcriptionally regulate *TFEB*,

TFE3 and lysosomal genes in multiple cell-lines (Annunziata et al., 2019). Here, the authors find differential transcriptional response to HDAC inhibition between *TFEB* and *TFE3* and hypothesise it due to differential transcript abundance. This is similar to our findings. Thus, we postulate that HDAC modulation of *TFEB* and *TFE3* transcription is not neuronal specific, but the selective regulation of one transcription factor or the other is facilitated by HDACs in different cell-types. In this case, HDACs repress *TFEB* in neurons. Regulation of downstream lysosomal genes is also likely to be controlled by HDACs in this manner too (Annunziata et al., 2019). Thus, the induction of CLEAR genes following HDACi can be due to the direct de-repression of CLEAR promoters, *and* indirect induction by increasing *TFEB* expression.

LAQ824 increased lysosomal GCase, however the compound also elevated lysosomal pH and reduced the number of proteolytic endolysosomes. This may be resultant of broadly targeting multiple HDACs, increasing the likelihood of off-target effects. Further study into which HDACs regulate *TFEB* expression in neurons is essential for reducing this toxicity.

H3K27me3 signal was heightened proximal to the *TFEB* TSS in neurons. This modification is considered antagonistic to H3K27 acetylation (van der Vlag and Otte, 1999). H3K27 methylation and facilitation of transcriptional repression is performed by the polycomb repressor complex 2 (PRC2). PRC2 cooperates in tandem with HDACs to modify target histones and repress transcription (van der Vlag and Otte, 1999; Varambally et al., 2002). One may hypothesise that PRC2 inhibition may also induce *TFEB* expression in neurons. The next chapter addresses this hypothesis, aiming to confirm HDACi as a functional modulator of *TFEB* expression, and identify novel mechanisms of *TFEB* transcriptional regulation in neurons through high-throughput screening bioactive compounds in a larger-scale qRT-PCR screen in collaboration with Biogen.

Chapter 4: High-throughput Bioactive compound- and CRISPRi-based-Screening for neuronal *TFEB* and *TFE3* transcriptional activators

4.1 Introduction

Chapter 3 alluded to a transcriptional regulation upon *TFEB* in neurons by HDACs. We further built upon this hypothesis using a ChIP-Seq dataset, suggesting PRC2 may co-operate with HDACs to regulate *TFEB* transcription.

This chapter aims to explore this hypothesis and further uncover mechanisms of neuronal *TFEB* regulation by developing a high-throughput qRT-PCR method and screening 3,000 bioactive compounds targeting numerous different classes of regulatory targets.

TFEB regulation has primarily revolved around post-translational modification and localisation of TFEB. As such, previous screening approaches assessed induction of TFEB nuclear localisation (Song et al., 2016; Kanfer et al., 2020; Carling et al., 2023). Lysomotropic compounds and other stressors stimulate TFEB nuclear translocation (Lu et al., 2017; Zhitomirsky et al., 2018). This has hindered identification of biologically relevant and pharmacologically viable hit targets in screens, although efforts have been made to surpass these confounding effects through counter-screening (Carling et al., 2023).

As TFEB expression is negligible in neurons, screening by TFEB localisation is not feasible. qRT-PCR screening for *TFEB* transcriptional induction in neurons provides a novel approach to pharmacologically activate TFEB in neurodegeneration, utilising a disease-relevant cellular model. qRT-PCR screening hits will be less confounded by lysomotropic agents in neurons than translocation screens, as demonstrated in

Section 3.8 when CQ treatment failed to significantly upregulate *TFEB* expression. Furthermore, this strategy will uncover novel regulatory biology upon *TFEB* in neurons, derived from our previous comprehensive characterisation of neuronal *TFEB* biology. Hits will bridge a gap between neuroprotective effects seen by *TFEB* overexpression, and a therapeutically viable strategy for *TFEB* activation in neurodegeneration. Several *TFEB* transcriptional regulators have previously been identified in different cell types (Table 1.3). Our approach builds upon this and benefits from a large-scale, unbiased approach, using a cellular model that better replicates the transcriptional landscape of neurons in the brain.

Here, we developed and applied a high-throughput qRT-PCR method in iPSC-Ngn2 neurons to assess regulation of HDACs and PRC2 on *TFEB* expression, before screening 3000 bioactive compounds (Appendix S.3) to identify novel *TFEB* transcriptional regulators.

We also applied CRISPRi to our qRT-PCR screening method aiming to genetically identify individual HDACs regulating *TFEB* transcription (Section 1.5). This was carried out utilising an iPSC-Ngn2 line genetically modified to contain dCas9-KRAB machinery in the CLYBL safe-harbour locus (Tian et al., 2019). Thus, through viral-mediated delivery of sgRNA, we aimed to stably knockdown HDAC expression in this neuronal cell line to induce *TFEB* transcription.

Aims of this chapter:

- Develop and optimise a high-throughput qRT-PCR method in iPSC-Ngn2 neurons
- Validate and examine the role of HDAC and PRC2 complex in *TFEB* transcription in neurons
- Screen for novel transcriptional regulators of neuronal *TFEB* and *TFE3*, using a 3000 bioactive compound set
- Use high-content lysosomal imaging assays to examine functional consequences of hit target modulation
- Use CRISPRi in iPSC-Ngn2 neurons to delineate which HDACs are responsible for *TFEB* transcriptional regulation in neurons

4.2 Optimisation of high-throughput 1-step qRT-PCR method

We first optimised and compared 1-step and 2-step high-throughput qRT-PCR methods for assessing *TFEB* mRNA induction. 2-step qRT-PCR uses the commercially available 'Cells-to-Ct kit' modified for 384-well format.

1-step qRT-PCR is an inexpensive method utilising a 'homebrew'-style lysis buffer (Shatzkes et al., 2014). Briefly, the 1-step qRT-PCR method involves direct cellular lysis using a simple non-ionic detergent buffer. Lysate was added directly to the terminal qPCR plate containing a 1-step qRT-PCR mix (with duplexed qPCR probes). Reverse transcription (RT) and inactivation was carried out before the qPCR reaction was carried out in the same plate (Figure 4.1 A i). The 2-step qRT-PCR reaction involves direct cell lysis before the addition of a STOP solution to abolish DNase I activity. Lysate was then diluted in RT buffer (+RT enzyme) in a new qPCR plate and RT takes place for 60 minutes before inactivation. cDNA is added to a 2-step qPCR reaction with duplexed probes and qPCR is undertaken (Figure 4.1 A ii). 1-step qRT-PCR is substantially cheaper than 2-step qRT-PCR, and easier to carry out (Figure 4.1 B).

1-step qRT-PCR Lysis buffer was modified to improve neuronal lysis. Previous buffers used 0.25% Igepal CA-630, which did not lyse iPSC-Ngn2 neurons sufficiently to obtain high RNA yield (data not shown, carried out by Biogen). Thus, a stronger detergent, 0.2% Triton X-100 was used. Basal *TFEB* Ct values were much lower in the optimised 1-step method compared to the 2-step method (32 and 35, respectively – Figure 4.1 C). The high Ct value following 2-step qRT-PCR falls outside the log-linear range of the *TFEB* probes, thus cannot be reliably used (Figure 3.5 C iii).

The 1-step qRT-PCR reaction does not include DNase I. Although the probes were designed not to amplify gDNA, we assessed whether adding DNase I increased 1-step Ct values. As expected, DNase I + STOP addition to the method did not change *TFEB* Ct values (Figure 4.1 D). This demonstrated gDNA amplification wasn't occurring in the 1-step reaction, and low 2-step Ct values were not resultant from residual DNase I activity following inhibition.

A 10-point dose response following 48-hour LAQ824 treatment was also used to compared *TFEB* mRNA variability between 1-step and 2-step methods. Both displayed

similar average dose-responses, but the 1-step method was far less variable than the 2-step method (Figure 4.1 E).

These data show 1-step qRT-PCR a simpler, cheaper and more reliable method for high-throughput screening of *TFEB* mRNA activators in neurons.

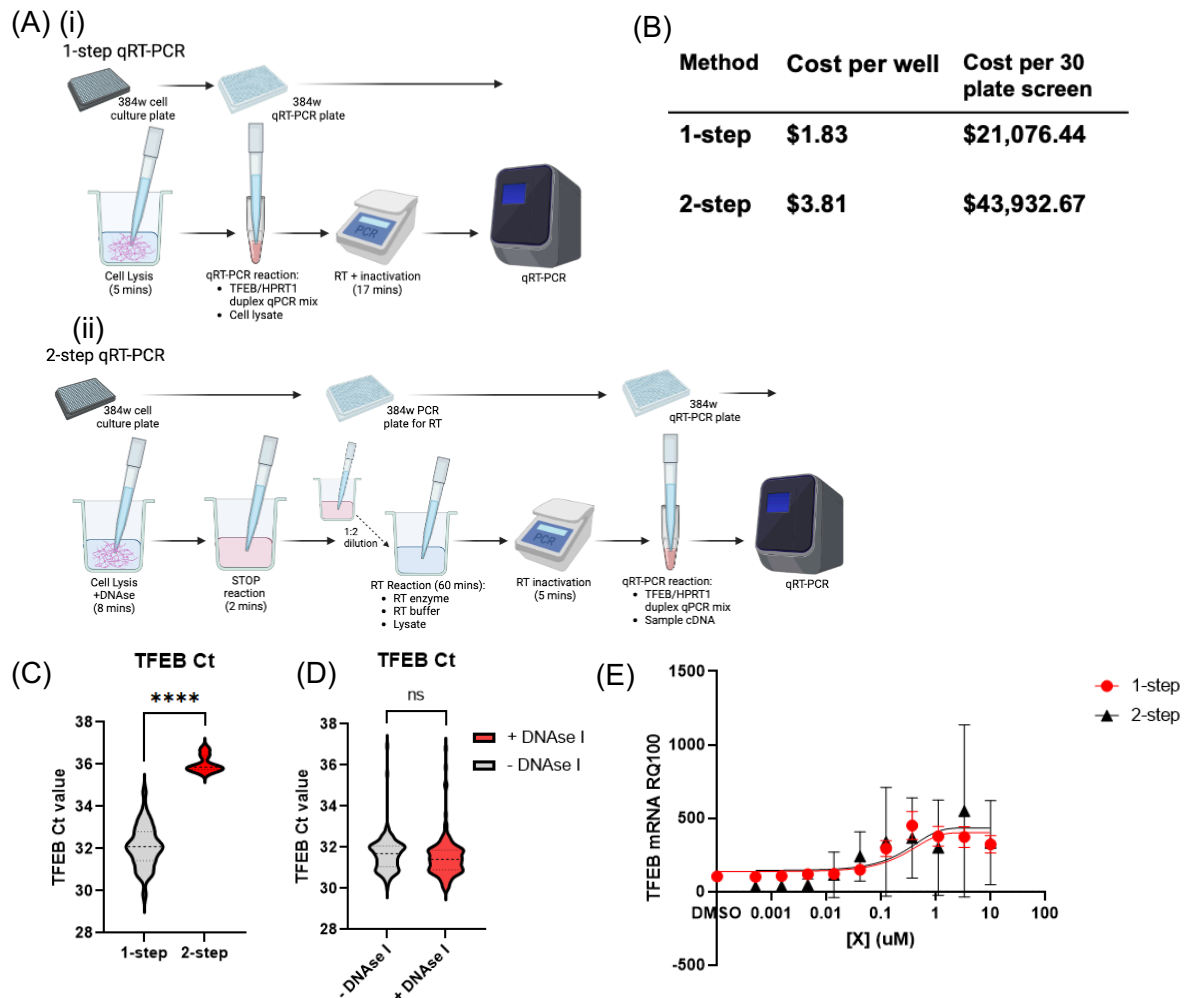


Figure 4.1 – 1-step vs 2-step qRT-PCR testing and optimisation.

(A) Schematic methodology of 384-well (i) 1-step qRT-PCR and (ii) 2-step qRT-PCR methods in iPSC-Ngn2 neurons. (B) Costs analysis of 1-step vs 2-step for a 30x 384w plate qRT-PCR screen and the cost per well reaction. (C) *TFEB* Ct values from a 384w plate processed via 1-step (grey) or 2-step (red). T-test; **** $p < 0.0001$. N=1 differentiation. (D) *TFEB* Ct values from wells treated with and without DNase I during the 1-step qRT-PCR process. N=1 differentiation. (E) 10-point dose response of *TFEB* RQ100 mRNA activity in response to LAQ824. Mean points $\pm$ standard deviation. Non-linear curve fitting represented by respective lines. N=2 differentiations.

4.3 Dose-response analysis of PRC2 and HDAC inhibitors on *TFEB* transcription

Using the 1-step qRT-PCR method, *TFEB* induction following treatment with 43 HDAC, Sirtuin, or PRC2-targeting compounds was assessed in iPSC-Ngn2. 10-point dose-responses, with 3-fold dilution from 10 μ M were carried out in duplicate in 384-well plates (Appendix S.2). Z-score (determines how many standard deviations a point lies from the baseline (DMSO) mean: $Z = \frac{mean_{Sample} - mean_{Control}}{SD}$) activity for *TFEB* mRNA induction was used instead of fold-change as this was more consistent across differentiations.

25 of 32 HDAC-targeting compounds displayed *TFEB* transcriptional activity (Figure 4.2 A; Table 4.2). Sirtuin- (Class III NAD⁺-dependent HDACs involved in both histone and protein deacetylation), and PRC2-targeting compounds did not increase *TFEB* expression (Figure 4.2 B-C; Table 4.2). Hence, HDACs robustly regulate neuronal *TFEB* expression, but Sirtuins or PRC2 components (EED/EZH2) alone do not.

Assessment of active/non-active compound targets dismisses Class II HDACs as likely *TFEB* regulators (Figure 4.2 A; Table 4.2). Robust induction of *TFEB* expression was found when compounds inhibited more than one of the Class I HDACs: HDAC1, HDAC2, or HDAC3. Inhibition of only one of these HDACs was not sufficient to produce a robust response (Figure 4.2 A; Table 4.2). Thus, Class I HDACs: HDAC1/2/3 are most likely candidates for *TFEB* transcriptional regulation in a partially redundant fashion.

(A) HDAC-targeting compounds

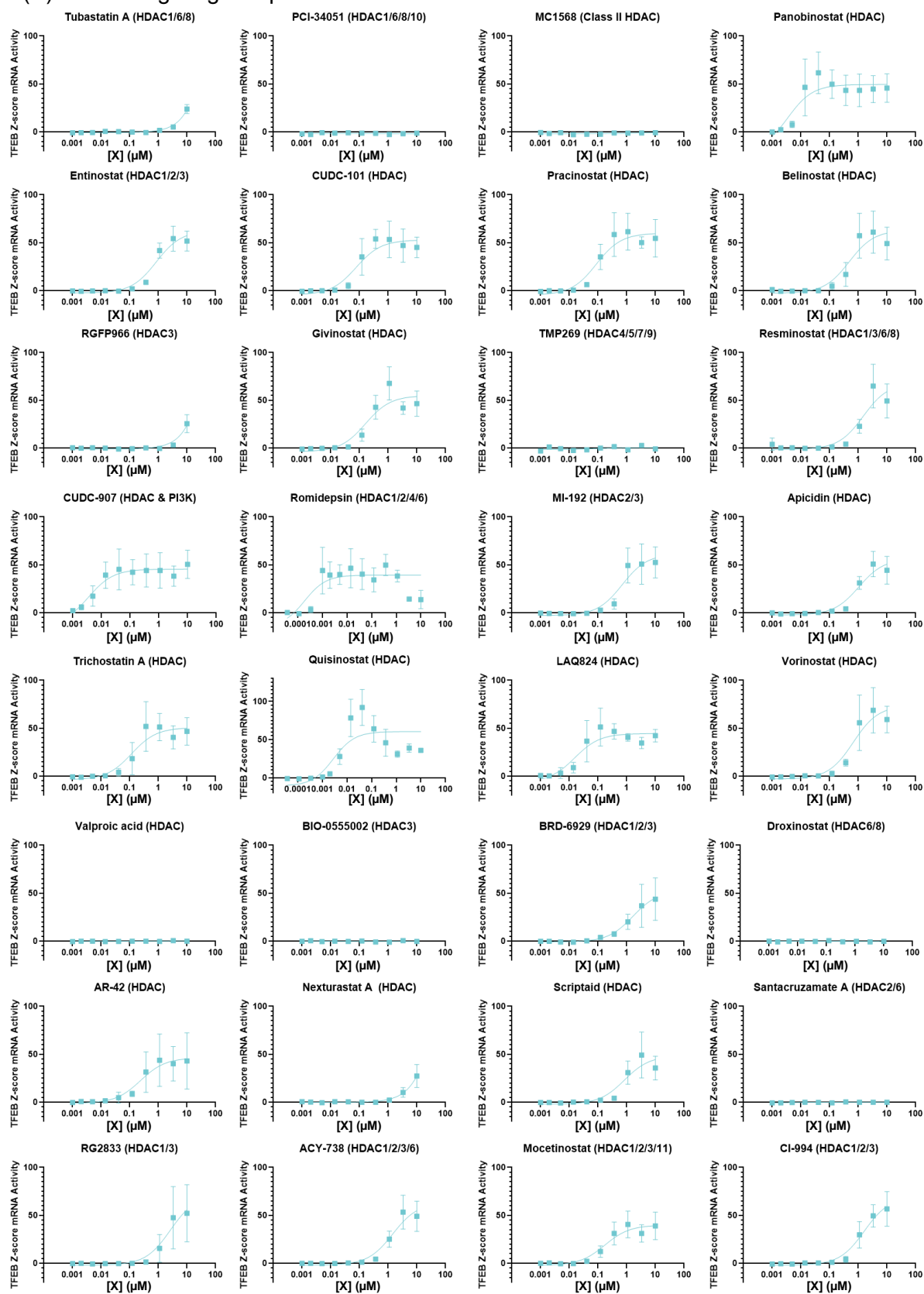
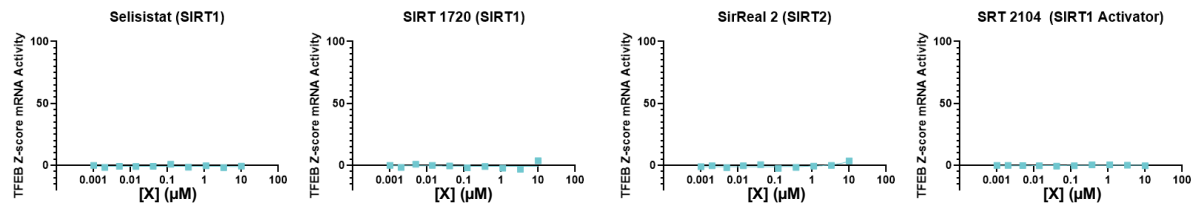


Figure 4.2 Continued...

(B) SIRT-targeting compounds



(C) PRC2-targeting compounds

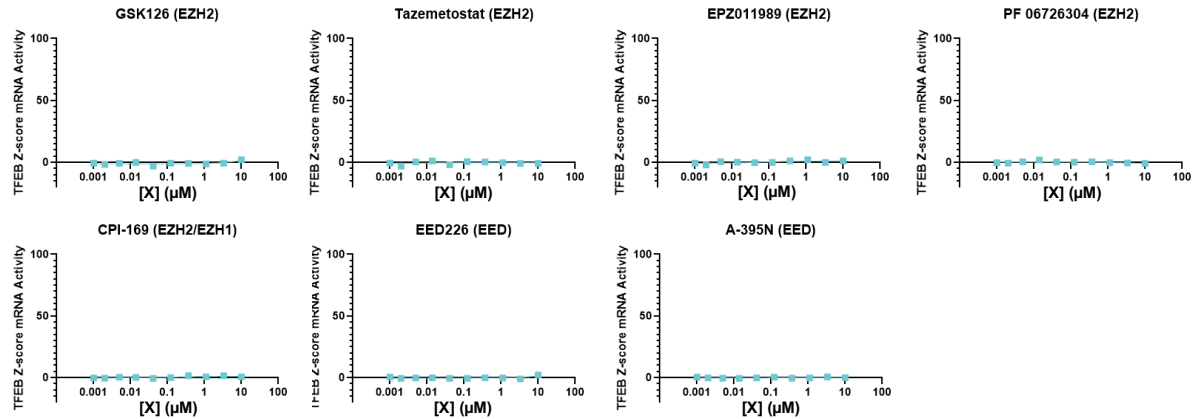


Figure 4.2 – Dose responses profiling HDAC, Sirtuin and PRC2 complex inhibitors for TFEB mRNA induction.

TFEB mRNA Z-score activity following 10-point dose-response of (A) HDAC-targeting (B) Sirtuin-targeting and (C) PRC2 complex-targeting compounds in iPSC-Ngn2 neurons. Mean $\pm$ S.D represented with non-linear fitted curve. Name of compound displayed in the title and primary targets displayed in brackets. N=2-4 differentiations.

Table 4.2 - HDAC-, SIRT-, PRC2-targeting compounds Activity upon *TFEB* mRNA expression and pharmacodynamics in iPSC-Ngn2

Compound Name	Target	Activity?	EC ₅₀	E _{max} (Z-score)
Tubastatin A	HDAC1/6/8/10	Yes	>10μM	N/A
PCI-34051	HDAC1/6/8/10	No	N/A	N/A
MC-1568	Class II HDACs	No	N/A	N/A
Panobinostat	Pan-HDAC	Yes	3.5nM	49.45
Entinostat	HDAC1/2/3	Yes	847.9nM	62.42
CUDC-101	Pan-HDAC	Yes	77.2nM	52.86
Pracinostat	Pan-HDAC	Yes	87.9nM	59.92
Belinostat	Pan-HDAC	Yes	484.6nM	62.98
RGFP966	HDAC3	No	N/A	N/A
Givinostat	Pan-HDAC	Yes	163.8nM	54.94
TMP269	HDAC4/5/7/9	No	N/A	N/A
Resminostat	HDAC1/3/6/8	Yes	1.505μM	68.23
CUDC-907	HDAC (&PI3K)	Yes	3.5nM	45.53
Romidepsin	HDAC1/2/4/6	Yes	0.187nM	39.39
MI-192	HDAC2/3	Yes	698.5nM	61.29
Apicidin	Pan-HDAC	Yes	1.055μM	56.35
Trichostatin A	Pan-HDAC	Yes	116.4nM	50.62
Quisinostat	Pan-HDAC	Yes	2.8nM	60.52
LAQ824	Pan-HDAC	Yes	15.6nM	44.47
Vorinostat	Pan-HDAC	Yes	670nM	73.55
Valproic acid	Pan-HDAC	No	N/A	N/A
BIO-0555002	HDAC3	No	N/A	N/A
Ci-994	HDAC1/2/3	Yes	1.639μM	69.29
BRD-6929	HDAC1/2/3	Yes	1.641μM	52.42

Droxinostat	HDAC6/8	No	N/A	N/A
AR-42 (HDAC-42)	Pan-HDAC	Yes	237.9nM	46.26
Nexturastat A	Pan-HDAC	Yes	>10μM	N/A
Scriptaid	Pan-HDAC	Yes	843.7nM	48.35
Santacruzamate A	HDAC2/6	No	N/A	N/A
RG2833	HDAC1/3	Yes	2.607μM	70.72
ACY-738	HDAC1/2/3/6	Yes	1.456μM	63.04
Mocetinostat	HDAC1/2/3/11	Yes	172.3nM	39.87
Selisistat	SIRT1	No	N/A	N/A
SIRT 1720	SIRT1	No	N/A	N/A
SirReal2	SIRT2	No	N/A	N/A
SRT 2104	SIRT1 (Activator)	No	N/A	N/A
GSK126	EZH2	No	N/A	N/A
Tazemetostat	EZH2	No	N/A	N/A
EPZ011989	EZH2	No	N/A	N/A
PF 06726304	EZH2	No	N/A	N/A
CPI-169	EZH1/EZH2	No	N/A	N/A
EED226	EED	No	N/A	N/A
A-395N	EED	No	N/A	N/A

4.4 Stringency testing the *TFEB* qRT-PCR method for high-throughput screening

We selected ACY-738 as a positive screening control and carried out several stringency assays. Z'-factor ($Z' = 1 - \frac{3(SD_{sample} + SD_{control})}{(mean_{sample} - mean_{control})}$) was used to determine assay quality as this assesses statistically how likely a positive control (3μM ACY-738) is different from the baseline (DMSO) by 3 standard deviations (99% confidence interval). Z' above 0.3 was required for plates to be accepted during screening. Z' with ACY-738 was 0.44±0.09. Consistent pharmacodynamic response was assessed by EC₅₀ variance across multiple dose-responses from ACY-738 and 21 other HDAC inhibitors. ACY-738 pEC₅₀ was extremely consistent (6.00±0.03), and all compounds, bar one, fell within 2 standard deviations of pEC₅₀ difference across multiple differentiations (Mean pEC₅₀ difference = 0.19) (Figure 4.2 A-B; Table 4.3).

False discovery rate and spatial biases were assessed using DMSO-treated plates. A checkerboard assay with ACY-738, DMSO and a reference compound (Scriptaid) was used to observe spatial bias, assay window and compound variability. Spatial bias was observed so DMSO correction plates were added to the screen (Table 4.3). GeneData Screener used these plates to correct compound plate spatial bias in the screen.

Duplicates on separate 384-w plates were found to produce a lower false discovery rate (FDR) than if adjacent on the same plate. Thus, compound replicates were designated to independent plates in the screen (Table 4.3).

A high coefficient of variation (CV%) following DMSO treatment was observed (50%). This can be attributed to low *TFEB* expression in neurons. As this screen intended to observe robust inducers of *TFEB*, a high CV% was acceptable (50% CV would require ~2.5-fold increase for hit compound identification). Positive and reference compound (ACY-738 and Scriptaid) CV% was satisfactory (~20% and ~17% respectively), therefore no further corrections were needed (Table 4.3).

Table 4.3 – qRT-PCR Stringency testing

Stringency Test	Rationale	Outcome	Solutions
DMSO only plates	Spatial biases False-discovery rate (FDR)	Minor spatial bias FDR = ~0.5% if adjacent duplicate	DMSO correction plates Duplicate points over 2 plates in screen = 0%FDR

Checkerboard positive control vs DMSO	Spatial bias CV% for DMSO and controls Z' across plate	Minor spatial bias High DMSO CV%	DMSO correction plates Low TFEB expression, only want robust inducers
320 compound mini-screen	Identify hit-rate and confirmation rate	Non-stringent hit rate (average duplicate > 3 Z-score) = 5%	Stringent hit rate (both duplicate >3 Z-score) = 1.3%
Plate Z' > 0.3	Appropriate assay window between - & + control	ACY-738 Z' plates > 0.3	None needed
EC ₅₀ variance	Consistent dose-response pharmacodynamics	EC ₅₀ variance within 2 S.D of positive control for 20/21 compounds	None needed

We next carried out a 320-compound mini high-throughput screen to identify expected compound hit rate. Non-stringent hits were classified as the average duplicate Z-scores response >3, whereas a stringent hit was identified where both duplicates display Z-score > 3. Following screening 320 compounds, expected non-stringent and stringent hit rates were 5% and 1.3%, respectively (Table 4.3). Thus, stringent hit identification was selected for the screen.

The full screening strategy is displayed in Figure 4.3 C. Briefly, the primary 3,000 compound screen was split over 2 differentiations, one week apart. Each compound was mapped in duplicate on separate plates and administered at 10 μ M to DIV14 iPSC-Ngn2 neurons for 48 hours. *TFEB* Z-score response was normalised within each plate (accounting for differential plate response) and spatial bias was corrected using DMSO correction plates. ACY-738 Z' for each plate was required to be above 0.3 to be accepted, or it would be re-screened. Stringent hit identification was used (both duplicate Z-score>3). Pharmacodynamic *TFEB* activation was confirmed by 10-point dose-response. Target enrichment analysis was used to define most likely *TFEB* regulators. Following, TFEB protein levels and lysosomal function was assessed in response to compounds modulating selected targets using high-content imaging.

TFEB is a CLEAR gene and TFE3 translocation may induce *TFEB* transcription in neurons. mTOR inhibition, lysosomal perturbation, ER stress and apoptotic stimuli have been shown to promote TFEB/TFE3 translocation (Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Martina et al., 2016; Brady et al., 2018; Yang et al., 2022). Thus, we assessed *TFEB* expression using our high-throughput qRT-PCR method following Torin1, CQ, Tunicamycin (promotes ER stress) and Staurosporine

(pan-kinase inhibitor that induces apoptosis) dose-responses. Torin1 had no effect on *TFEB* induction, whereas high concentrations of CQ, and tunicamycin mildly induced expression to 4-fold and 2-fold, respectively (Figure 4.3 D). Staurosporine also induced *TFEB* expression 6-fold, before depleting at higher concentrations. Importantly, *TFEB* induction following tool compound stimuli was minimal compared to HDAC inhibitor, ACY-738 (Figure 4.3 D). Weaker hit compounds were susceptible to being confounded by stress, however this screen was substantially less prone to cellular stress bias than previous translocation screens.

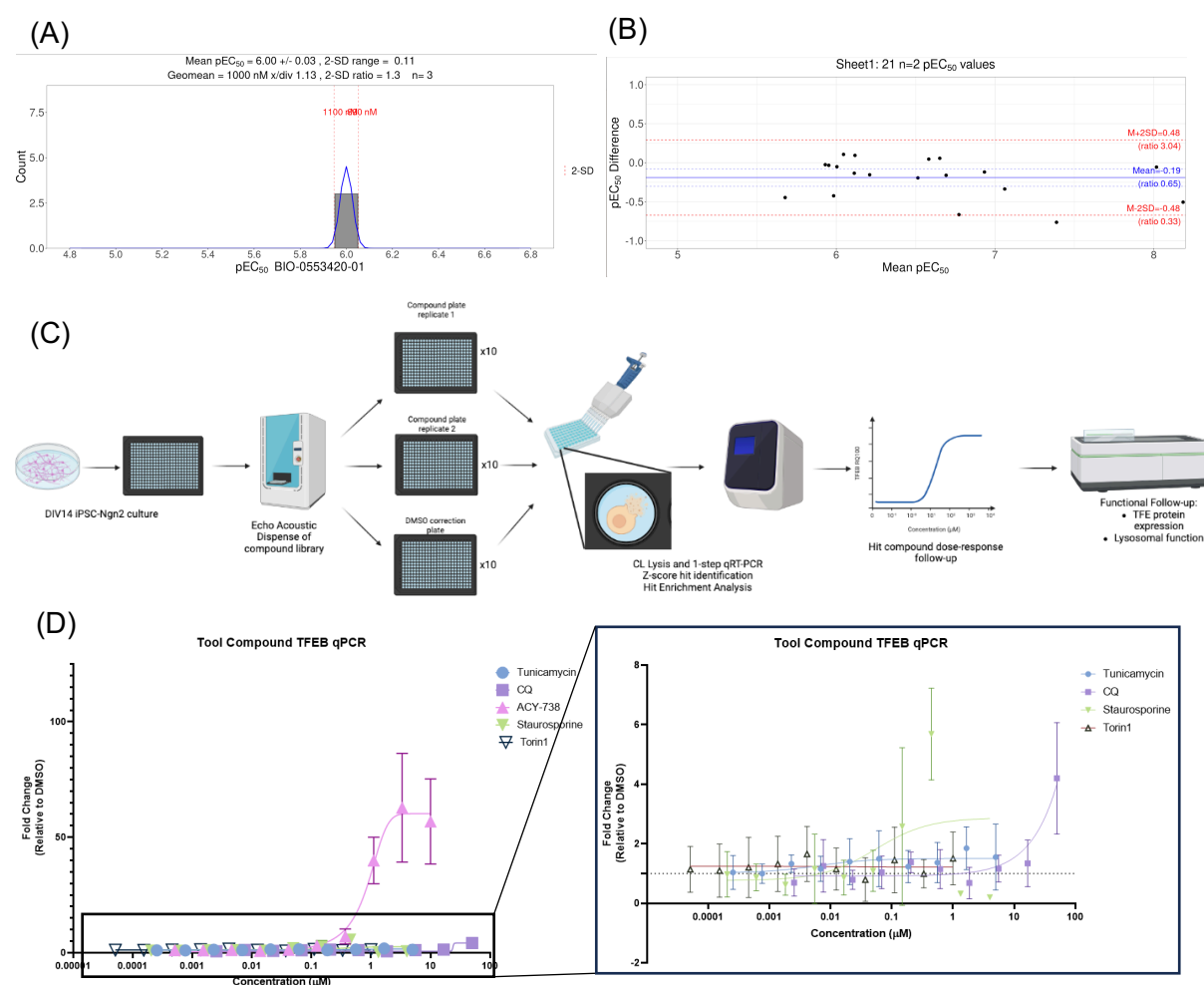


Figure 4.3 – Stringency testing the 1-step qRT-PCR high-throughput method.

(A) Histogram of the pEC₅₀ of positive compound, ACY-738 over 3 dose responses. Red lines represent 2 S.Ds either side of the mean. N=3 differentiations. (B) Variation in pEC₅₀ of 21 compounds following 2 dose-responses. Blue line indicates the average pEC₅₀ change from all 21 compounds. Red line represents 2 standard deviations of pEC₅₀ differential from all compounds. N=21 compounds, 2 differentiations. (C) Schematic of the screen layout following stringency testing. (D) *TFEB* mRNA fold induction following treatment with Torin1 (brown - hollow triangles), Tunicamycin (blue - dotpoint), CQ (purple – Square), ACY-738 (pink – triangle) and Staurosporine (green – inverse triangle). Mean fold change ± standard deviation with non-linear fitted curve. Expanded box represents zoomed in view of less-potent stressors Torin1, CQ, Staurosporine and Torin1. N=2 differentiations.

4.5 Screening bioactive compounds to activate *TFEB* transcription in iPSC-Ngn2 Neurons

Following primary screening of 3,000 bioactive compounds, 91 stringent hits were identified for dose-response follow-up. Of these 91 compounds, 62 were confirmed to activate *TFEB* transcription by dose-response (Figure 4.4 A). Enrichment analysis was calculated as the logarithmic of proportion of hits in the library on-target of interest, relative to the proportion of all compounds in the library on-target of interest (Figure 4.4 B). Compound class (Table S.8) enrichment analysis identified chaperone, epigenetic and ATPase-targeting compounds most enriched (Figure 4.4 C). The fraction above each point shows: $\frac{\text{No. of target hits}}{\text{No. of targeting compounds}}$ for each target of interest. Unsurprisingly, enrichment analysis of primary compound targets found HDAC-targeting compounds most enriched for *TFEB* modulation, with 20/25 compound hits (Figure 4.4 D). Noteworthy, all screened HDAC-targeting compounds responded as expected considering previous dose-response experimentation, except for romidepsin. Romidepsin was a potent *TFEB* activator, but at higher concentrations (particularly 10 μM) transcription falls drastically, thus its absence from hit identification is not surprising (Figure 4.2 A). B2 receptor and NSD2-targeting compounds were next most-enriched but only 1 compound was screened. Interestingly, HSP90 and proteasome inhibitors displayed high enrichment with 7/12 and 5/6 hit compounds, respectively (Figure 4.4 D). We averaged the maximum fold-change from dose-responses for each confirmed compound to assess the magnitude of response elicited by modulating each target (Figure 4.4 E). Robust induction of *TFEB* could be seen following modulation of most targets. Of particular interest were the 3 highly enriched targets with multiple targeting compounds – HDACs, proteasome and HSP90 (Figure 4.4 E).

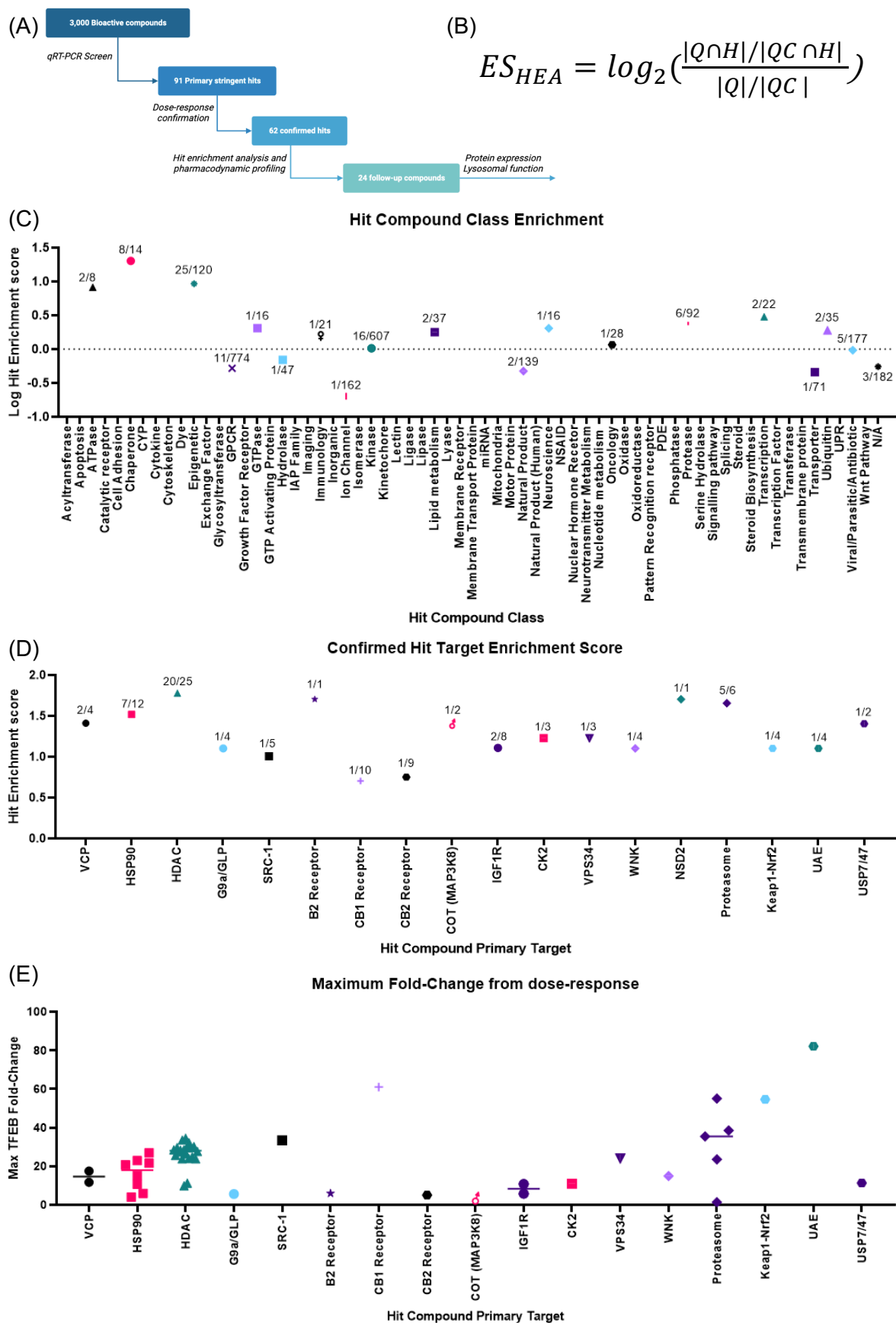


Figure 4.4 – Bioactive compound qRT-PCR screening for *TFEB* transcriptional inducers in neurons.

(Figure 4.4 continued...) (A) Flowchart of screening process. 3,000 bioactive compounds were screened. 91 stringent hit activators were identified, 62 of which were confirmed by 2 dose-response follow-up differentiations. Hit enrichment analysis identified most likely TFEB regulating targets, and pharmacodynamic assessment of hit compounds led to the selection of 24 follow-up compounds assessed for protein induction and lysosomal function. (B) Enrichment analysis equation, where Q = Input compounds targeting protein or class of interest, Qc = Input compounds not targeting protein or class of interest, and H = hit compounds. (Adapted from Park et al., 2019). (C) Enrichment score for all compound classes in the screen. Confirmed hits used for enrichment. (D) Enrichment score for all primary targets of confirmed compound hits in screen. Fraction above point indicates (hit compounds)/(total compounds). (E) Maximum TFEB RQ100 observed in dose-responses from confirmed hit compounds relating to their targets. N=2 differentiations.

STRING was used to visualise interactions between hit targets, revealing HSP90 as a central protein linking many targets (Figure 4.5 A). KEGG pathway analysis was also used to identify enriched pathways (Figure 4.5 B). Due to the complex nature of the proteasome and the unidentified effector HDACs, pathway analysis was skewed toward functions relating to these proteins. Interestingly, pathways associated with neuropathological disorders (spinocerebellar ataxia, Parkinson's disease, Prion disease, Huntington disease, Amyotrophic lateral sclerosis and Alzheimers disease) were enriched, although this enrichment was mainly associated with proteasomal subunits (Figure 4.5 B).

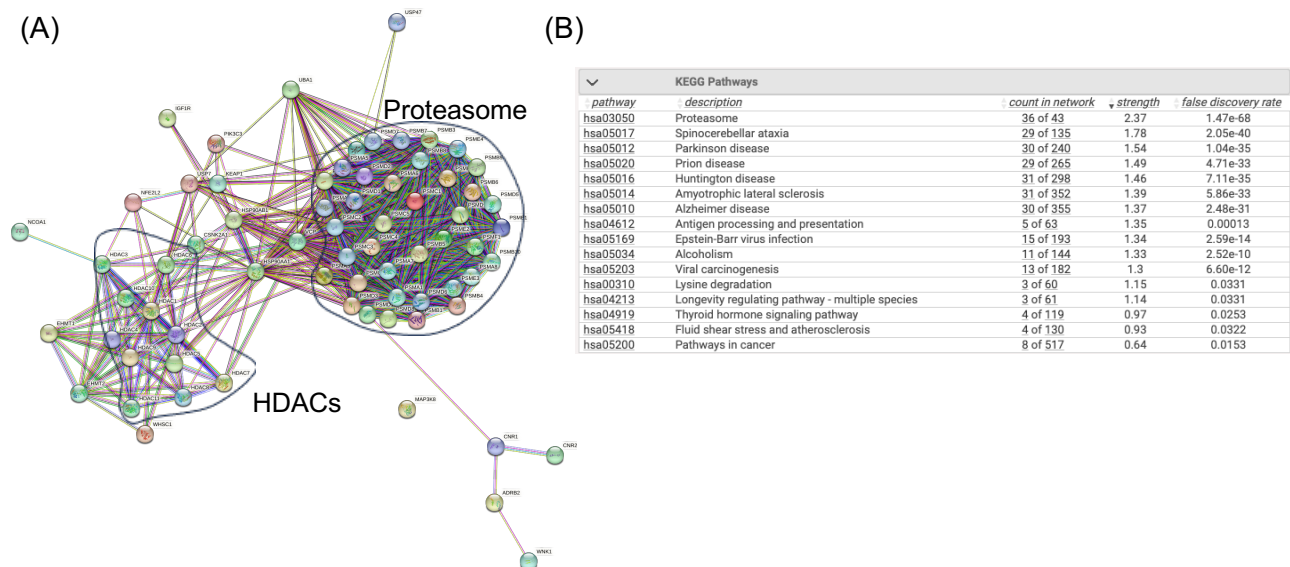


Figure 4.5 – Connectivity and pathway analysis of all confirmed hit targets from *TFEB* qRT-PCR targets.
 (A) STRING connectome analysis of all primary *TFEB* targets relating to confirmed compound hits. Histone deacetylases and proteasomal subunits are labelled and encircled. (B) KEGG Pathway enrichment analysis of hit *TFEB* regulatory targets.

4.6 High-content imaging to examine TFEB protein expression using qRT-PCR Screening hits

We next assessed whether increased *TFEB* mRNA translated to increased protein expression following modulation of selected targets in iPSC-Ngn2 neurons. We carried out a 10-point dose-response on compounds targeting HDACs, proteasome and HSP90; and immunocytochemically stained for TFEB in iPSC-Ngn2 neurons. Correlation between *TFEB* mRNA and TFEB intensity was plotted to observe the relationship between mRNA induction and protein induction. We found HDAC inhibition robustly induced TFEB protein expression in all HDAC compounds tested (Figure 4.6 A-B). A strong and significant correlation was seen between TFEB mRNA and protein expression for HDAC inhibition (Figure 4.6 C).

Proteasome inhibitors did not robustly induce TFEB intensity in any compounds tested (Figure 4.7 A). No correlation was observed between mRNA levels and protein intensity with proteasome inhibitors (Figure 4.7 B).

HSP90 inhibitors displayed lower *TFEB* mRNA induction than HDAC and proteasome inhibitors, thus little induction of TFEB intensity was observed (Figure 4.7 C). Only 4 of 7 compounds demonstrated increased TFEB intensity. Despite this, a significant positive correlation between TFEB mRNA and protein expression was observed (Figure 4.7 D).

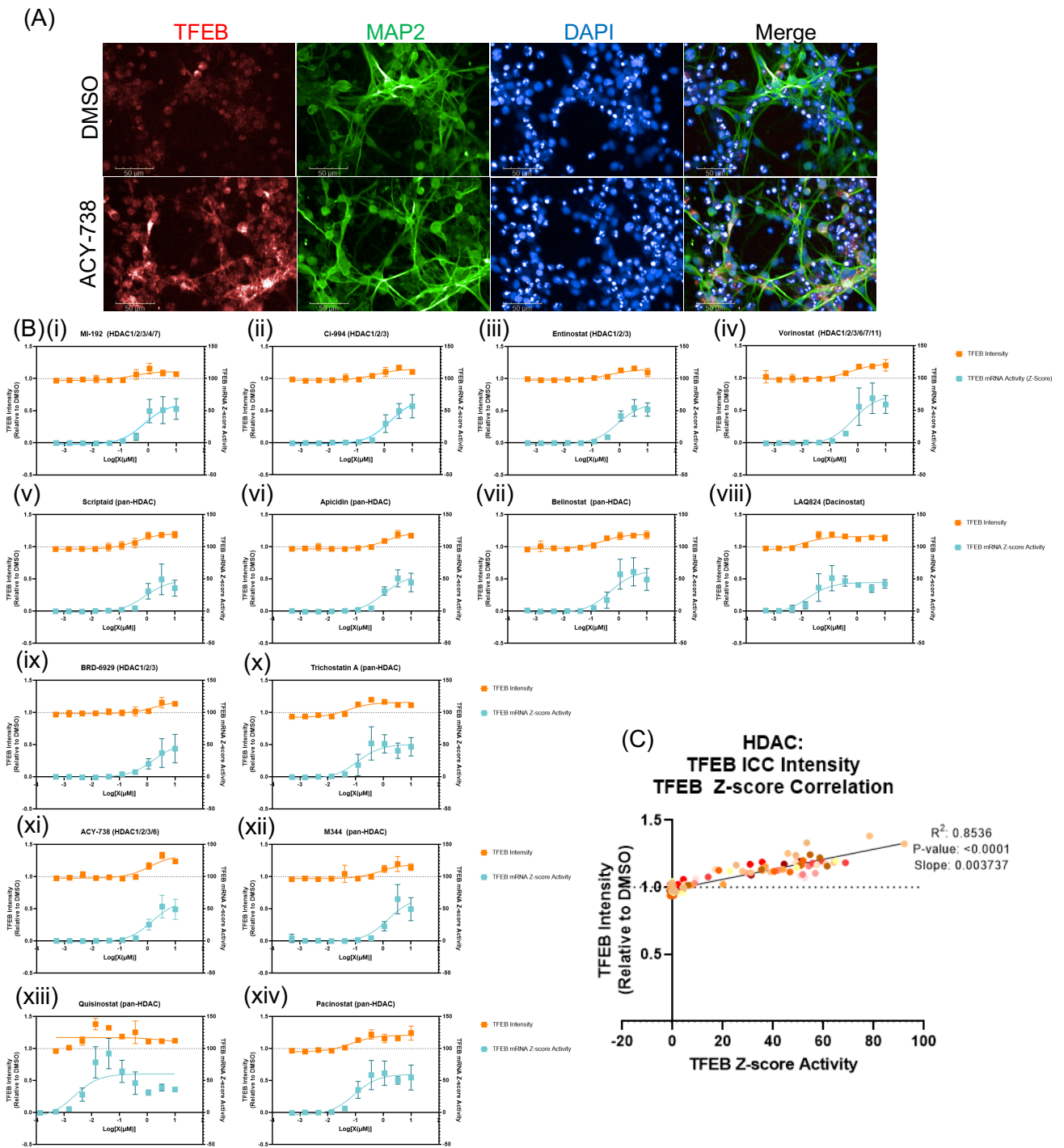


Figure 4.6 – TFEB protein induction following HDAC inhibition.

(A) Representative images of iPSC-Ngn2 neurons treated with DMSO and 3 μ M ACY-738 immunostained for TFEB (red), MAP2 (green) and DAPI (blue). (B) (i-xiv) 10-point dose-responses of *TFEB* mRNA Z-score activity (teal – right y axis) and fold change TFEB cellular intensity relative to DMSO (orange – left y axis) in iPSC-Ngn2 neurons treated with follow-up hit HDAC inhibitors. Primary targeted HDACs are in brackets next to compound names above the graphs. Mean TFEB mRNA Z-score and TFEB intensity plotted $\pm$ standard deviation, with non-linear fitted curve. (C) Correlation of TFEB mRNA Z-score activity plotted against TFEB cellular intensity relative to DMSO. Each colour represents a different HDAC-targeting compound. Simple linear regression analysis was used and R-squared goodness of fit, p-value and fitted line gradient (slope) was stated on the graph N=2 differentiations.

These data show induction of *TFEB* mRNA following HSP90 and HDAC inhibition translates to increased protein levels in iPSC-Ngn2 neurons, but not following proteasome inhibition.

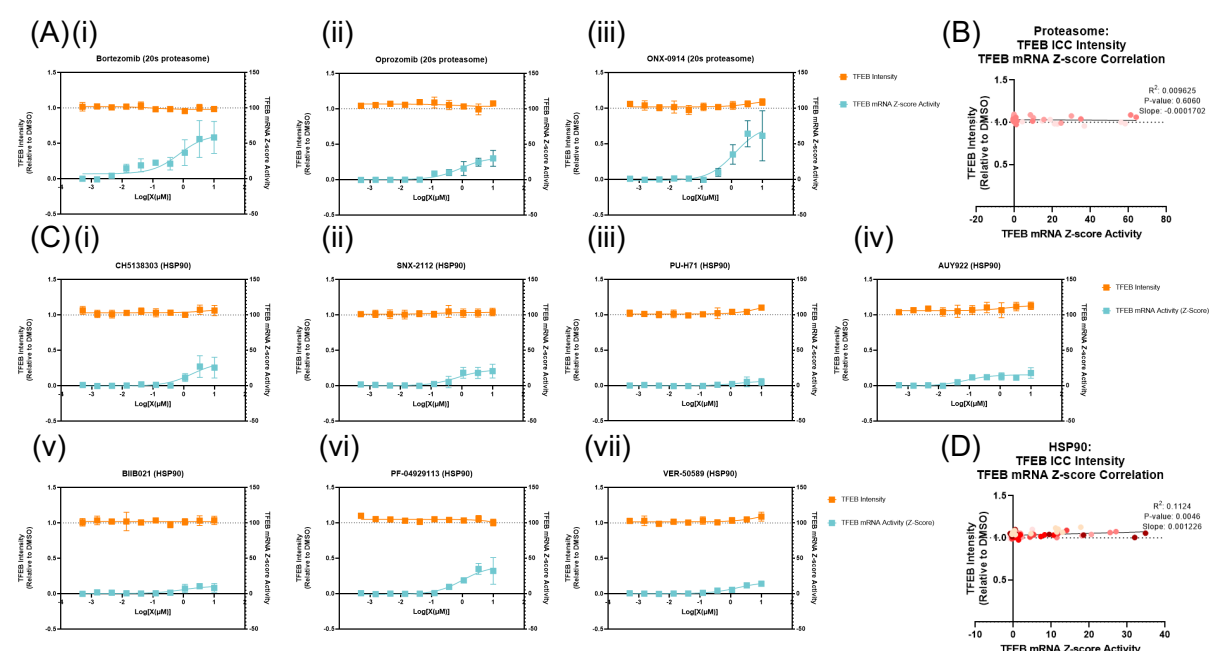


Figure 4.7 – TFEB protein induction following proteasomal and HSP90 inhibition. 10-point dose-responses of *TFEB* mRNA Z-score activity (teal – right y axis) and fold change TFEB cellular intensity relative to DMSO (orange – left y axis) in iPSC-Ngn2 neurons treated with follow-up hit (A (i-iii)) proteasome and (C (i-vii)) HSP90 inhibitors. Mean TFEB mRNA Z-score and TFEB intensity plotted $\pm$ standard deviation, with non-linear fitted curve. Correlation analysis of TFEB mRNA Z-score activity plotted against TFEB cellular intensity relative to DMSO for (B) proteasome and (D) HSP90 inhibitors. Each colour represents a different hit compound. Simple linear regression analysis was used and R-squared goodness of fit, p-value and fitted line gradient (slope) was stated on the graph. N=2 differentiations.

4.7 Functional lysosomal follow-up of selected

TFEB qRT-PCR hits

Increased *TFEB* expression should promote lysosomal biogenesis and function in neurons. We used high-content lysosomal imaging assays, MDW-941 and DQ-BSA to examine the effects of *TFEB* activators upon lysosomal GCase and endolysosomal proteolysis, respectively. The same compounds were used as in Section 4.6, assayed in tandem.

Most HDAC-targeting compounds displayed a robust increase in MDW-941 spot intensity, and less obvious changes in spot number (Figure 4.8 A). Increases in MDW-941 spot intensity appeared following a slight lag after mRNA induction. A significant positive correlation was observed between mRNA induction and both MDW-941 spot number and intensity following HDAC inhibition, albeit spot intensity induction was far greater than spot number (Figure 4.8B).

DQ-BSA responses varied following HDAC-targeting compound treatment (Figure 4.9 A). Frequently, DQ-BSA spot intensity was elevated relative to DMSO at concentrations preceding *TFEB* mRNA induction but decreased at high concentrations. A significant negative correlation between *TFEB* expression and DQ-BSA spot intensity was observed, although this was varied and intensity was mostly above baseline, before declining (Figure 4.9 B ii). No correlation was observed between DQ-BSA spot number and *TFEB* expression following HDAC inhibition (Figure 4.9 B i). Thus, HDAC inhibition stimulates an increased level of lysosomal GCase within neurons that correlates with the induction of *TFEB*, however, higher compound concentrations tend to mildly inhibit endolysosomal proteolysis.

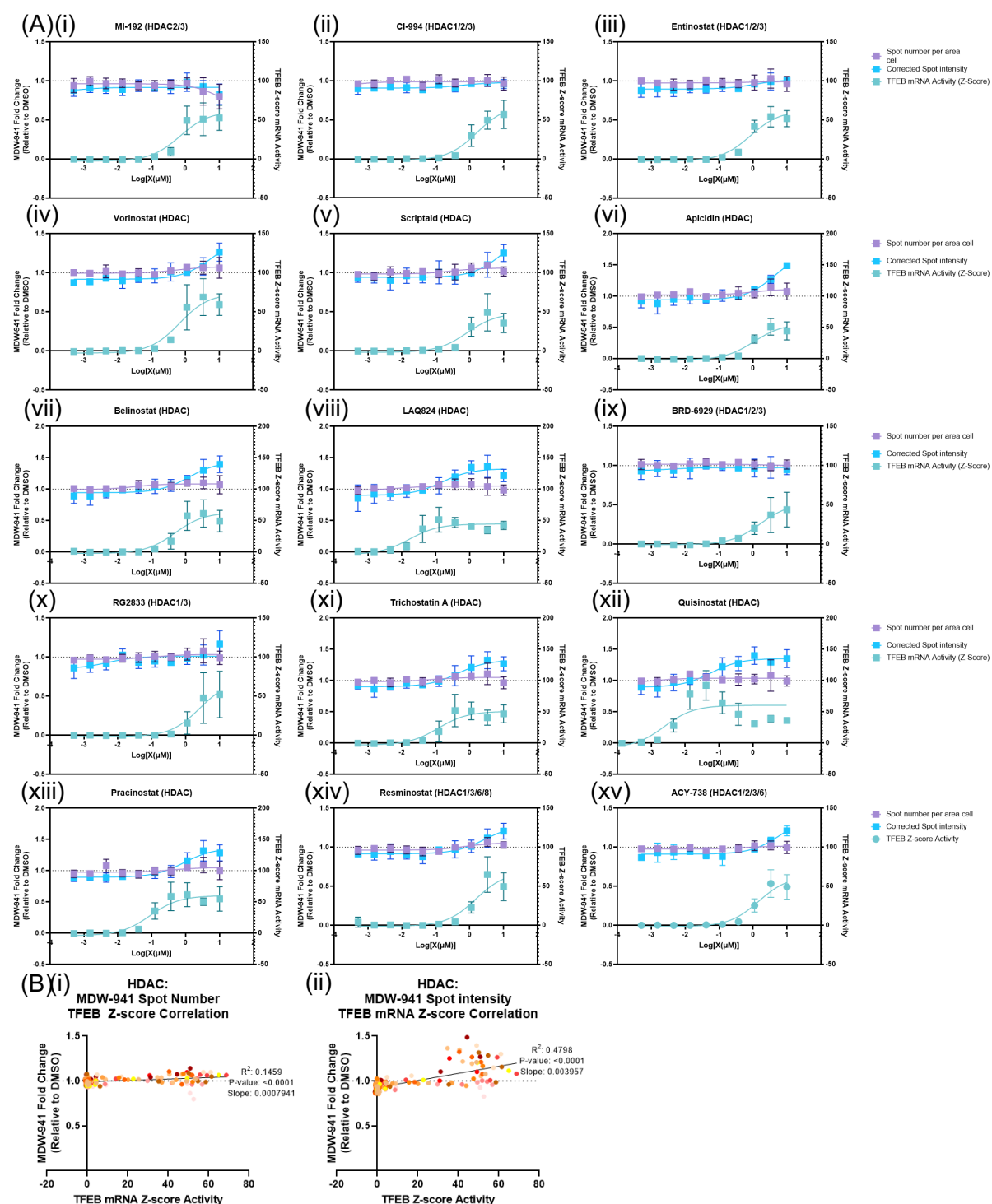


Figure 4.8 – HDAC inhibition upregulates lysosomal GCase expression in iPSC-Ngn2 neurons.

(A) (i-xv) 10-point dose response curves of *TFEB* mRNA Z-score activity (teal – right y-axis), fold change MDW-941 spot number per area cell (purple square – left y-axis) and MDW-941 spot intensity (blue square – left –axis) in iPSC-Ngn2 neurons following treatment of HDAC-targeting compounds. Mean values $\pm$ standard deviation plotted with non-linear fitted curve. (B) Correlation analysis of *TFEB* mRNA Z-score activity with MDW-941 (i) spot number per area cell, and (ii) Spot intensity. Simple linear regression analysis carried out and best fitting line plotted. R-squared goodness of fit, p-value and gradient (slope) of fitted line displayed on the graphs. Colours of points represent each HDAC-targeting compound. N=2 differentiations.

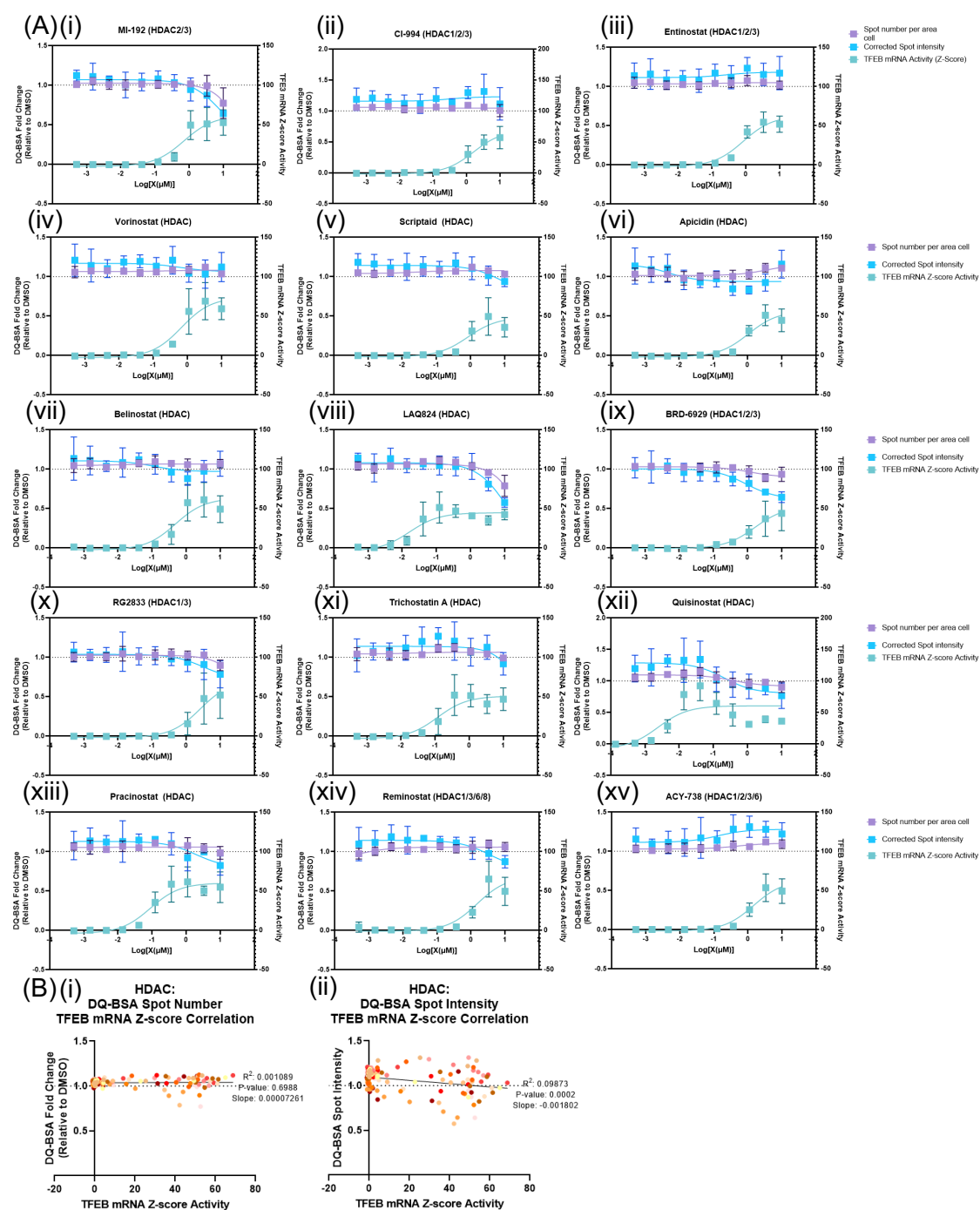


Figure 4.9 – Endolysosomal proteolysis function following HDAC inhibition in iPSC-Ngn2 neurons.

(A) (i-xv) 10-point dose response curves of *TFEB* mRNA Z-score activity (teal – right y-axis), fold change DQ-BSA spot number per area cell (purple square – left y-axis) and DQ-BSA spot intensity (blue square – left –axis) in iPSC-Ngn2 neurons following treatment of HDAC-targeting compounds. Mean values $\pm$ standard deviation plotted with non-linear fitted curve. (B) Correlation analysis of *TFEB* mRNA Z-score activity with DQ-BSA (i) spot number per area cell, and (ii) Spot intensity. Simple linear regression analysis carried out and best fitting line plotted. R-squared goodness of fit, p-value and gradient (slope) of fitted line displayed on the graphs. Colours of points represent each HDAC-targeting compound. N=2 differentiations.

Most HSP90 inhibitors robustly increased MDW-941 spot intensity, but not spot number (Figure 4.10 A). Compounds increasing MDW-941 spot intensity displayed mild reductions in spot number. Likewise, a robust positive correlation could be seen between induction of *TFEB* and MDW-941 spot intensity, but a weak negative correlation between *TFEB* induction and MDW-941 spot number was observed following HSP90 inhibition (Figure 4.10 B). A dose-dependent decrease in DQ-BSA spot number and intensity was seen with most HSP90 inhibitors (Figure 4.11 A). As such, significant negative correlations in both spot number and intensity were observed (Figure 4.11 B).

Proteasome inhibition did not increase MDW-941 (except bortezomib between 0.1-1 μ M), and no correlation with *TFEB* mRNA was observed (Figure 4.10 C-D). Proteasome inhibitors also displayed robust endolysosomal toxicity by DQ-BSA, with a significant negative correlation observed between *TFEB* induction and DQ-BSA intensity (Figures 4.11 C-D).

These data demonstrate variable lysosomal response to *TFEB* transcriptional activators. HSP90 and HDAC inhibition robustly increased GCase within the lysosome, correlating with *TFEB* induction. DQ-BSA is less uniform, and toxicity frequently occurs at higher concentrations. Proteasome inhibition did not upregulate lysosomal function, concordant with the inability to upregulate *TFEB* protein expression in Section 4.6.

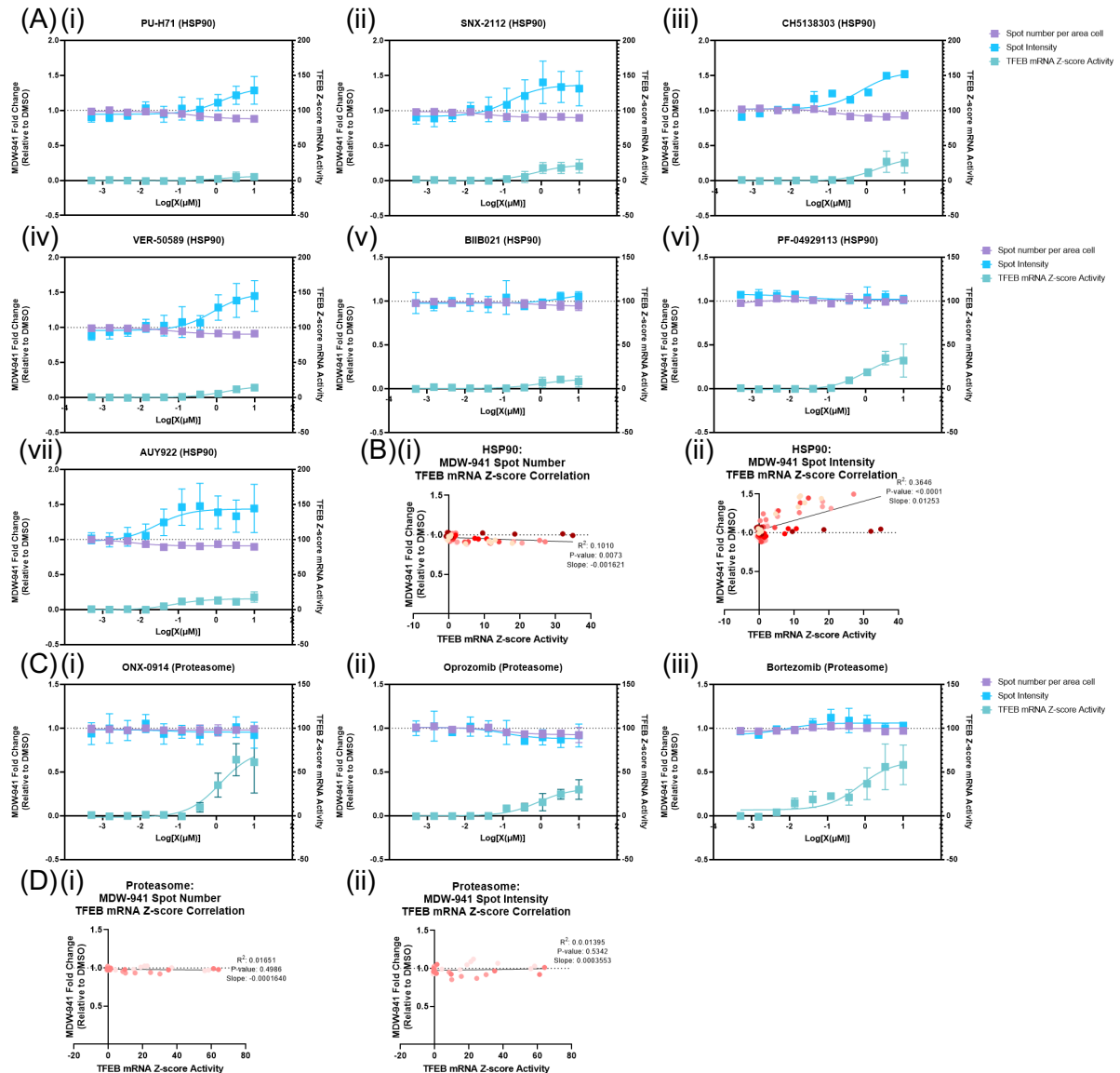


Figure 4.10 – Lysosomal GCase expression measured by MDW-941 in iPSC-Ngn2 neurons treated by HSP90 and proteasome inhibitors.

10-point dose response curves of *TFEB* mRNA Z-score activity (teal – right y-axis), fold change MDW-941 spot number per area cell (purple square – left y-axis) and MDW-941 spot intensity (blue square – left –axis) in iPSC-Ngn2 neurons following treatment of (A i-vii) HSP90- and (C i-iii) proteasome-targeting compounds. Mean values $\pm$ standard deviation plotted with non-linear fitted curve. Correlation analysis assessing (B) HSP90- and (D) proteasome inhibition on the relationship between *TFEB* mRNA Z-score activity with MDW-941 (i) spot number per area cell, and (ii) Spot intensity. Simple linear regression analysis carried out and best fitting line plotted. R-squared goodness of fit, p-value and gradient (slope) of fitted line displayed on the graphs. Colours of points represent each HSP90- or proteasome-inhibiting compound on their respective graphs. N=2 differentiations.

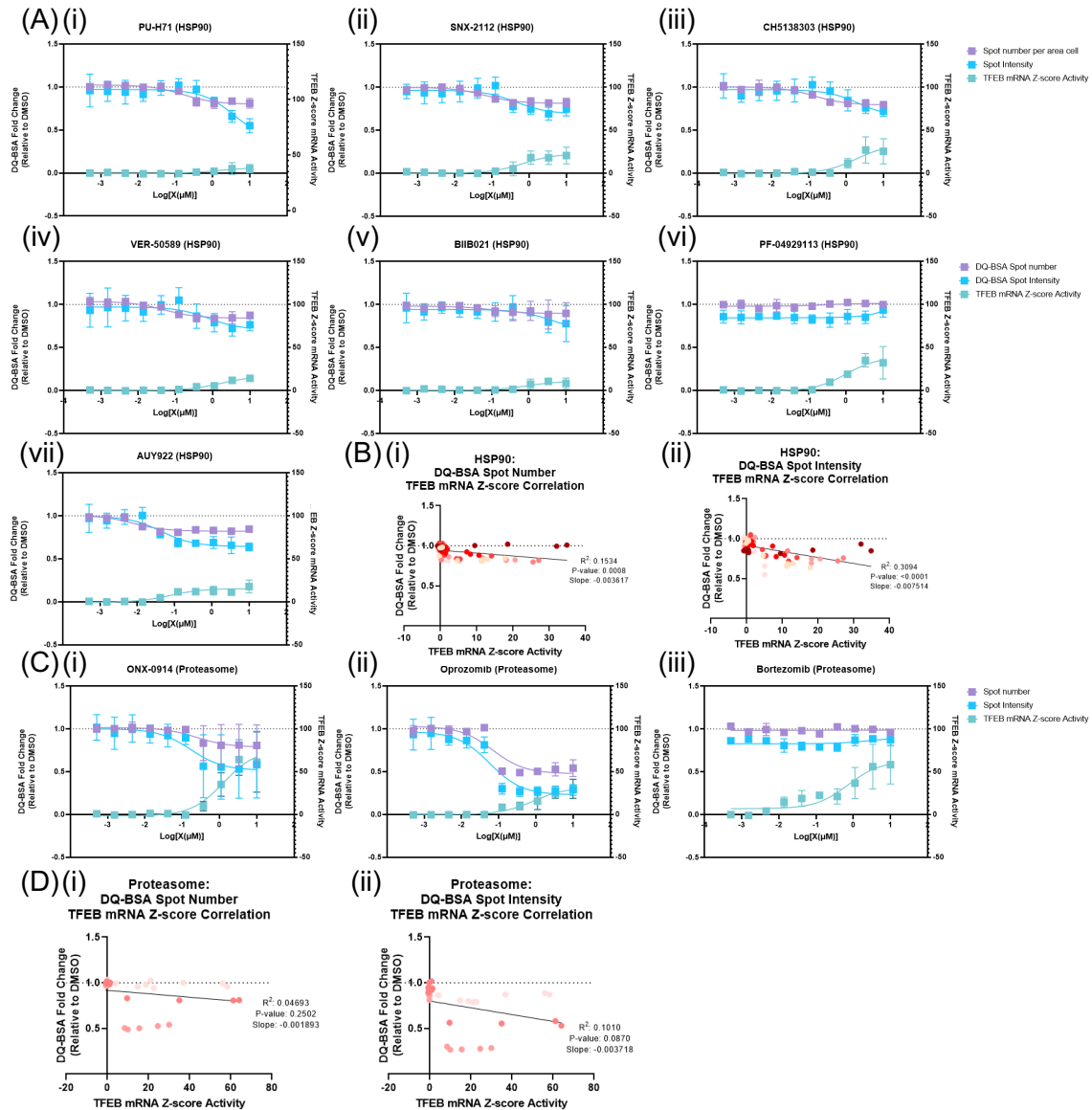


Figure 4.11 – Endolysosomal proteolysis measured by DQ-BSA in iPSC-Ngn2 neurons treated by HSP90 and proteasome inhibitors.

10-point dose response curves of *TFEB* mRNA Z-score activity (teal – right y-axis), fold change DQ-BSA spot number per area cell (purple square – left y-axis) and MDW-941 spot intensity (blue square – left –axis) in iPSC-Ngn2 neurons following treatment of (A i-vii) HSP90- and (C i-iii) proteasome-targeting compounds. Mean values $\pm$ standard deviation plotted with non-linear fitted curve. Correlation analysis assessing (B) HSP90- and (D) proteasome inhibition on the relationship between *TFEB* mRNA Z-score activity with DQ-BSA (i) spot number per area cell, and (ii) Spot intensity. Simple linear regression analysis carried out and best fitting line plotted. R-squared goodness of fit, p-value and gradient (slope) of fitted line displayed on the graphs. Colours of points represent each HSP90- or proteasome-inhibiting compound on their respective graphs. N=2 differentiations.

4.8 Re-screening the qRT-PCR library for activators of neuronal *TFE3* transcription

Re-screening was carried out using the same lysate from the *TFEB* screen. Before screening, *TFE3* transcriptional induction following Torin1, staurosporine, tunicamycin and CQ treatment was assessed. CQ did not increase *TFE3* expression, however Torin1, tunicamycin and staurosporine upregulated expression by 1.5-fold, 2-fold and 3-fold, respectively (Figure 4.12 A). DMSO CV% for *TFE3* was 13% (hit identification requires ~1.4-fold upregulation). Hits may therefore be confounded by stimuli such as ER stress or apoptosis.

Following the primary screening, 166 stringent hits were identified. 86 hits were confirmed following dose-response (Figure 4.12 B). Enrichment analysis of compound classes (Table S.8) identified chaperones (6/12 hit compounds) and Dyes (1/2 hit compounds) as most enriched (Figure 4.12 C). Kinases were much more enriched in the *TFE3* screen (46 hits) compared to the *TFEB* screen (16 hits), and epigenetic compounds were less enriched (*TFE3*: 6 hits; *TFEB*: 24 hits) (Figures 4.4 C and 4.12 C).

Enrichment analysis of hit targets found Rac1, BMX, GCN2 and GLK all to be the most enriched, although only containing 1 screened compound (Figure 4.12 D). ALK, IGF1R and HSP90-targeting compounds were next most enriched. Other noteworthy kinases such as mTOR, PI3K and AKT were also enriched (Figure 4.12 D). Taking the average maximum response from confirmed compound dose-response curves, we observed *TFE3* expression to be primarily 2-4-fold increased (Figure 4.12 E).

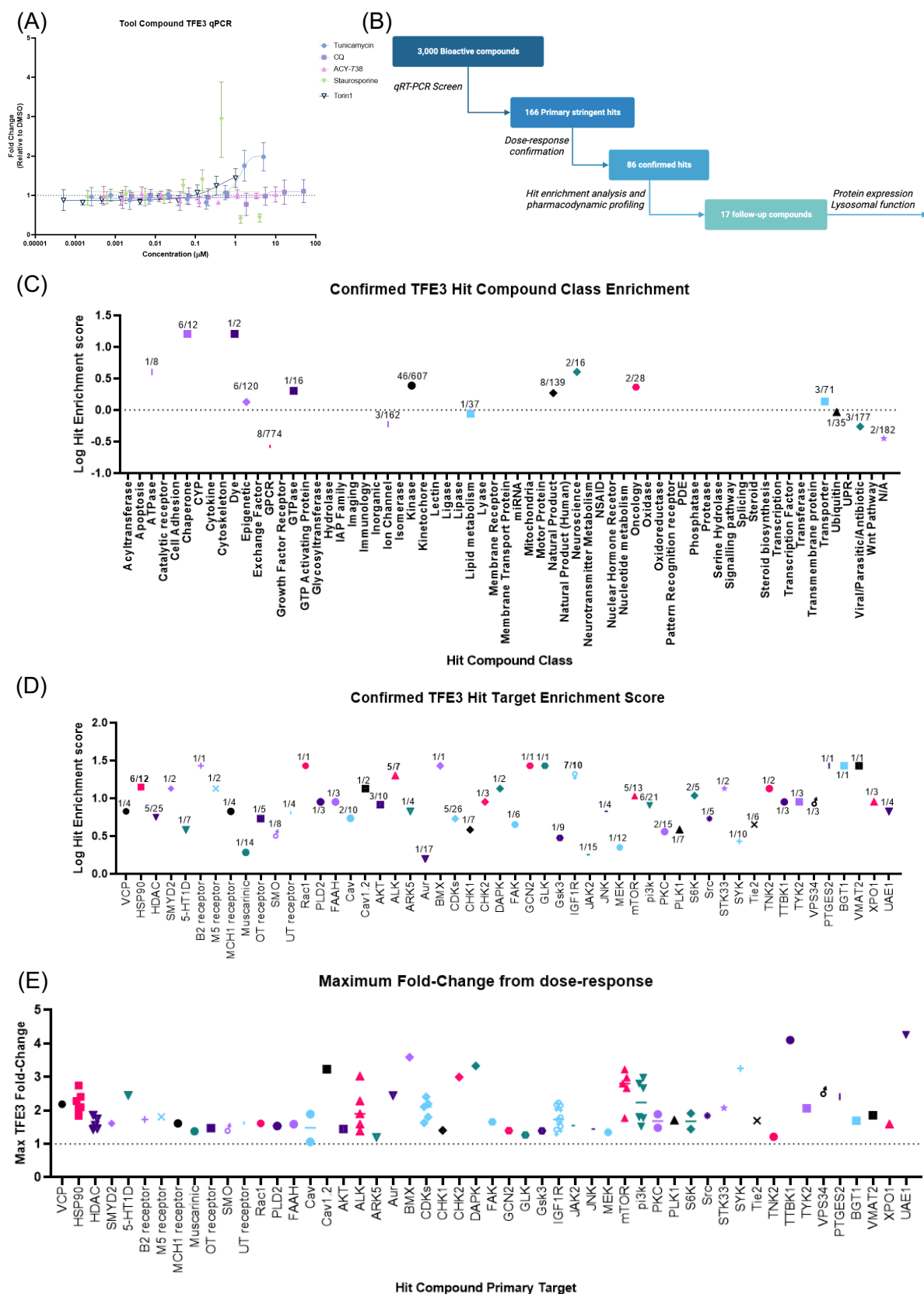


Figure 4.12 - Bioactive compound qRT-PCR screening for *TFE3* transcriptional activators in neurons.

(Figure 4.12 continued...) (A) *TFE3* mRNA fold change in iPSC-Ngn2 following treatment of Torin1 (brown hollow inverted-triangles), Tunicamycin (blue circles), CQ (purple squares), ACY-738 (pink triangles), and Staurosporine (green inverted triangles). Mean fold *TFE3* change plotted $\pm$ standard deviation with non-linear fitted curve for the respective compounds. N=2 differentiations. (B) Flowchart of screening process. 3,000 bioactive compounds were screened. 166 stringent hit activators were identified, 86 of which were confirmed by 2 dose-response follow-up differentiations. Hit enrichment analysis identified most likely *TFE3* regulating targets, and pharmacodynamic assessment of hit compounds led to the selection of 17 follow-up compounds processed for protein induction and lysosomal functional assaying. (C) Enrichment score for all compound classes in the screen. Confirmed hits used for enrichment. (D) Enrichment score for all primary targets of confirmed compound hits in screen. Fraction above point indicates (hit compounds)/(total compounds). (E) Maximum *TFE3* RQ100 observed in dose-responses from confirmed hit compounds relating to their targets. N=2 differentiations

STRING analysis showed a complex connectome with HSP90 as a central interacting node, along with AKT1, mTOR, CDKs and PI3K (Figure 4.13 A). KEGG analysis found the PI3K-Akt pathway enriched (hsa04151 - Figure 4.13 B). Indeed, several hit targets reside within this pathway (red boxes – Figure 4.13 C). Interestingly, the highly enriched targets IGF1R and ALK, lie upstream as signal transducing receptor tyrosine kinases.

To further probe this association, we hypothesised PTEN should antagonise this pathway and PTEN inhibition will reduce neuronal *TFE3* expression (blue square – Figure 4.13 C). Due to the low CV% of the *TFE3* screen, we were able to identify hit knockdown compounds from the screen. Interestingly, the only screened PTEN inhibitor (SF 1670) reduced expression to 55%. We further assessed this through dose-response treatment of SF 1670, VO-Ohpic tetrahydrate (another PTEN inhibitor) and Oroxin B (a PTEN activator) in iPSC-Ngn2 neurons. SF1670 reproduced the knockdown phenotype observed and Oroxin B upregulated *TFE3* expression (Figure 4.13 D). VO-Ohpic did not modulate *TFE3* expression however (Figure 4.13 D). Thus, we confirmed directionality of PI3K-Akt regulation upon *TFE3* expression in neurons, although not all compounds evoke a response.

Interestingly, Alzheimer's disease KEGG pathway was once again enriched, this time for *TFE3* hits (Figure 4.13 B). Proteins driving this enrichment include PI3K, AKT, mTOR, GSK3B, CaV_{1.2}, JNK1/2, and muscarinic receptors.

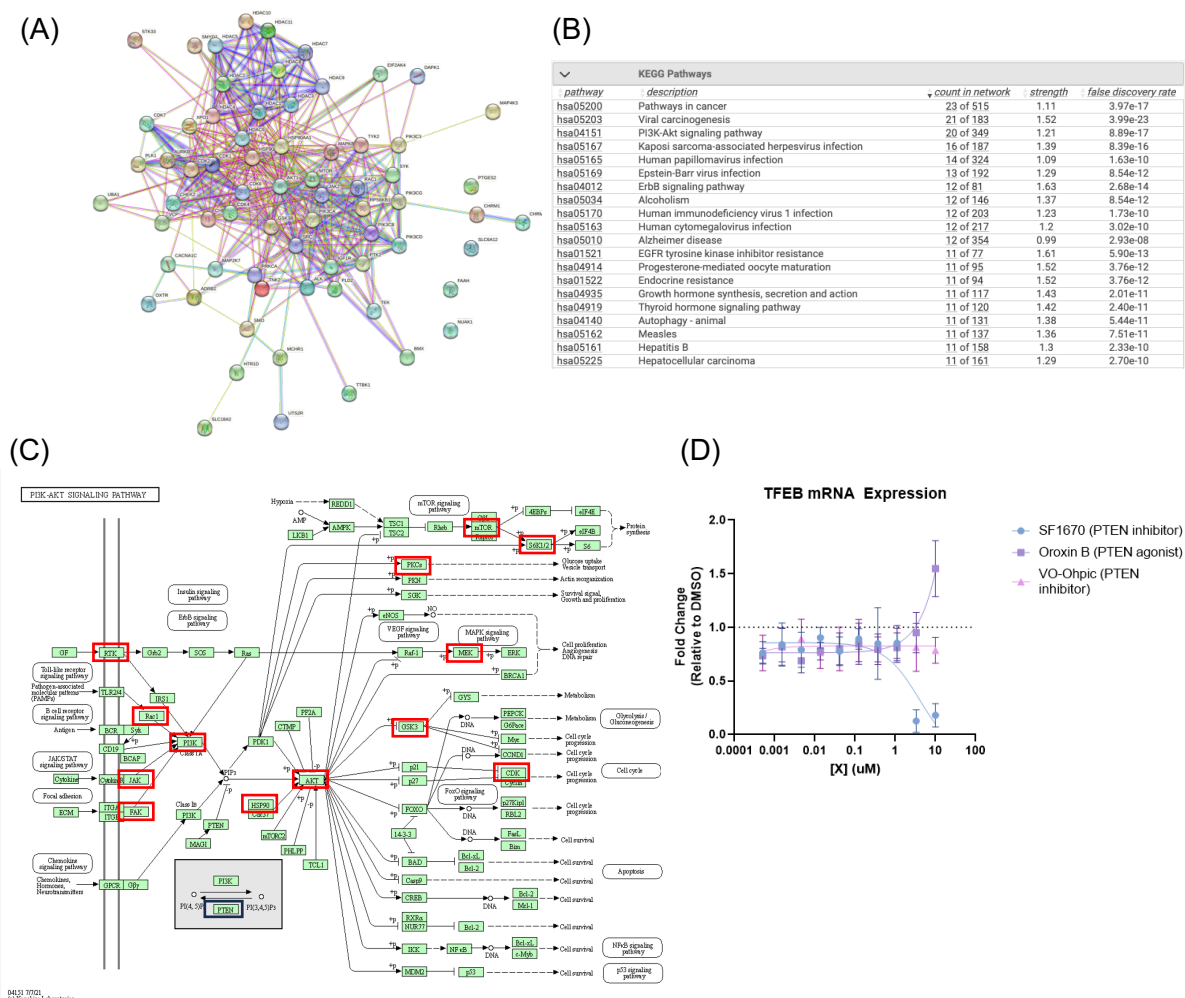


Figure 4.13 – Connectivity and pathway analysis of all confirmed hit targets from *TFE3* qRT-PCR screen identify PI3K-Akt pathway as a potential regulator of expression.

(A) STRING connectome analysis of all primary *TFE3* targets relating to confirmed compound hits. (B) KEGG pathway enrichment analysis of top 20 pathways associated with *TFE3* transcriptional regulation ordered by number of hits within the family. (C) PI3K-Akt signalling KEGG pathway. Hit targets identified by qRT-PCR screen are surrounded by red square. PTEN (a knockdown hit) is surrounded by a blue square. (D) *TFE3* mRNA fold change following 10-point dose-response treatment of SF1670 (PTEN inhibitor – blue circle), Oroxin B (PTEN activator – purple square) and VO-Ohpic (PTEN inhibitor – pink triangle). Mean *TFE3* fold change $\pm$ standard deviation plotted with non-linear fitted curve. N=2 differentiations.

4.9 Assessing TFE3 protein induction with hit compounds

Next, TFE3 protein levels were assessed by immunocytochemical staining. Compounds displaying robust induction of *TFE3* mRNA were selected.

Of the HSP90 inhibitors, PU-H71 increased TFE3 intensity similarly to mRNA induction, however the remaining HSP90 inhibitors did not replicate this (Figure 4.14 A). Nonetheless, a significant weak-positive correlation was observed between TFE3 intensity and *TFE3* mRNA following HSP90 inhibition (Figure 4.9 B).

ALK inhibitors did not robustly elevate TFE3 protein levels (Figure 4.9 C). As such, no significant correlation was observed between TFE3 mRNA and protein (Figure 4.9 D).

Of the 2 IGF1R inhibitors, (-)-podophyllotoxin increased TFE3 protein levels mirroring mRNA levels, however BMS-536924 did not (Figure 4.9 E). Expectedly, no significant correlation was observed between TFE3 protein and mRNA levels following IGF1R inhibition (Figure 4.9 F).

3 mTOR inhibitors were assessed. 2 also targeted PI3K (PF-04691502 and Omipalisib). Torin2 and Omipalisib upregulated TFE3 protein levels in response to treatment but PF-04691502 did not evoke a response (Figure 4.9 G). No correlation was seen following mTOR inhibition between TFE3 mRNA and protein expression (Figure 4.9 H).

PI3K inhibition assessment using Buparlisib and the aforementioned mTOR/PI3K inhibitors displayed a significant negative correlation of TFE3 intensity with mRNA induction (Figure 4.9 I-J). SRC inhibitor, KX2-391 upregulated TFE3 protein expression, whereas AKT inhibitor, afuresertib and CaV1.2 channel inhibitor, Nisoldipine did not increase TFE3 expression (Figure 4.9 K-M).

Overall, most compounds did not seem to translate enhanced TFE3 mRNA expression to protein expression with the exception of a few, but these compounds did not conform to a specific target. PI3K inhibition even displayed negative correlation between mRNA and protein levels.

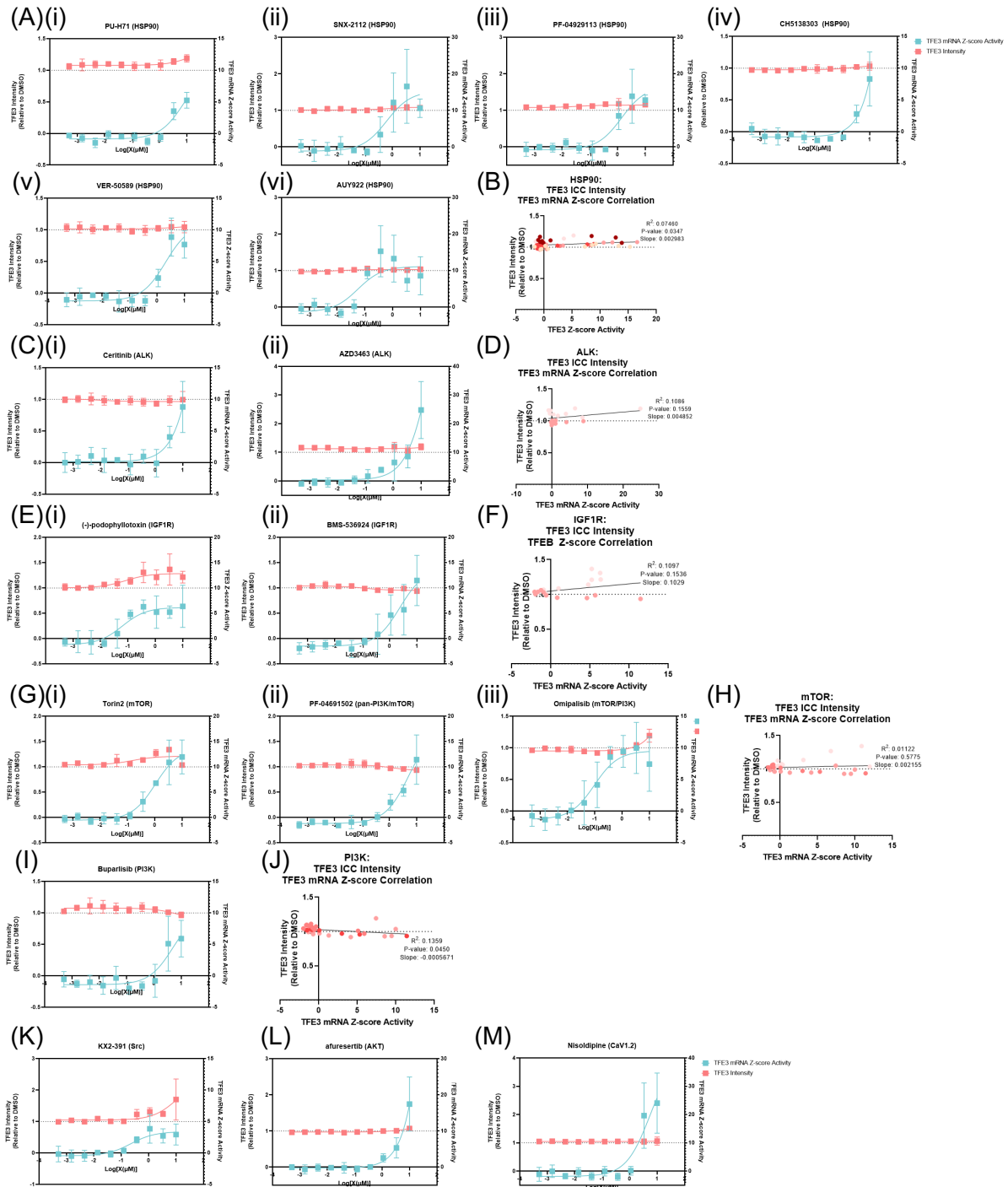


Figure 4.14 – TFE3 protein induction following treatment with hit compounds.

10-point dose-responses of *TFE3* mRNA Z-score activity (teal – right y axis) and fold change TFE3 fluorescence intensity measured by immunocytochemistry, relative to DMSO (red – left y axis) in iPSC-Ngn2 neurons treated with follow-up hit compounds targeting (A) HSP90, (C) ALK, (E) IGF1R, (G) mTOR, (I) PI3K, (K) Src, (L) AKT, and (M) CaV1.2. Mean *TFE3* mRNA Z-score and TFE3 intensity plotted $\pm$ standard deviation, with non-linear fitted curve.

Correlation analysis of relationship between *TFE3* mRNA Z-score activity and TFE3 immunofluorescent intensity relative to DMSO following treatment of (B) HSP90, (D) ALK, (F) IGF1R, (H) mTOR and (J) PI3K. Each colour represents a different hit compound targeting the protein of interest. Simple linear regression analysis was used and R-squared goodness of fit, p-value and fitted line gradient (slope) was stated on the graph. N=2 differentiations.

4.10 Functional cellular and lysosomal imaging assay follow-up of select *TFE3* screen hits

We also assessed lysosomal function following treatment of iPSC-Ngn2 neurons using the selected *TFE3* qRT-PCR hits in Section 4.9 by MDW-941 and DQ-BSA.

The same MDW-941 and DQ-BSA dose-response data from HSP90 inhibition used for comparison with *TFEB* mRNA was applied to *TFE3* mRNA. MDW-941 spot intensity induction observed by 5/6 HSP90 inhibitors preceded the induction of *TFE3* mRNA (Figure 4.15 A). Nonetheless, a significant positive correlation was observed between MDW-941 spot intensity and *TFE3* mRNA expression, whereas a significant negative correlation was found between *TFE3* mRNA and MDW-941 spot number (Figure 4.15 B). As HSP90 inhibition perturbed DQ-BSA signal, a negative correlation with *TFE3* induction was also observed (Figure 4.16 A-B).

One of 2 ALK-targeting compounds (AZD3463) increased MDW-941 spot intensity, however inhibited DQ-BSA (Figures 4.15 C and 4.16 C). Ceritinib did not elicit any response by either lysosomal assay (Figures 4.15 C i and 4.16 C i). No significant correlation was seen between MDW-941 and *TFE3* induction following ALK inhibition (Figure 4.15 D). A significant negative correlation between *TFE3* mRNA and DQ-BSA was present (4.16 D).

IGF1R inhibition produced opposing MDW-941 responses from each compound (Figure 4.15 E). Surprisingly, (-)-podophyllotoxin treatment reduced MDW-941 spot intensity and increased *TFE3* protein expression (Figure 4.14 E i and 4.15 E i). No correlation between MDW-941 and *TFE3* mRNA expression were observed for IGF1R inhibition (Figure 4.15 F). Both IGF1R inhibitors perturbed DQ-BSA signal, thus a negative correlation between *TFE3* mRNA and DQ-BSA was observed with these compounds (Figures 4.16 E-F).

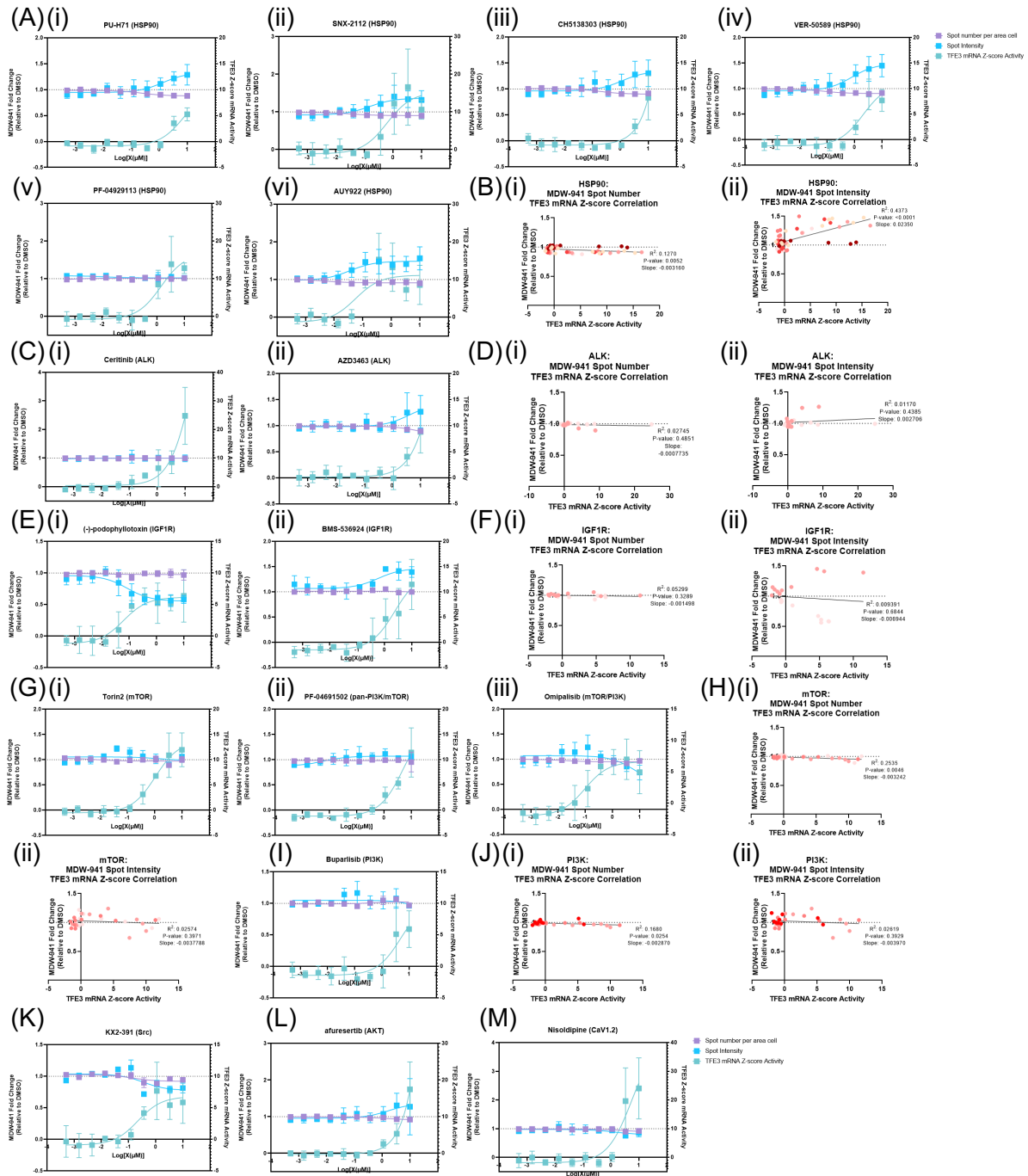


Figure 4.15 – Lysosomal GCase expression measured by MDW-941 in iPSC-Ngn2 neurons treated by selected *TFE3* qRT-PCR hits.

10-point dose response curves of *TFE3* mRNA Z-score activity (teal – right y-axis), fold change MDW-941 spot number per area cell (purple square – left y-axis) and MDW-941 spot intensity (blue square – left y-axis) in iPSC-Ngn2 neurons following treatment of (A) HSP90, (C) ALK, (E) IGF1R, (G) mTOR, (I) PI3K, (K) Src, (L) AKT, (M) CaV1.2-targeting compounds. Mean values $\pm$ standard deviation plotted with non-linear fitted curve.

Correlation analysis assessing (B) HSP90, (D) ALK, (F) IGF1R, (H) mTOR and (J) PI3K inhibition on the relationship between *TFE3* mRNA Z-score activity with MDW-941 (i) spot number per area cell, and (ii) Spot intensity. Simple linear regression analysis carried out and best fitting line plotted. R-squared goodness of fit, p-value and gradient (slope) of fitted line displayed on the graphs. Each colour represents a different hit compound targeting the protein of interest. N=2 differentiations.

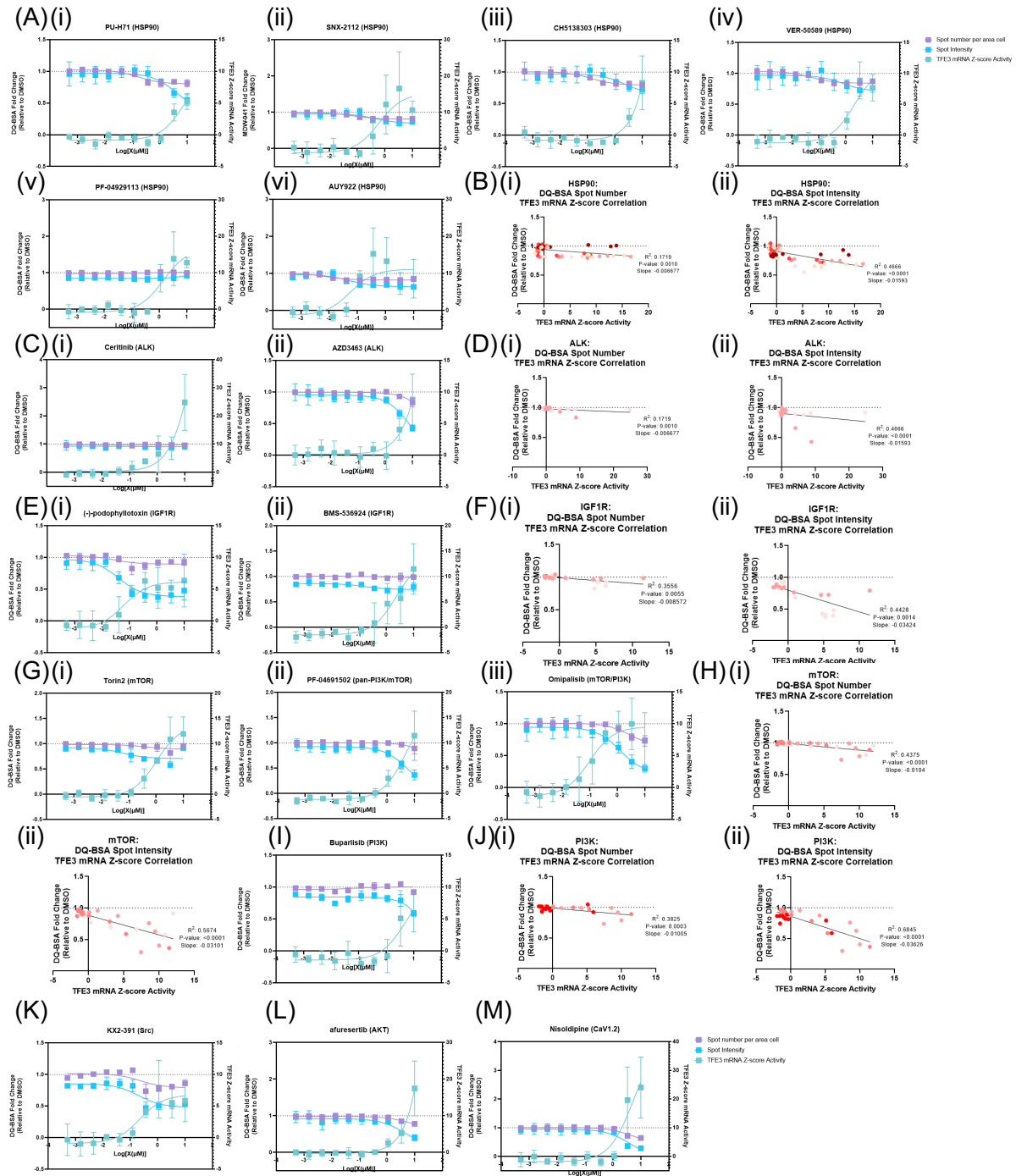


Figure 4.16 – Endolysosomal proteolysis measured by DQ-BSA in iPSC-Ngn2 neurons treated by selected *TFE3* qRT-PCR hits.

10-point dose response curves of *TFE3* mRNA Z-score activity (teal – right y-axis), fold change DQ-BSA spot number per area cell (purple square – left y-axis) and DQ-BSA spot intensity (blue square – left –axis) in iPSC-Ngn2 neurons following treatment of (A) HSP90, (C) ALK, (E) IGF1R, (G) mTOR, (I) PI3K, (K) Src, (L) AKT, (M) CaV1.2-targeting compounds. Mean values $\pm$ standard deviation plotted with non-linear fitted curve.

Correlation analysis assessing (B) HSP90, (D) ALK, (F) IGF1R, (H) mTOR and (J) PI3K inhibition on the relationship between *TFE3* mRNA Z-score activity with DQ-BSA (i) spot number per area cell, and (ii) Spot intensity. Simple linear regression analysis carried out and best fitting line plotted. R-squared goodness of fit, p-value and gradient (slope) of fitted line displayed on the graphs. Each colour represents a different hit compound targeting the protein of interest. N=2 differentiations.

Minimal changes in MDW-941 were induced by compounds targeting mTOR or PI3K (Figure 4.15 G and I). There was no correlation between *TFE3* mRNA expression and MDW-941 spot intensity, but a weak negative correlation was present between *TFE3* mRNA and MDW-941 spot number, for mTOR or PI3K inhibitors (Figures 4.15 H (i-ii) and J (i-ii)). All compounds inhibiting mTOR or PI3K perturbed DQ-BSA signal (Figure 4.16 G and I). Indeed, negative correlations were seen in relation to both DQ-BSA parameters with *TFE3* induction for mTOR and PI3K inhibitors (Figure 4.16 H and J). KX2-391 (Src-targeting compound) caused dose-dependent decreases in both DQ-BSA and MDW-941 (Figures 4.15 K and 4.16 K). Afuresertib (AKT inhibitor) increased in MDW-941 Spot intensity but reduced DQ-BSA signal in a dose-dependent fashion (Figures 4.15 L and 4.16 L). Nisoldipine (CaV1.2 inhibitor) also reduced both MDW-941 and DQ-BSA signal in iPSC-Ngn2 neurons (Figures 4.15 M and 4.16 M). Collectively, inconsistent lysosomal responses between compounds both within, and between targets was observed for compounds upregulating *TFE3* mRNA. HSP90 inhibition upregulated lysosomal GCase but at concentrations preceding *TFE3* induction suggesting a less relevant response to *TFE3* mRNA induction. DQ-BSA toxicity was observed following most compound treatments. Thus, pharmacological inhibition of targets activating *TFE3* mRNA expression did not compellingly enhance lysosomal function.

4.11 CRISPR-interference screening HDACs for *TFEB* regulators in i³ neurons

Table 4.4 - sgRNA sequences for CRISPRi targeting HDACs

Target of interest	sgRNA Name	sgRNA Sequence
HDAC1	HDAC1-1	GACCGACTGACGGTAGGGAC (Courtesy of S. Szunyogh)
	HDAC1-2	GGCAGACGCAGGGCACCCGG (Courtesy of S. Szunyogh)
HDAC2	HDAC2-1	GGGCCTCCACGGGGGAGGAA (Courtesy of S. Szunyogh)
	HDAC2-2	GGAAAGCTCGGAATCGGAGG (Courtesy of S. Szunyogh)
HDAC3	HDAC3-1	GGGGTCGTAGAAATAGGCCA (Courtesy of S. Szunyogh)
	HDAC3-2	GGTTCGTCTAGCTCTCACCCC (Courtesy of S. Szunyogh)
HDAC4	HDAC4-1	GCCTGGCTCGACGCTGGAGT (Courtesy of Anna Nelke)
	HDAC4-2	GTCAACCGCGAAGCACCCAG (Courtesy of Anna Nelke)
HDAC5	HDAC5-1	GGGAGGAGCCGCGCAAAGA
	HDAC5-2	GGGCTCCTCCATCTTTGCGG
HDAC6	HDAC6-1	GGAGGGAGAAGGCCTGAGAG
	HDAC6-2	GACCGGTCCGGCCCGATAAG
HDAC7	HDAC7-1	GCTGCAGAGCGACTGTGAGA
	HDAC7-2	GTGTGAGACGGCGAGAGGAA
HDAC8	HDAC8-1	GGGACGTCCAATGGACAGGT
	HDAC8-2	GCTCCTCTGATCGGCCGCAG
HDAC9	HDAC9-1	GATAGGAGAAGGGCACCGGC
	HDAC9-2	GTGGGTAGGTGGGAGAACCA
HDAC10	HDAC10-1	GCGCTGCCGCAGGCGATCCA
	HDAC10-2	GCCCAGGCGCGCAGAAGGCA
HDAC11	HDAC11-1	GCCGCGGCACTCACATCCCG
	HDAC11-2	GCCAGTGGCTGCCTCCGTGG
SIRT1	SIRT1-1	GCAGCGGTGGCTCCCGAGAT
	SIRT1-2	GCAGAGAGGCAGTTGGAAGA
SIRT2	SIRT2-1	GCGGTCCGTGACAGCCTCAA
	SIRT2-2	GGGTGACGGGACAGAGCGGT
SIRT3	SIRT3-1	GCACAGCCGGAGGGCTGCCG
	SIRT3-2	GAGCCAAGTGCGCGGGAAGT
SIRT4	SIRT4-1	GGTGTCCGTAGAGCTGTGAG
	SIRT4-2	GATCAGACGGTCCCCTGTG
SIRT5	SIRT5-1	GCGTGGGAGACTGTATTCCG
	SIRT5-2	GGCGCTCCGGACCTGAGCCA
SIRT6	SIRT6-1	GTGTCGCCGTACGCGGACAA
	SIRT6-2	GAGACGCGCTCACCTCCGGG
SIRT7	SIRT7-1	GCGACGGGCGGTCCCGGGTG
	SIRT7-2	GCAGCTGCTCCCGAAACCGG

HDAC inhibition reproducibly and robustly upregulated *TFEB* expression in neurons. To identify effector HDACs regulating *TFEB* transcription, we used CRISPRi to individually knockdown each human HDAC, using a previously engineered iPSC-Ngn2 line containing CRISPRi machinery (i³ neurons) (Tian et al., 2019). Lentiviral-mediated delivery of sgRNAs specific to HDACs regulating *TFEB* transcription, should repress HDAC expression and induce *TFEB* expression in i³ neurons (Figure 4.17 A).

HDAC-targeting sgRNA sequences were selected using a published genome-wide sgRNA library (Horlbeck et al., 2016). We cloned 2 guides for all 18 HDACs into a published EF1 α -Puro-T2A-2xmycNLS-mTagBFP2-U6-sgRNA lentiviral backbone (Table 4.4, Figure 4.17 B-C) (Tian et al., 2019). Cloning and lentiviral production are described in Section 2.4 and 2.5.

Following lentiviral cloning and production, a functional titre by measurement of % cells transduced with each virus according to volume was assessed. Transduced cells were BFP-positive in the nucleus. Viruses were serially titred onto i³ neurons using a Labcyte Echo to acoustically dispense virus. 3 days following, cells were co-stained with NucRed Live nuclear marker and % BFP+ nuclei was assessed (Figure 4.18 A). An exemplar titration heatmap of %BFP+ cells is displayed in Figure 4.18 B. %Transduction was plotted against volume for each virus and an interpolation of 80% was calculated (Figure 4.18 C). One plate was dropped by the robotic system; hence titre was reduced. Due to time constraints, viral titre was estimated by multiplying titres of the dropped plate by a coefficient that produced an average titre equal to that of the other titration plates.

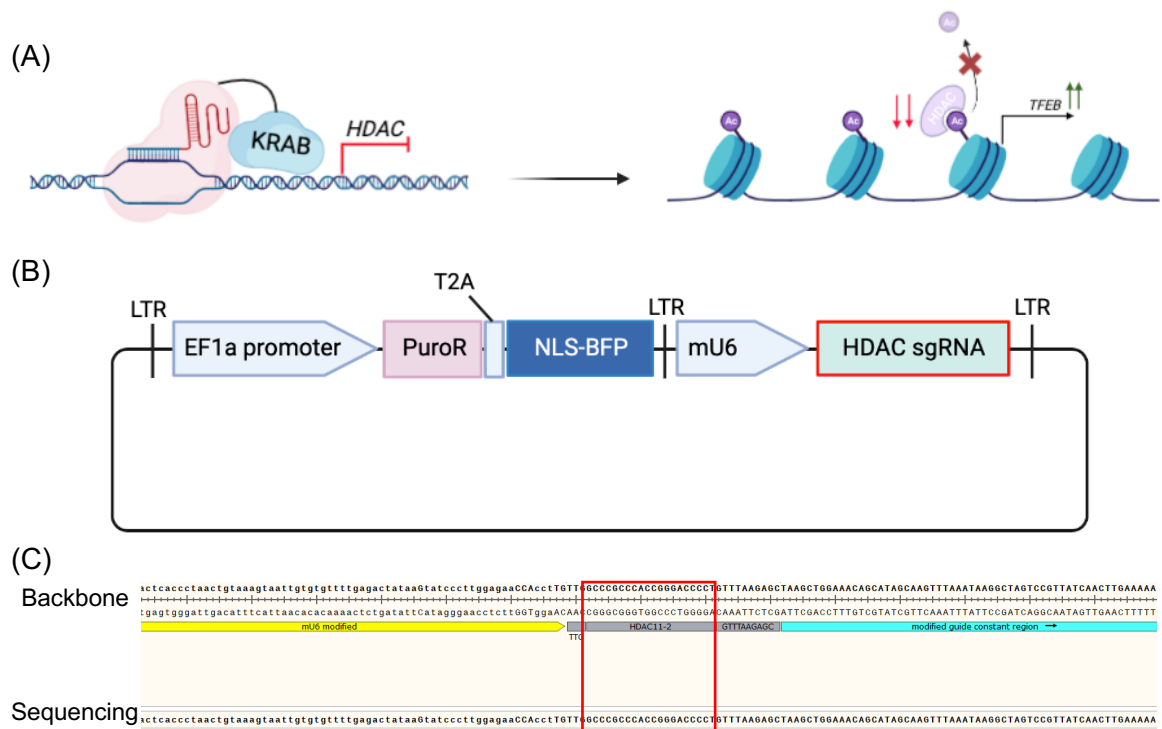


Figure 4.17 – CRISPR-interference to study HDAC knockdown on *TFEB* induction in *i*³ neurons.

- (A) Schematic hypothesis for HDAC knockdown inducing *TFEB* expression. In short, a lentivirus encoding an sgRNA is transduced into the neuron. The sgRNA then targets dCas9 (pink)-KRAB (blue) complex to the target *HDAC* locus and inhibits transcription. Reduction of an effector HDAC should increase histone acetylation at the *TFEB* locus and induce transcription of *TFEB*. (B) Schematic of the lentiviral backbone used to transduce neurons with the specifically designed sgRNA (HDAC sgRNA). Puromycin resistance (PuroR) allows for selection of neurons containing the guide sequence. The T2A separates translation of PuroR and NLS-BFP (blue fluorescent protein, tagged with a nuclear localisation signal). LTRs allow integration into the host genome. EF1a and mU6 are different promoters facilitating transcription of the constructs of interest. Adapted from Tian et al., 2019. (C) Example of a guide sequence cloned into the lentiviral backbone. Top sequence is the expected sequence. Bottom sequence is the results from sanger sequencing following cloning. The guide sequence is encircled by a red square as reference.

The half-life of target HDACs was assessed to facilitate functional reductions in protein expression following transcriptional repression by CRISPRi. Data was provided by Biogen in iPSC-Ngn2 neurons. HDAC1 had the longest half-life of 6.78 days (Figure 4.19 A; Table 4.5). Thus, we cultured neurons for 17 days post-transduction (2 weeks post-maximal expression of sgRNA) before collection, to allow ample time for reduction of the protein (Figure 4.19 B). Following, the 1-step qRT-PCR method was carried out.

TFEB expression was unchanged following individual knockdown of each HDAC (Figure 4.19 C). These data preliminarily suggest knockdown of only one HDAC in

neurons is not sufficient to prompt *TFEB* expression, likely due to redundancy between HDACs.

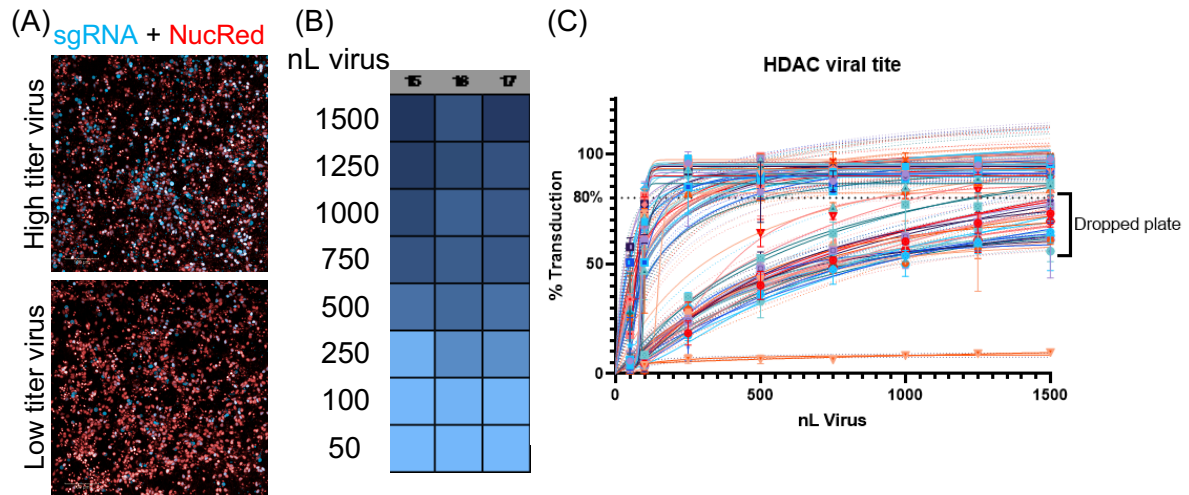


Figure 4.18 – Functional titration of lentivirus encoding HDAC-targeted sgRNAs.

(A) Representative images of neuronal nuclei (stained with nucled - red), transduced with high titre (top) and low titre (bottom) volumes of lentivirus expressing the sgRNA of interest and NLS-BFP (blue). (B) Exemplar heatmap of % cells transduced (BFP+ nuclei) following lentiviral titration of sequential volumes of virus (left) in triplicate. Deepness of blue represents higher % transduction. (C) Quantification of % transduction for each volume of virus used. Each curve represents a single lentivirus assayed. Mean % transduction plotted $\pm$ standard deviation, with non-linear fit curves. Interpolation at 80% was used to calculate the titer of each virus. The bracket indicates the viruses in which the plate was dropped by the robot.

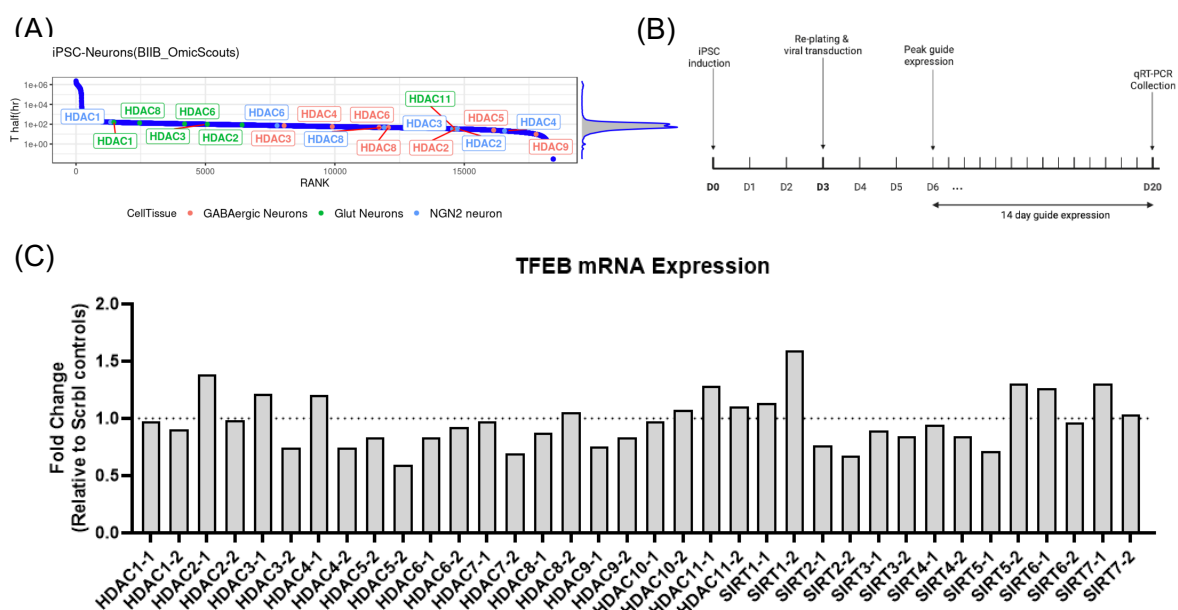


Figure 4.19 – CRISPRi-mediated knockdown of histone deacetylases in i^3 neurons.

(A) Half-life of protein turnover in iPSC-derived GABAergic (red), glutamatergic (green) and Ngn2 (blue) neurons, ranked according to protein longevity. HDACs identified by the assay have been labelled. Courtesy of Biogen. (B) Timeline for HDAC screening CRISPRi study. iPSCs were induced at D0 and replated into 384 well plates at D3. Lentiviruses encoding sgRNA were transduced using the pre-calculated 80% titre at D3 re-plating. Peak expression of the lentivirus was expected at D6 (3-days post transduction). i^3 neurons were cultured to D20 (14 days post—peak transduction) and 1-step qRT-PCR was carried out to observe *TFEB* expression. (C) *TFEB* mRNA fold induction for sgRNA-guides targeting knockdown of HDACs in i^3 neurons relative to Scrambled control sgRNAs. 2 guides were used per HDAC target.

Table 4.5 - HDAC half-life in iPSC-Ngn2 neurons

Gene Name	T_Half Life (hr)	T_Half Life (days)
HDAC1	162.72	6.78
HDAC2	33.39	1.3913
HDAC3	34.43	1.4346
HDAC4	21.28	0.8867
HDAC5	24.74	1.0308
HDAC6	73.56	3.065
HDAC7	N/A	N/A
HDAC8	47.17	1.9654
HDAC9	N/A	N/A
HDAC10	N/A	N/A
HDAC11	N/A	N/A
HDAC12	N/A	N/A

4.12 Discussion

4.12.1 *TFEB* qRT-PCR screening

Many publications have demonstrated a neuroprotective function following increased *TFEB* expression, through viral-mediated overexpression (Decressac et al., 2013; Polito et al., 2014; Torra et al., 2018; Arotcantera et al., 2019). *TFEB* transcriptional activation could bridge the gap between these studies and providing a pharmacological modality facilitating neuroprotection through *TFEB* induction.

In this chapter, we developed a cheap and effective high-throughput 1-step qRT-PCR method for screening transcriptional inducers of *TFEB* in iPSC-Ngn2 neurons.

Through dose-response analysis of HDAC, Sirtuin and PRC2-targeting compounds, we validated HDACs regulation upon *TFEB* transcription in iPSC-Ngn2 neurons. There was no evidence for regulation by Sirtuins or PRC2 components, however. The absence of *TFEB* induction following PRC2 repression may suggest that HDAC function is sufficient to maintain transcriptional repression through deacetylation of H3K27 (and other substrates), without H3K27 methylation by PRC2 further facilitating repression. Co-treatment of HDAC and PRC2 inhibitors would be an interesting approach to observe whether dual inhibition may have synergistic effects on *TFEB* expression, however from our data it seems unlikely PRC2 plays a causal role.

HDACs may act indirectly upon *TFEB* expression through de-repression of an intermediate transcription factor. However, previous reports have shown HDAC2 to bind, deacetylate and transcriptionally repress the *TFEB* promoter (with Myc) in multiple cell-types (Annunziata et al., 2019). The authors speculate genetic inhibition of HDAC2 alone is sufficient to de-repress lysosomal gene regulation. Our data suggest this not to be the case for *TFEB* in neurons. Our results find inhibition of 2 or more class I HDACs: HDAC1, HDAC2 or HDAC3, is required for *TFEB* induction. Compounds inhibiting only one of these HDACs did not elicit a robust response. Further, CRISPRi-mediated knockdown of individual HDACs could not induce *TFEB* expression in i³ neurons, although knockdown still needs to be validated. This discrepancy between our results and previous reports may be resultant from the use of different cell-types displaying different regulation upon the *TFEB* locus, underpinning the benefits of using specifically a neuronal model here. Alternatively, Annunziata and colleagues did not display the effects of HDAC2 knockdown on *TFEB*

expression itself, only assessing 4 lysosomal genes (2019). Thus, *TFEB* may be regulated more stringently by HDACs than downstream lysosomal genes.

The 3,000 bioactive compound screen for *TFEB* inducers identified all expected HDAC inhibitors as hits. This validates the reproducibility of the screen. Three primary targets of interest were identified: HDACs, the proteasome and HSP90. Interestingly, Myc inhibition was not identified as a hit, despite being previously reported to function in tandem with HDAC2 (Annunziata et al., 2019), owing to differential regulation upon *TFEB* in neurons.

STRING analysis of hit targets indicated HSP90 may have a central role in *TFEB* regulation, linking many hit targets and two major inducers of *TFEB*, the proteasome and HDACs. Indeed, there are overlapping functions between the proteasome, HSP90 and HDACs. For example, HDAC inhibition and proteasome inhibition indirectly prompts HSP90 release from cells by ROS generation and caspase 10 activation, thereby inhibiting its function (Park et al., 2021).

Increases in mRNA expression often does not necessarily correlate with protein levels, however (Edfors et al., 2016). Hit compounds may activate transcription or prevent transcript degradation, increasing abundance without necessarily increasing translation. This seems the case for proteasome inhibitors, as *TFEB* protein expression did not increase concomitantly with mRNA following treatment, suggesting translation of now abundant *TFEB* transcripts had been perturbed.

Homeostatically, it is plausible that inhibition of protein degradation by the proteasome is compensated by reduced protein translation, particularly for proteasome-targeted proteins. Indeed, *TFEB* is a protein targeted for proteasomal degradation (Sha et al., 2017). Further, proteasome inhibition has been shown to reduce translation in neurons (Ding et al., 2006). How mRNA transcription is activated without the induction of protein levels is more paradoxical.

The proteasome regulates a multiplicity of functions relating to gene expression beyond protein degradation. The proteasome has been implicated in modulating co-repressor and co-activator activity; histone modification; transcriptional termination at the 3'UTR; mRNA decay; and mRNA export from the nucleus for translation (Laroia et al., 1999; Kwak et al., 2011). Perhaps proteasomal inhibition stabilises a co-activator of *TFEB* transcription or prevents *TFEB* mRNA decay, while inhibiting the export and translation of this mRNA, leading to mRNA abundance.

HSP90 and HDAC inhibitors both induced TFEB protein level expression, albeit HDAC inhibitors more robustly. From STRING analysis, HSP90 may have a co-operative role with HDACs, converging to regulate *TFEB* transcription. HSP90 has bidirectional relationships with multiple HDACs. HDAC6 and HSP90 can co-regulate one another (Liu et al., 2021). This relationship is thought to be involved in triggering aggresome formation through several mechanisms, one of which is dissociation from HSF1 (derepressing the transcription factor) following aggregate sensing (Boyault et al., 2007). HSF1 can activate PGC1a (a transcriptional activator of *TFEB*), thus HDAC6 or HSP90 inhibition may indirectly induce *TFEB* transcription (Tsunemi et al., 2012; Ma et al., 2015; Table 4.1). HDAC6 is a less likely candidate for *TFEB* induction from our results, suggesting this may be a less significant mechanism. There is a bidirectional facilitatory relationship between HSP90 and HDAC3, a more likely candidate for *TFEB* regulation (Kotwal and Subbarao, 2020). Thus, HSP90 inhibition may impair HDAC3 function, promoting *TFEB* de-repression.

Surprisingly, HSP90 was recently shown to bind the *TFEB* promoter and upregulate expression in Hep2G cells, contradictory to our results (Wang et al., 2020). Cell-type specificity may cause this disparity with our observations, by which different HSP90 clients may be associated when binding to the *TFEB* locus, forming a repressive complex in neurons and not other cell types.

Further study by ChIP and genetic modulation will help elucidate the exact regulatory mechanisms of *TFEB* transcription in neurons to better manipulate these pathways therapeutically.

4.12.2 *TFE3* qRT-PCR screening

Re-screening with the same iPSC-Ngn2 lysate allowed us to identify transcriptional regulators of neuronal *TFE3*. Unlike the *TFEB* screen, *TFE3* screening was hindered by the assay window responding to a similar magnitude as several cellular stressors. Thus, qRT-PCR screening in this context seemed best suited to examine the robust induction of lowly expressed genes.

Interestingly, *TFE3* hits displayed higher kinase enrichment to *TFEB* and fewer epigenetic hits. This represents a differential regulatory landscape upon the expression of each transcription factor and suggests a more transcriptionally repressive regulation upon *TFEB* in neurons, corroborating our findings of minimal *TFEB* expression.

6 HDAC inhibitors upregulated *TFE3* transcription albeit at low fold induction. HDACs have been reported to bind to the *TFE3* locus previously (Annunziata et al., 2019). Online neuronal ChIP data from Section 3.12 shows higher H3K27Ac at the *TFE3* TSS. Thus, while HDACs may bind both *TFEB* and *TFE3*, HDAC function may be reduced at the *TFE3* locus. The regulatory mechanism defining selective repression upon *TFEB* and not *TFE3* by HDACs is an interesting avenue to be explored.

IGF1R, ALK and HSP90 were amongst the highest enriched targets with multiple compound hits. IGF1R and ALK are both receptor tyrosine kinases with functional and structural similarity, feeding into similar downstream pathways. One of these pathways, PI3K-Akt signalling, was enriched in KEGG analysis for *TFE3* regulation (LeRoith et al., 1995; Wulf et al., 2021). This pathway contains several kinases known to regulate *TFE3* localisation, and our data suggests this regulation may also be transcriptional. The PI3K-Akt pathway plays crucial roles in cellular growth, survival, protein synthesis and proliferation (Vivanko and Sawyers, 2002). Interestingly, *TFE3*-activation in hepatocytes directly upregulates elements of the IRS-1/PI3K/AKT pathway involved in glucose homeostasis and insulin signalling (Nagakawa et al., 2006). Thus, PI3K/Akt inhibition may enhance *TFE3* transcription (and translocation), to upregulate its own pathway, allowing tight homeostatic control and regulation of glucose sensing and metabolism. We displayed directionality of this effect by modulating PTEN, the PI3K/AKT pathway antagonist. The PTEN inhibitor SF1670 was a knockdown hit in the *TFE3* screen, which reproduced by dose-response. PTEN agonist Oroxin B, increased *TFE3* expression also, displaying correct directionality to modulation of the PI3K-Akt pathway upon *TFE3* transcription.

HSP90 also plays a functional role within the PI3K/AKT pathway, perhaps identifying differential pathways for the regulation of *TFEB* and *TFE3* transcription.

An important caveat is that *TFE3* expression was upregulated following ER-stress, apoptosis, functions that are closely related to PI3K/AKT function (Hyoda et al., 2006; Jeong et al., 2008; Dai et al., 2010). Thus, cellular stress responses cannot be ruled out as the primary reason for *TFE3* induction.

TFE3 protein expression through immunofluorescence saw varying results. HSP90 inhibition displayed the only significant positive relationship between *TFE3* mRNA and protein levels, although this may be due to higher powering compared to other targets. Despite this, *TFE3* protein induction was sporadic regarding which compounds

elevated expression. It was difficult to delineate the relationship between target engagement and *TFE3* induction.

4.12.3 Functional assays in response to *TFEB* and *TFE3* activation

Lysosomal assays were used to identify compounds that enhanced lysosomal response and draw relationships between *TFEB* or *TFE3* induction and lysosomal function. DQ-BSA frequently displayed toxicity associated with increased compound concentrations, thus negatively correlating with the induction of *TFEB* or *TFE3*. DQ-BSA fluorescence is the result of many convergent pathways including endocytosis, vesicle trafficking, and lysosomal fusion. This makes the assay prone to inhibition by several modulatory effects (on- or off-target). Modulation of endocytosis is known to activate *TFEB/TFE3*, thus assessment of transcription following endocytic modulation (through treatment with Dynasore) should be carried out to examine if this may confound results.

A better correlation was seen between MDW-941 and induction of *TFEB*. This assay directly probes levels of correctly folded GCase in the lysosome, thus is a more direct readout for the lysosomal biogenesis activity of *TFEB* and *TFE3*. Further, compounds promoting *TFEB* protein expression robustly upregulated MDW-941 in neurons. This indicates positive regulation upon lysosomal function when targeting inducers of *TFEB* protein expression, as seen by GCase induction following inhibition of HSP90 and HDACs but not the proteasome.

TFE3 mRNA enhancing compounds were unconvincing in the upregulation of lysosomal GCase except for HSP90. We demonstrated *TFE3* hits could be confounded by cellular stressors such as staurosporine or tunicamycin, thus *TFE3* induction may not be directly resultant from 'on-target' compound effects, rather by off-target cellular stress responses that may inhibit lysosomal enzyme synthesis/biogenesis. It is important to note that due to the high-throughput nature of the assays carried out in this screening pipeline (for both *TFEB* and *TFE3* screens), positive controls demonstrating correct compound activity on-target was not examined. Thus, one cannot rule out a lack of lysosomal response due to inactivity of a compound assessed in some assays.

An assay for understanding how *TFEB/TFE3* mRNA induction can directly induce functional activity of *TFEB/TFE3* following compound addition may be a CLEAR-luciferase reporter assay, removing many off-target variables associated with the

lysosomal assays used. However, this would not provide information as to exactly which transcription factor is acting to upregulate CLEAR genes following compound addition.

The causality of lysosomal activation by TFEB or TFE3 following compound addition could be assessed through genetic knockdown of TFEB or TFE3. This would directly unpick the functional role of TFEB or TFE3 induction on lysosomal function induced by treatment. This same approach may help decipher the magnitude of the role played by induction of TFEB and/or TFE3 following the treatment of HSP90 inhibitors. CRISPRi-mediated knockdown of TFEB may be difficult as dCas9 can be blocked by nucleosomes (Horlbeck et al., 2016). As TFEB is likely transcriptionally repressed, a repressive chromatin formation may be present, thus treatment would require robust chromatin remodelling to allow access to the dCas9 machinery. Anti-sense oligonucleotides may provide a viable alternative here.

4.12.4 HDAC regulation upon TFEB and CRISPRi-screening

HDAC inhibitors were the best compounds for induction of TFEB and enhancing lysosomal GCase (with the least deficits in DQ-BSA). This was best represented by ACY-738, a HDAC1, 2, 3 and 6 inhibitor. ACY-738 induced TFEB mRNA and protein levels, in addition to activating lysosomal GCase and DQ-BSA. This compound therefore seems most optimal to follow-up to enhance lysosomal function in PD. EC₅₀ for this compound from our qRT-PCR assay was calculated to be 1.45µM. Reported ACY-738 cell-free IC₅₀ values are: HDAC1 – 94nM; HDAC2 – 128nM; HDAC3 – 218nM; and HDAC6 1.7nM. Given the EC₅₀ proximity to the HDACs1,2 and 3 cell-free IC₅₀, this provides more indication of these HDACs to be the effectors in TFEB expression, particularly in the context of ACY-738.

We did not observe knockdown of any specific HDAC to induce *TFEB* expression. Knockdown of each HDAC still needs to be confirmed, however this finding aligns with the delineation of our pharmacological data. Class I HDACs seem the most likely functional regulators of *TFEB* transcription, with redundancy between them. We hypothesise 2 of the 3 HDACs must be inhibited to produce a robust *TFEB* induction. HDAC1 and HDAC2 are highly similar and have frequently been known to have redundant functions, whereas HDAC3 is less known for this (Jurkin et al., 2011; Moser et al., 2013). Nonetheless, HDAC3 may act upon a converging pathway co-operating with HDAC1/2 to aid in the inhibition of *TFEB*.

Since CRISPRi represses transcription, expression is merely knocked down not ablated. This partial knockdown may not be sufficient to reduce the regulation upon *TFEB* expression. Pharmacological inhibition benefits over genetic knockdown in this manner as compounds can successfully modulate the function of the protein itself. Thus, to address redundancy between HDACs, future experimentation will co-knockdown multiple HDACs to identify redundantly acting HDACs. We will also knockdown HDACs in the presence of specific inhibitors at EC₅₀ concentration. This aims to combine the specificity of genetic modulation, with the modulatory benefits of compound treatment. Thus, if knockdown of a specific protein is not sufficient to reduce its complete regulation upon *TFEB*, knockdown should sensitise the cells to compound co-treatment thereby lowering the EC₅₀ (and in this case, increasing *TFEB* induction at EC₅₀).

4.12.5 Summary

In summary, a high throughput qRT-PCR approach identified novel *TFEB* transcriptional regulators in neurons, such as HSP90, and confirmed the modulatory effects of HDACs upon *TFEB*, both of which induced *TFEB* protein expression in neurons. Proteasome inhibition elevated *TFEB* mRNA but this did not translate to protein expression. qRT-PCR screening for *TFE3* transcriptional activators was hindered by potential confounding stressors but identified PI3K/AKT and HSP90 as potential hits.

HDAC and HSP90 inhibition robustly upregulated lysosomal GCase expression in neurons, which correlated well with *TFEB* expression. *TFE3* hits were less successful in upregulating protein expression and hit target engagement did not convincingly enhance lysosomal function. Further work needs to be carried out to identify regulatory HDACs upon *TFEB* expression by genetic means, however through analysis of pharmacological data, Class I HDACs appear most likely candidates.

Chapter 5: Pharmacological and genetic modulation of TFE3 and TFEB in iPSC-DaNs

5.1 Introduction

We discussed in Chapter 3 that the discovery of TFE3 as the primary neuronal transcription factor between TFEB and TFE3 led to 2 avenues of research. The first was assessed in Chapter 3 and 4 by exploration of transcriptional regulation upon *TFEB* (and *TFE3*) in neurons. Chapter 5 and 6 will address the second avenue in understanding how TFE3 modulation may affect lysosomal function in the context of PD.

Pharmacological stimulation of the CLEAR network is often studied regarding TFEB translocation. In PD, pharmacological TFEB activation has been studied frequently in cell-lines or rodent models but scarcely in human neuronal cultures (Table 1.4). Further, TFE3 is frequently overlooked for conferring functional benefits in neurodegenerative models following treatment of these compounds despite being regulated in a similar manner (Section 1.3.6).

Our results suggest that TFE3 is more highly expressed than TFEB in neurons and is therefore a more relevant therapeutic target for lysosomal function in neurodegeneration. Thus, efforts to understand how TFE3 play a role in neurodegeneration, both through genetic and pharmacological means should be undertaken.

Lysosomal Ca^{2+} is of great interest in neurodegeneration, both in pathogenesis as well as for therapeutic benefit. Findings have shown defective lysosomal calcium storage and release in various neurodegenerative models including PD (Feng and Yang, 2016). As discussed in Section 1.2.3.1.1, lysosomal Ca^{2+} release has a profound function toward lysosomal signalling transduction, lysosomal biogenesis, fusion exocytosis, acidification and autophagy (Medina et al., 2021). Lysosomal Ca^{2+} release by TRPML1 activates the protein phosphatase calcineurin, which can dephosphorylate TFEB and TFE3, inducing nuclear translocation thereby upregulating

lysosomal biogenesis (Medina et al., 2015; Martina et al., 2016). TRPML1 activation has previously been shown to confer neuroprotection in neurodegenerative models, mostly by aiding cellular clearance pathways (Pollmanns et al., 2022; Somogyi et al., 2023). We sought to assess TFE3 activation and functional lysosomal responses to the TRPML1 agonist, ML-SA5 in control iPSC-DaNs.

Genetically modulating TFE3 (and TFEB) expression allows us to specifically elucidate their roles upon the lysosomal network and other functions. TFEB overexpression has been well reported to benefit neurodegenerative models, but less has been done in the context of TFE3 overexpression. We aimed to assess the effects of genetically overexpressing TFE3 and TFEB in iPSC-DaNs and knocking down TFE3 using *i*³ neurons, upon lysosomal function in neurons to understand the therapeutic role TFE3 may have in neurons compared to TFEB. We also sought to understand specific roles for TFEB and TFE3 in iPSC-DaNs using RNA-Seq following overexpression to define the transcriptional changes associated with each transcription factor in this cell-type.

Aims of this chapter:

- Assess TFE3 translocation and lysosomal function following lysosomal Ca²⁺ release, through ML-SA5 treatment in control iPSC-DaNs.
- Examine CLEAR gene and lysosomal responses to TFE3 overexpression and knockdown in iPSC-DaNs and *i*³ neurons, respectively.
- Assess effects of TFEB overexpression on CLEAR genes and lysosomal function in iPSC-DaNs
- Undertake RNA-Seq to identify the transcriptional responses to TFEB or TFE3 overexpression in iPSC-DaNs.

5.2 TRPML1-mediated lysosomal Ca^{2+} release effects on TFE3 and lysosomal function in iPSC-DaNs

We first assessed TFE3 activation following lysosomal Ca^{2+} release using TRPML1-agonist, ML-SA5. ML-SA5-mediated TRPML1 activation should prompt localised Ca^{2+} release, calcineurin activation, subsequent TFE3 dephosphorylation and nuclear translocation (Figure 5.1 A). We confirmed localised Ca^{2+} release proximal to the lysosome following ML-SA5 treatment using a LAMP1-fused genetically encoded Ca^{2+} indicator (GECI), LAMP1-GCaMP7. iPSC-DaNs transduced with LAMP1-GCaMP7 were acutely treated with ML-SA5 (90 μM). Fluorescence was used to measure lysosomal Ca^{2+} release and was assessed over 1 minute following treatment (Figure 5.1 B i-ii). ML-SA5 evoked increased Ca^{2+} in a 2-part response, one immediate small peak followed by a greater, slower elevation in Ca^{2+} (Figure 5.2 B iii).

We next assessed TFE3 activation following TRPML1 activation. To do this, we observed %DaNs with TFE3 nuclear localisation following ML-SA5 treatment. We also assessed established TFE3 activators CQ and Torin1, as a comparison (Figure 5.1 C i). All 3 treatments stimulated robust nuclear TFE3 localisation in iPSC-DaNs, with ML-SA5 inducing translocation comparably to mTOR inhibition (Torin1), but lower than CQ (Figure 5.1 C ii). Significant increases in CLEAR genes *LAMP1*, *BECN1* and *ATP6V0A1* were observed by qRT-PCR, as well as trending increases in *GBA*, *CTSD* and *SQSTM1* (Figure 5.1 D). Western blotting of TFE3 and lysosomal proteins displayed no significant expression changes following ML-SA5 treatment, although LAMP1 expression showed minor trends in upregulation (Figure 5.1 E).

Next, we assessed lysosomal function following ML-SA5 treatment in control iPSC-DaNs, by MDW-941 (lysosomal GCase inhibitory), 4-MUG (GCase activity assay), DQ-BSA (endolysosomal proteolysis), lysosomal pH and LAMP1+ puncta. MDW-941 binds near the active pocket of lysosomal GCase, thus is suggested to measure GCase activity, however it does not measure the substrate catabolic activity of GCase. 4-MUG is a lower resolution assay than MDW-941 (which can be assessed at single cell level), but 4-MUG measures substrate cleavage and therefore GCase catabolic activity in culture. We used both assays to gain a greater perspective of GCase activity within neurons. ML-SA5 treatment significantly increased MDW-941 spot intensity and

near significantly increased MDW-941 spot number (Figure 5.1 F). ML-SA5 robustly increased 4-MUG in all 3 control iPSC-DaN lines but differential magnitudes of response produced a lack of significance (Figure 5.1 G). A significant increase in LAMP1-positive puncta was also observed, demonstrating increased endolysosomes following treatment (Figure 5.1 H). ML-SA5 treatment significantly increased lysosomal pH (Figure 5.1 I). Despite this, DQ-BSA spot number and intensity were unchanged (Figure 5.1 J i-ii). Collectively, lysosomal Ca^{2+} release activated TFE3 and subsequent lysosomal biogenesis/function but may perturb luminal acidification.

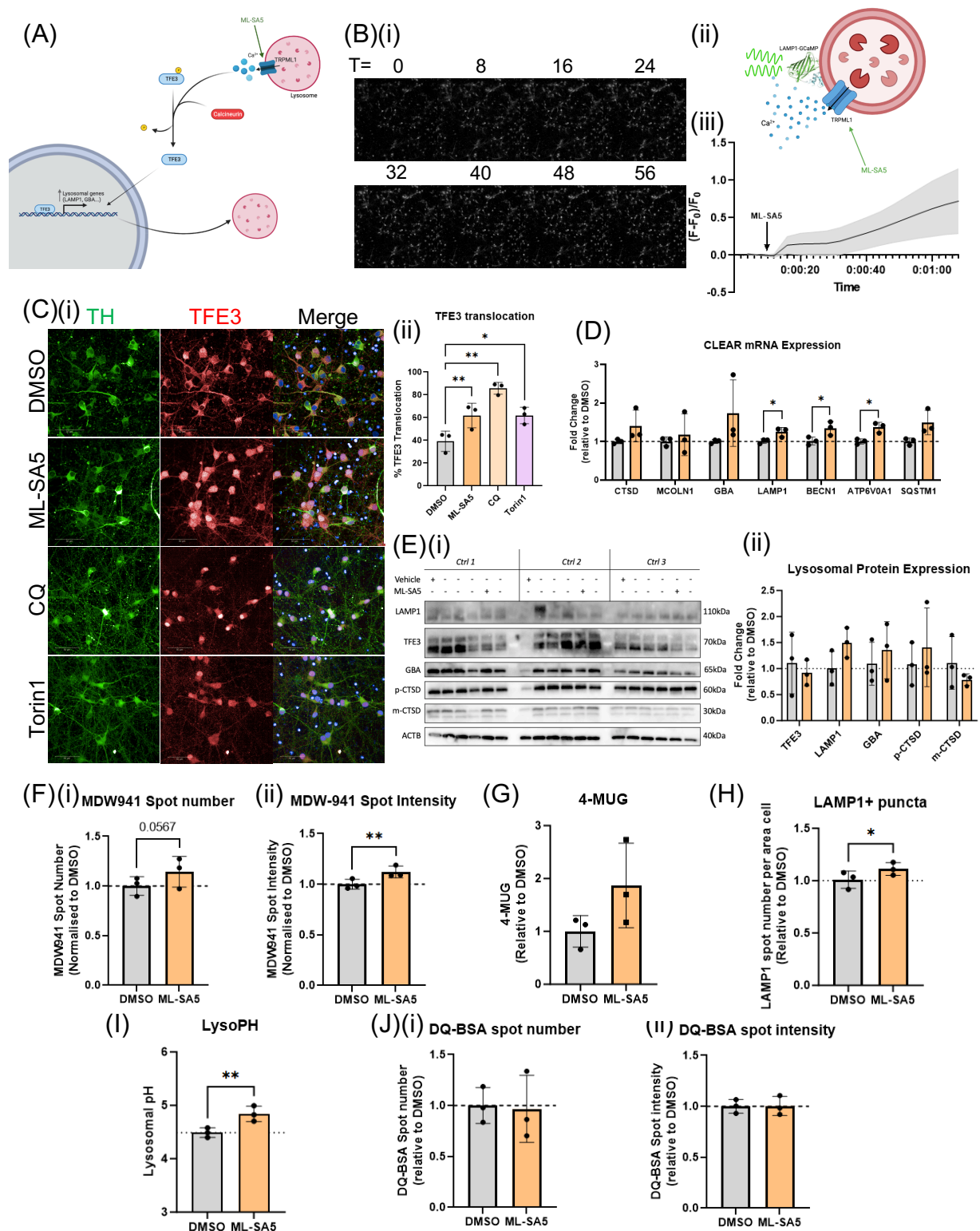


Figure 5.1 – ML-SA5 stimulates lysosomal calcium release and activates TFE3 translocation in iPSC-DaNs.

(A) Schematic of ML-SA5 function. Briefly, ML-SA5 activates TRPML1-mediated lysosomal Ca^{2+} release. Increased Ca^{2+} proximal to the lysosome activates Calcineurin (red) that dephosphorylates TFE3, causing translocation to the nucleus and upregulation of lysosomal gene expression, thereby enhancing lysosomal function. (B) ML-SA5 treatment causes lysosomal calcium release, assessed by fluorescence of LAMP1-GCaMP7-transduced iPSC-DaNs. (i) Representative images displaying increased fluorescence of LAMP1-GCaMP7 in

(Figure 5.1 continued...) iPSC-DaNs following treatment of ML-SA5. T (time with respect to addition of ML-SA5 in seconds). (ii) Schematic displaying calcium release at the lysosome induces fluorescence from LAMP1-GCaMP7 proximal to TRPML1. (iii) Trace showing average $(F-F_0)/F_0$ change in fluorescence intensity of the lysosomal GECI after ML-SA5 treatment in 3 control iPSC-DaN lines over 1 minute. (C) (i) Representative image and (ii) quantification of TFE3 translocation (red) in dopaminergic neurons (stained with TH, green) following treatment of 10 μ M ML-SA5, 20 μ M CQ, and 1 μ M Torin1. (D) Fold change mRNA expression of CLEAR genes by qRT-PCR following ML-SA5 treatment of control iPSC-DaNs, relative to DMSO control. (E) (i) Representative blots and (ii) fold change quantification of lysosomal proteins by western blot in control iPSC-DaNs following ML-SA5 treatment relative to DMSO (N=2 differentiations). Fold change (F) MDW-941 and (i) spot number and (ii) spot intensity in control iPSC-DaNs after ML-SA5 treatment relative to DMSO. (G) 4-MUG measured in iPSC-DaNs following ML-SA5 treatment. (H) Number of LAMP1-positive puncta per area cell measured by ICC in iPSC-DaNs treated with ML-SA5. (I) Lysosomal pH changes following ML-SA5 treatment. (J) Fold change DQ-BSA (i) spot number and (ii) spot intensity following ML-SA5 treatment in iPSC-DaNs. Paired t-test undertaken for all sections except (C) in which a paired One-way ANOVA was carried out with Dunnett's multiple comparisons. * $p < 0.05$, ** $p < 0.01$. N= 3 control lines, 3-4 differentiations unless stated.

5.3 Far-red lentiviral TFEB-iRFP670 and TFE3-iRFP670 vectors for overexpression of TFEB and TFE3 in iPSC-DaNs

To examine TFEB or TFE3 overexpression in iPSC-DaNs, we designed lentiviral constructs that placed TFEB or TFE3 under the regulation of the strong promoter EF1 α , and attached a far-red fluorescent reporter, iRFP670 to TFEB or TFE3 via a linker (Figure 5.2 A). The far-red fluorophore allows live-cell imaging of TFEB/TFE3 overexpression constructs, compatible with the fluorescent lysosomal assays we have established. The production of the lentiviral constructs by gBlock design and cloning is discussed in Methods; Section 2.4.1.

Following lentiviral production, iPSC-DaNs were transduced with TFEB-iRFP670 or TFE3-iRFP670 or iRFP670 (control vector) (Figure 5.2 A). TFEB and TFE3 expression was confirmed following transduction of their respective constructs by both immunocytochemistry and western blotting (Figure 5.2 B-E). iRFP670 fluorescent signal was observed by immunofluorescence in cells transduced with each construct, albeit weaker in TFEB and TFE3 constructs (Figure 5.2 B and D).

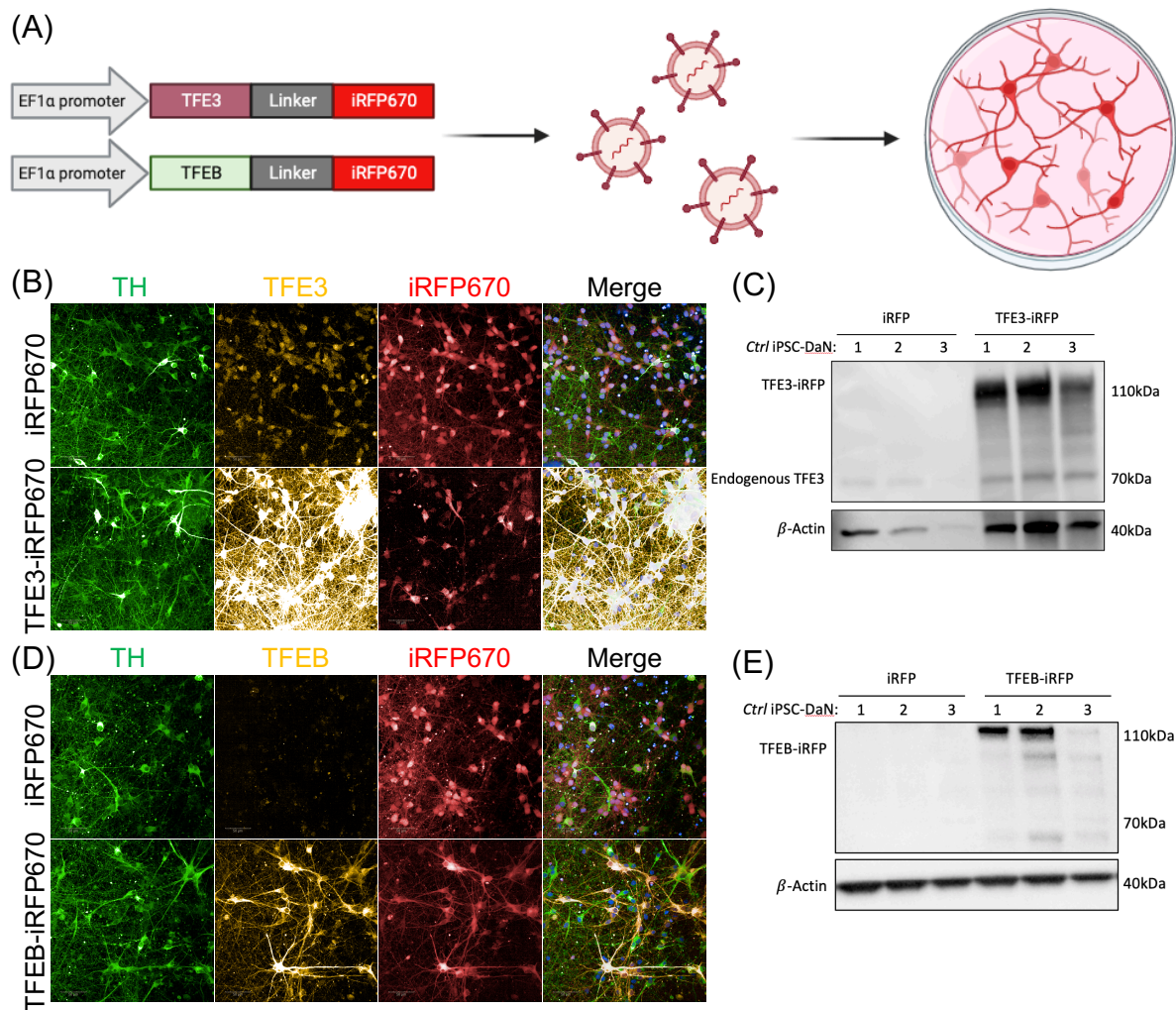


Figure 5.2 – Production and transduction of iPSC-DaNs with far-red TFE3 and TFE3 lentiviral overexpression vectors.

(A) Schematic of far-red lentiviral TFE3-iRFP670 and TFE3-iRFP670 transduction into neurons. Representative images of TFE3 overexpression by (B) immunocytochemistry in a control iPSC-DaN line, and (C) Western blot in 3 control iPSC-DaN lines transduced with iRFP670 control vector or TFE3-iRFP670. Representative images of TFE3 overexpression by (D) immunocytochemistry in a control iPSC-DaN line and (E) Western blot in 3 control iPSC-DaNs transduced with control iRFP670 vector or TFE3-iRFP670.

5.4 Effects of TFEB and TFE3 overexpression upon autophagic/lysosomal function in iPSC-DaNs

We then assessed downstream effects upon the autophagic-lysosomal system following TFEB or TFE3 overexpression in control iPSC-DaNs.

5.4.1 Lysosomal function following TFE3 overexpression in iPSC-DaNs

TFE3-iRFP670 overexpression in control iPSC-DaNs significantly upregulated *CTSD* and *GBA* expression by qRT-PCR, and trending increases in other genes (such as *LAMP1* and *MCOLN1*) were observed (Figure 5.3 A). Lysosomal protein expression following TFEB or TFE3 overexpression was assessed by western blot (Figure 5.3 B i). TFE3 overexpression significantly upregulated GCase and mature CTSD (m-CTSD) protein levels and trending elevation of LAMP1, but not immature CTSD (p-CTSD) (Figure 5.3 B ii). The ratio of m-CTSD to p-CTSD increased significantly, suggesting improved CTSD maturation by TFE3 overexpression (Figure 5.3 B ii). LC3-II and p62 flux was assessed using Bafilomycin treatment to inhibit autophagosomal-lysosomal fusion, preventing LC3-II and p62 turnover (Figure 5.3 C i). LC3-II significantly increased following bafilomycin treatment and TFE3 overexpression prompted trending increases compared to iRFP670 transduced iPSC-DaNs (Figure 5.3 C ii). Bafilomycin + TFE3 did not robustly upregulate LC3-II further. As such, a mild trending but not significant decrease in LC3-II flux was observed (Figure 5.3 C iii). Trending increases of p62 were observed following TFE3-overexpression or bafilomycin treatment of iRFP-transduced iPSC-DaNs (Figure 5.3 C iv). Bafilomycin exacerbated the p62 increase seen following TFE3 overexpression. Thus, TFE3 overexpression prompted trending but not significant increases in p62 flux (Figure 6.2 C v).

TFE3 overexpression displayed significant upregulation of 4-MUG and near significant increases in MDW-941 spot number and intensity, indicative of enhanced GCase function (Figure 5.3 D and E).

Near significant increases in LAMP1-positive puncta were also observed (Figure 5.3 F). TFE3 overexpression significant increased DQ-BSA spot number but not spot

intensity (Figure 5.3 G). No significant changes in Lysosomal pH were observed (Figure 5.3 H). Overall, these results display a positive relationship between lysosomal function in neurons and TFE3 overexpression, displaying increased lysosome number, GCase function and CTSD maturation. It is uncertain whether TFE3 overexpression affects autophagic flux.

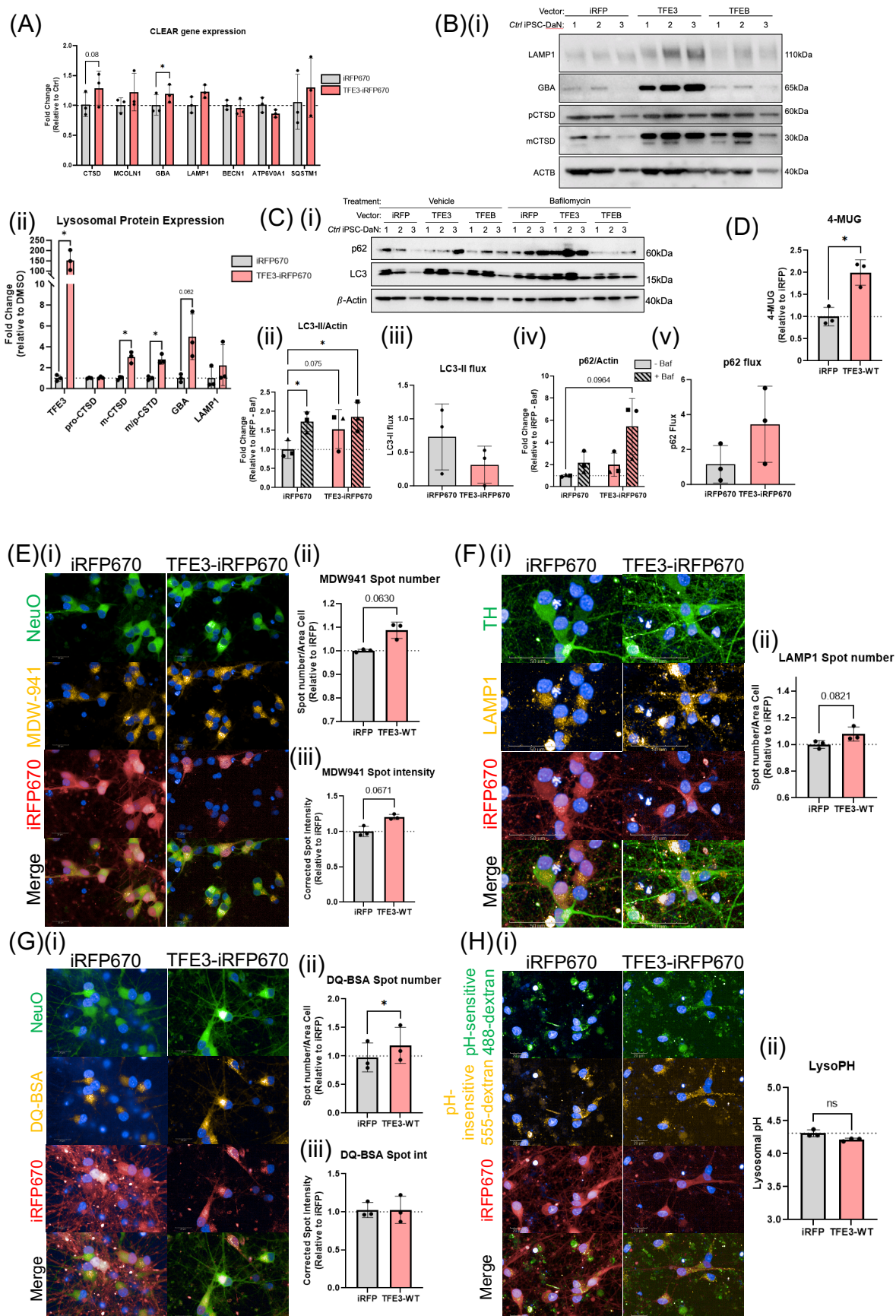


Figure 5.3 – TFE3-iRFP670 overexpression in control iPSC-DaNs upregulates lysosomal function and CLEAR expression.

(Figure 5.3 continued...) (A) CLEAR mRNA expression by qRT-PCR, following overexpression of TFE3 or control far-red vectors. (B) (i) Representative western blot of iRFP670, TFE3, and TFEB overexpression in 3 control iPSC-DaNs. (ii) Quantification of TFE3 overexpression upon lysosome-related proteins. (C) (i) Representative blots of LC3 and p62 levels in iPSC-DaNs overexpressing TFEB/TFE3 $\pm$ v-ATPase inhibitor, Bafilomycin A1 to measure flux. Quantification of (ii) LC3-II/Actin, (iii) LC3-II flux, (iii) p62/Actin and (iv) p62 flux following TFE3 overexpression. N=2 differentiations. (D) 4-MUG GCase activity assay following overexpression of iRFP670 or TFE3 in control iPSC-DaNs. (E) (i) Representative images of TFE3 overexpression (iRFP670, red) on MDW-941 (orange) in neurons (green). Quantification of TFE3 overexpression upon (ii) MDW-941 spot number per area cell and (iii) corrected spot intensity relative to control vector overexpression. (F) (i) Representative LAMP1 immunostaining images and (ii) quantification of LAMP1-positive puncta per area cell in control iPSC-DaNs overexpressing iRFP670 and TFE3. (G) (i) Representative DQ-BSA images in iRFP670 or TFE3 overexpressing iPSC-DaNs; and quantification of DQ-BSA (ii) spot number per area cell and (iii) corrected spot intensity. (H) (i) Representative lysosomal pH images of pH-sensitive 488 dextrans (green) and pH-insensitive 555 dextrans (orange) in control iPSC-DaNs overexpressing iRFP670 or TFE3–iRFP670 (red). (ii) Quantification of lysosomal pH. N=3 control lines, 3 differentiations unless previously stated. Paired t-test undertaken in all sections, except for (C) (ii) LC3-II/Actin and (iv) p62/Actin, in which a paired 2-way ANOVA with Šídák's multiple comparisons. * $p < 0.05$. p-value stated above comparisons with $p < 0.1$ that did not reach significance.

5.4.2 Lysosomal Function following TFEB overexpression in iPSC-DaNs

Surprisingly, TFEB overexpression significantly decreased *GBA*, *BECN1*, and *ATP6V0A1* mRNA expression (Figure 5.4 A). Western blot revealed near-significant increases in m-CTSD following TFEB overexpression but no significant changes in lysosomal protein expression were observed (Figure 5.4 B). LC3-II shows trending increases basally following TFEB overexpression or Bafilomycin treatment but no changes in autophagic flux (Figure 5.4 C i and ii). p62 displayed trending increases following bafilomycin treatment in iRFP670 cells and increased in 2 of 3 iPSC-DaNs following TFEB overexpression (Figure 5.4 C iii). However, Bafilomycin did not alter p62 levels following TFEB overexpression (Figure 5.4 C iii). As such, non-significant but trending decreases in p62 flux was observed. 4-MUG was significantly increased, but MDW-941 in fact displayed near-significant reductions in spot number following TFEB overexpression (Figures 5.4 D-E). No significant changes were seen in LAMP1-positive puncta, DQ-BSA, or lysosomal pH following TFEB overexpression (Figure 5.4 F-H). With the exception of GCase substrate cleavage, no functional enhancement of lysosomal parameter was observed following TFEB overexpression in iPSC-DaNs.

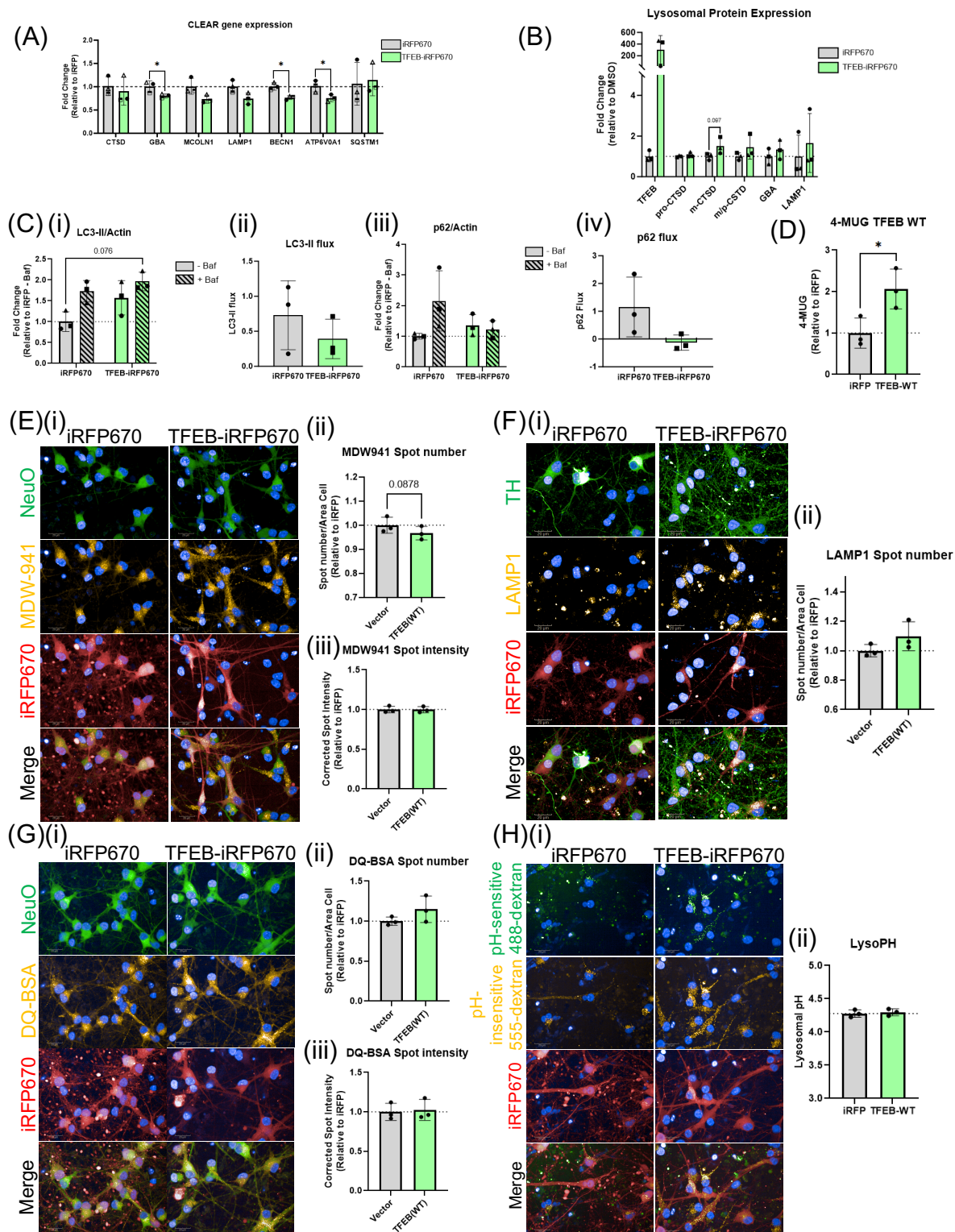


Figure 5.4 - Autophagic/lysosomal function and CLEAR gene expression following TFEB-iRFP670 overexpression.

(A) CLEAR mRNA expression by qRT-PCR, following overexpression of TFEB or control far-red vectors. (B) Quantification of TFEB overexpression upon lysosome-related proteins in iPSC-DaNs by western blot. (C) Quantification of (i) LC3-II/Actin, (ii) LC3-II flux, (iii) p62/Actin and (iv) p62 flux following TFEB overexpression. N=2 differentiations. (D) 4-MUG GCase activity assay following overexpression of iRFP670 or TFEB in control iPSC-DaNs. (E) (i) Representative images of TFEB overexpression

(Figure 5.4 continued...) (iRFP670, red) on MDW-941 (orange) in neurons (green). Quantification of TFEB overexpression upon (ii) MDW-941 spot number per area cell and (iii) corrected spot intensity relative to control vector overexpression. (F) (i) Representative LAMP1 immunostaining images and (ii) quantification of LAMP1-positive puncta per area cell in control iPSC-DaNs overexpressing iRFP670 and TFEB. (G) (i) Representative DQ-BSA images in iRFP670 or TFEB overexpressing iPSC-DaNs; and quantification of DQ-BSA (ii) spot number per area cell and (iii) corrected spot intensity. (H) (i) Representative lysosomal pH images of pH-sensitive 488 dextrans (green) and pH-insensitive 555 dextrans (orange) in control iPSC-DaNs overexpressing iRFP670 or TFEB–iRFP670 (red). (ii) Quantification of lysosomal pH. N=3 control lines, 3 differentiations unless otherwise stated. Paired t-test undertaken in all sections, except for (C) (i) LC3-II/Actin and (iii) p62/Actin, in which a paired 2-way ANOVA with Šídák's multiple comparisons. * $p < 0.05$. p-value stated above comparisons with $p < 0.1$ that did not reach significance.

5.5 CRISPRi-mediated knockdown of *TFE3* in i3 Neurons

TFE3 knockdown perturbed autophagic-lysosomal gene/protein expression and led to DaN neurodegeneration in mice, demonstrating an essential role for *TFE3* in DaN homeostasis (He et al., 2021). We examined CLEAR expression and lysosomal function following *TFE3* knockdown by CRISPRi in i³ neurons. We cloned 2 *TFE3*-targeted sgRNA lentiviral plasmids (Sequences in Table 5.1). qRT-PCR revealed robust knockdown of *TFE3* to near knockout levels (Figure 5.5 A). This was confirmed by western blot (Figure 5.5 B). Immunocytochemistry revealed less robust knockdown but reduced protein nonetheless, possibly due to non-specific antibody binding (Figure 5.5 D). *TFE3* knockdown demonstrated small but significant decreases in *MCOLN1*, *BECN1*, and *SQSTM1* mRNA expression, and trending reductions in the remaining genes (Figure 5.5 C). Knockdown was unable to produce robust perturbation of MDW-941 or DQ-BSA in i³ neurons however (Figure 5.5 E-F). Increasing the number of guides used will allow for a better insight into the functional consequences of *TFE3* knockdown, however it is evident knockdown of *TFE3* in neurons mildly perturbs the CLEAR network.

Table 5.1 - *TFE3* sgRNA guide sequences

Target of interest	sgRNA Name	sgRNA sequence
TFE3	TFE3-1	GCCGCGCAGTGCTAGCTCCA (<i>Courtesy of P. Carling</i>)
	TFE3-2	GCCCCGCTAGACGCAGTCAA (<i>Courtesy of P. Carling</i>)

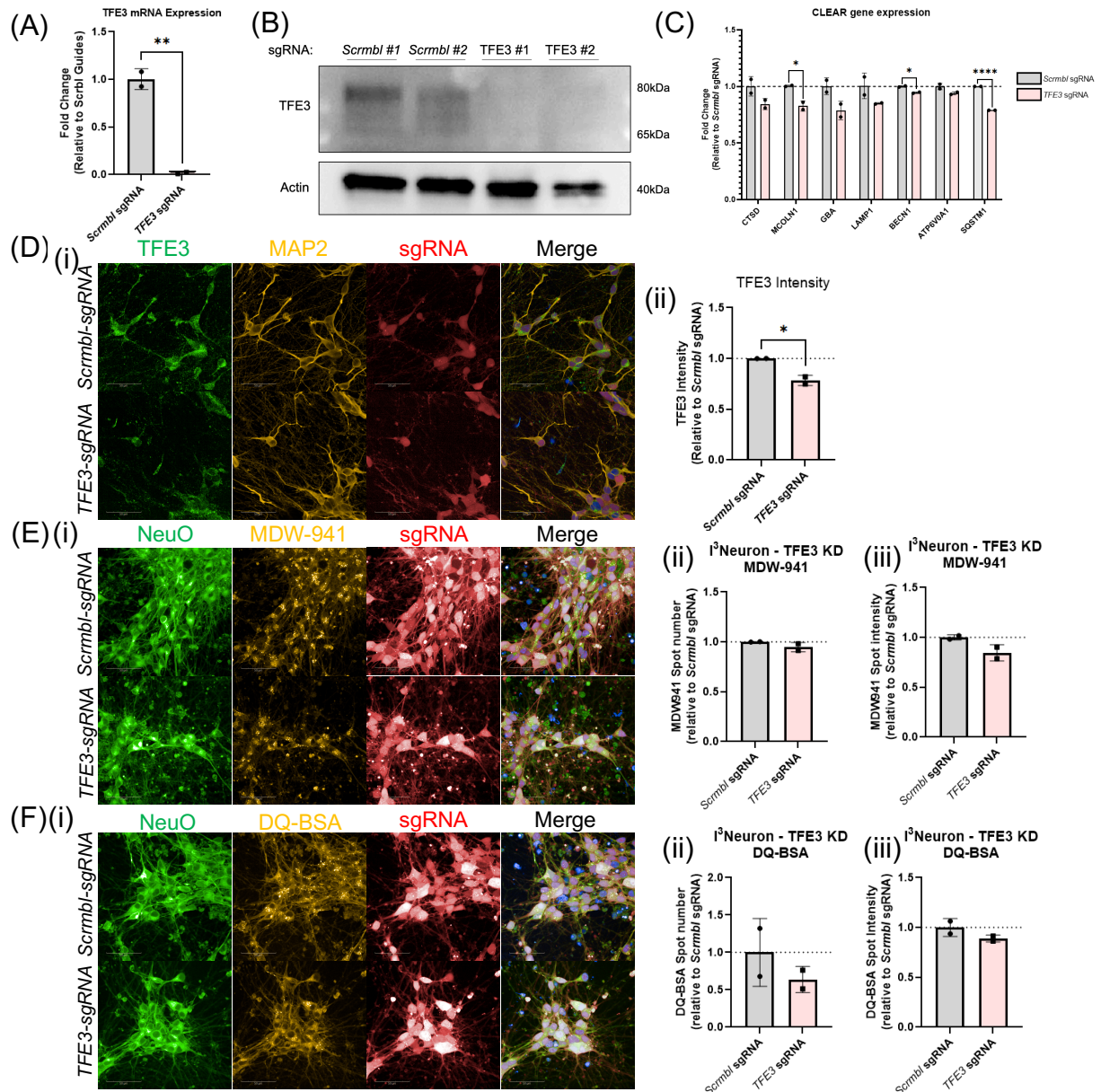


Figure 5.5 – TFE3 knockdown in i^3 Neurons.

(A) *TFE3* expression by (A) qRT-PCR and (B) Western blot following *TFE3* knockdown. (C) CLEAR gene mRNA expression in i^3 neurons with *TFE3* knockdown (light red). (D) (i) Representative images and (ii) quantification of TFE3 immunofluorescence intensity (green) following knockdown by far-red *TFE3* sgRNA vectors in i^3 neurons (green). (E) (i) Representative images of MDW-941 (orange) in i^3 neurons (NeuO – green) transduced with *Scrambled* or *TFE3* sgRNA (red). Quantification of *TFE3* knockdown on MDW-941 (ii) spot number per area cell, and (iii) spot intensity. (F) (i) Representative images of DQ-BSA (orange) in i^3 neurons (green) transduced with *Scrambled* or *TFE3* sgRNA vectors (red). Quantification of *TFE3* knockdown on DQ-BSA (ii) spot number per area cell and (iii) spot intensity. N=2 sgRNA vectors, 1 differentiation (B, E, F); 2 differentiations (A, C, D). T-test: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

5.6 Transcriptomic profiling TFE3 and TFEB overexpression in iPSC-DaNs

We found considerably different lysosomal responses to overexpression of TFEB and TFE3 in iPSC-DaNs, with TFE3 overexpression better at elevating CLEAR expression and lysosomal biogenesis/function than TFEB overexpression. We used RNA-Seq to examine transcriptomic changes following TFEB or TFE3 overexpression in iPSC-DaNs and identify potentially distinct roles for TFEB and TFE3 in this cell-type. This approach will allow us to better predict the cellular effects of TFEB re-introduction into neurons by pharmacological means, such as HDAC inhibition.

We transduced 3 control iPSC-DaN lines with control vector (mApple), TFEB-iRFP, or TFE3-iRFP, before RNA-sequencing. Following sequencing, quality control using FASTQC, transcript indexing/quantification using Salmon, and differential expression analysis was undertaken using DESeq2 (Section 2.7.3.2). We carried out Principle Component Analysis (PCA) using regularized logarithm (rlog) transformation to reduce weighting bias associated with different gene abundance. TFE3 and TFEB overexpression segregated from mApple in a similar direction for each line with respect to the principle component axes (Figure 5.6 A). This gave us confidence that all lines responded to overexpression in a similar manner and a substantial effect is present. TFEB overexpression differentially expressed 3895 genes (1959 up, and 1936 down), and TFE3 overexpression differentially expressed 1194 (800 up and 194 down).

5.6.1 CLEAR gene expression changes following TFEB and TFE3 overexpression in iPSC-DaNs

Table 5.2 - TFE3 overexpression differentially expressed CLEAR genes

CLEAR gene	Log ₂ FC	P-adj
ATP6V0D2	2.97161891	7.83E-10
ATG9B	2.01109485	6.60E-11
PPARGC1A	1.66715565	2.06E-13
RRAGD	1.57751931	2.52E-13
CTSA	1.22276059	1.47E-31
ASAH1	1.04614212	7.74E-18
CLCN7	0.97464327	1.61E-07
TPP1	0.95945658	5.12E-10
VPS18	0.8557621	5.46E-12
CTNS	0.83333917	0.00060717
WIPI1	0.73995244	0.00328059
CD63	0.69023149	3.46E-12
BCL2	0.62957189	0.00059543
ATP6V1C1	0.62661897	0.00116657
HEXA	0.61041767	0.00000309
CTSD	0.59358096	0.0000813
RRAGC	0.5905344	0.0000112
NAPA	0.57563233	0.00052261
ATP6V1D	0.5565982	0.00133379
ATP6V0E1	0.51094742	0.01077379
MCOLN1	0.49821718	0.00688204
GNS	0.48642985	0.0000406
HEXB	0.47792994	0.00426685
NPC1	0.45299201	0.03621602
RAB7A	0.44797215	0.0000199
SPNS1	0.4469924	0.00695981
ATP6V0D1	0.44187591	0.0000263
SLC36A1	0.43674587	0.01975258
IGF2R	0.42702461	0.00483393
GBA1	0.42563395	0.00302665
VPS35	0.39996147	0.00525851
ATP6V1E1	0.3875126	0.03506258
ATP6V1B2	0.34654128	0.00185436

Table 5.3 - TFEB overexpression differentially expressed CLEAR genes (red text: downregulated)

CLEAR gene	Log ₂ FC	P-adj
IFI30	4.15736113	0.02002058
ATP6V0D2	3.70009357	3.13E-16
TFEB	3.50576203	5.86E-11
PPARGC1A	2.62190731	1.67E-34
RRAGD	1.46090516	3.90E-12
BCL2	1.35225331	6.27E-19
ATG9B	1.3490877	1.53E-05
ATG4D	0.94379869	7.09E-06
FUCA2	0.91965575	0.00082774
ATP6V1D	0.84366638	1.02E-08
PRKAG2	0.6517972	6.08E-05
ATP6V1H	0.64692788	0.00012164
PPARA	0.64204455	0.04811342
WIPI1	0.61147468	0.00864769
AP1S2	0.53155467	1.08E-06
CTSD	0.51930366	0.00026663
CTSA	0.49807286	1.00E-05
NAPA	0.39981754	0.01355867
ATP6V0B	0.39092124	0.00061064
ATP6V1E1	0.38823074	0.0132358
ATP6V0D1	0.34004299	0.00087195
CD63	0.27220274	0.01376388
ATP6V0C	0.26108306	0.01646601
PLBD2	-0.4138725	0.02189867
M6PR	-0.4728079	0.00062872
SGSH	-0.4940724	0.00320933
GALNS	-0.5075408	0.01757419
SLC12A4	-0.5203183	0.04210162
GALC	-0.6175168	0.00302739
CLCN5	-0.6298634	0.00189772
SUMF1	-0.7022511	0.00107199
CD164	-0.7054512	2.17E-07
TPP1	-0.7784415	4.69E-07
NAGLU	-0.9940089	3.52E-08

We next assessed transcriptional changes on 110 CLEAR genes following TFEB or TFE3 overexpression. A heatmap displayed TFE3 overexpression to generally upregulate CLEAR genes, but few changes were observed following TFEB overexpression (Figure 5.6 B). Of the 110 CLEAR genes examined, 33 were differentially expressed by TFE3 overexpression, and all upregulated (Table 5.2).

TFEB overexpression differentially regulated 34 CLEAR genes, but 11 were downregulated (Table 5.3).

Exemplar differentially expressed CLEAR genes are displayed on volcano plots of TFE3 and TFEB overexpression (Figure 5.6 C-D; Green boxes = upregulated, black boxes = downregulated). Overexpression of both transcription factors upregulated several of the same genes including v-ATPase subunits, cathepsins and others such as *Pgc1a*, showing functional overlap still occurs in DaNs (Tables 5.2 - 5.3). *RRAGD* was also significantly upregulated in both (as was *FLCN* – data not shown), demonstrating negative feedback following overexpression.

TFEB overexpression reduced expression of genes such as *M6PR* and *GALC*. *MCOLN1* and *GBA1* were specifically upregulated by TFE3 and not TFEB, similar to previous qRT-PCR data (Figures 5.3 A and 5.4 A). Other interesting TFE3-specific genes include *TPP1*, *NPC1*, *RAB7A* and more. Collectively, these data show TFE3 overexpression to have a more unanimous positive effect on CLEAR genes than TFEB overexpression.

5.6.2 PD-related gene expression following TFEB and TFE3 overexpression in iPSC-DaNs

Interestingly, several PD-associated genes were differentially expressed following TFEB or TFE3 overexpression in iPSC-DaNs. Both TFEB and TFE3 overexpression robustly upregulated *GPNUMB*, *GRN*, *SNCA*, *ELOVL7*, *ITPKB* and *CAMK2D* (Table 5.4-5.5). TFE3 overexpression-specific DEGs included *VPS35*, *GBA1* and *RAB29* (Table 5.4; Figure 5.6 E). TFEB overexpression upregulated genes such as *PINK1* and *TMEM163*, but downregulated many such as *SCARB2*, *DNAJC6*, *GALC* and *CLCN3* (Table 5.5; Figure 5.6 F).

Table 5.4 - TFE3 overexpression differentially expressed PD-related genes (red text: downregulated gene)

PD-associated gene	Log ₂ FC	P-adj
GPNMB	5.02190046	1.88E-19
ITPKB	1.4231701	1.35E-17
ELOVL7	1.86471073	0.0000514
CSMD1	1.71650758	0.03197435
GRN	1.02322684	3.5E-15
MBNL2	0.8988332	0.0000321
RAB29	0.73230131	0.01327437
SNCA	0.53503939	0.0070723
CAMK2D	0.44880586	0.00813531
GBA1	0.42563395	0.00302665
STK39	0.40586793	0.04277566
VPS35	0.39996147	0.00525851
IP6K2	-0.3586959	0.02074041
ZNF608	-0.490341	0.0050527

Table 5.5 - TFEB overexpression differentially expressed PD-related genes (red text: downregulated gene)

PD-associated gene	Log ₂ FC	P-adj
GPNMB	5.74809098	2.6152E-26
GRN	1.21470777	2.4384E-22
ELOVL7	1.92710354	5.4506E-06
SIPA1L2	1.16637811	1.8565E-05
ITPKB	0.99510093	3.7095E-09
SNCA	0.94172044	4.702E-09
SH3GL2	0.6701107	8.5124E-06
STK39	0.62858942	5.5197E-05
IGSF9B	0.61717175	0.00755502
HIP1R	0.52665972	0.00117241
BAG3	0.4138815	0.03862629
TMEM163	0.37445875	0.01179218
CAMK2D	0.35588716	0.02354181
PINK1	0.28557492	0.02946038
DCTN1	0.2731555	0.03140549
FYN	-0.343622	0.02612585
DNAJC6	-0.388752	0.00033554
RNF141	-0.3912736	0.01467207
SCARB2	-0.4051153	0.0008204
MED13	-0.4414605	0.00784243
CLCN3	-0.4768086	0.00028679
CHD9	-0.4777133	0.0163929
RIMS1	-0.529442	0.02091362
GALC	-0.6175168	0.00302739
RAB29	-0.787212	0.00297171
DLG2	-0.817955	1.4324E-05

LCORL	-0.8672602	2.7696E-08
SATB1	-1.0053953	3.5198E-06
LRP10	-1.3305404	4.9048E-15
ITGA8	-4.5282921	0.00865043

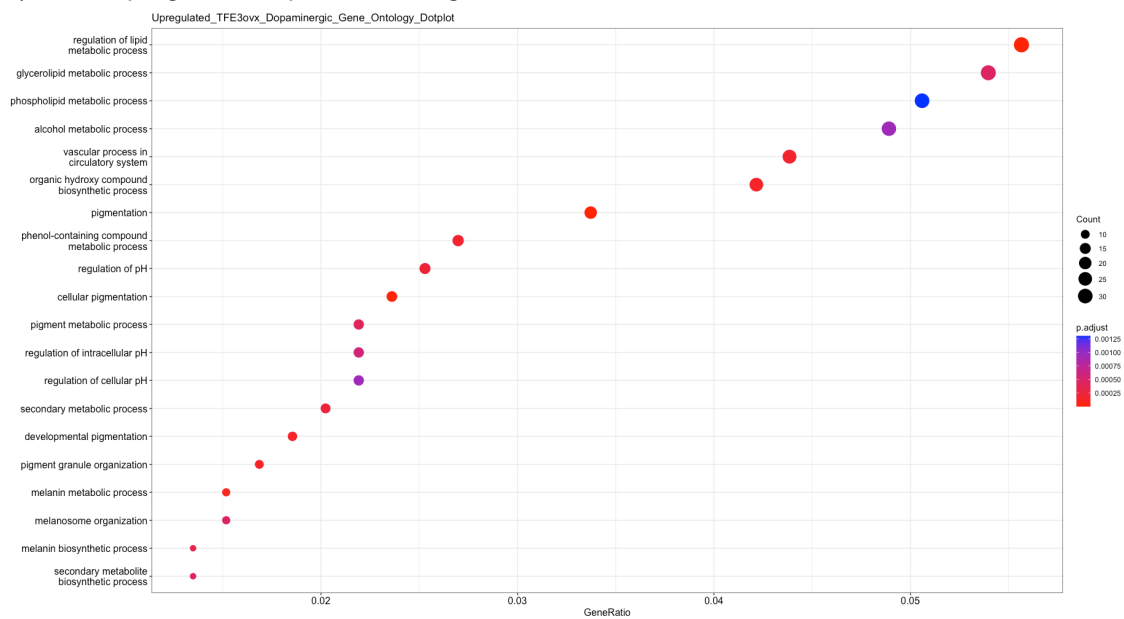
5.6.3 Pathway analysis of TFEB and TFE3 overexpression

Gene Ontology (GO) analysis of upregulated genes by TFE3 overexpression demonstrated lipid metabolism, regulation of pH and pigmentation to be among the highest enriched pathways (Figure 5.7 A). Strikingly, TFEB overexpression upregulated numerous mitochondrial pathways relating to energy production and respiration, suggesting more mitochondria-related role for TFEB in DaNs (Figure 5.7 B). This aligns with the significant upregulation of PINK1 and Pgc1a previously mentioned.

We took 70 genes relating to mitochondrial function (encoding proteins such as mitochondrial enzymes, electron transport chain complexes, tricarboxylic acid cycle proteins) and assessed transcriptional changes following TFEB or TFE3 overexpression by heatmap. There was a striking difference between TFEB overexpression compared to TFE3 overexpression or the control vector, suggesting a specific role for TFEB in mitochondrial function in DaNs (Figure 5.8 A). TFE3 overexpression mildly increased expression of these genes but the effect was much smaller. A volcano plot was used to visualise the fold change of some significantly upregulated mitochondria-related genes following TFEB overexpression (Figure 5.7 B).

Overall, TFE3 overexpression upregulated more CLEAR genes in iPSC-DaNs than TFEB overexpression, although several were upregulated by both. Intriguingly, many PD-related genes were differentially regulated following TFEB and TFE3 overexpression. Finally, TFEB may have a specific role in DaN mitochondrial function following its overexpression.

(A) TFE3 Upregulated Top 20 GO categories



(B) TFEB Upregulated Top 20 GO categories

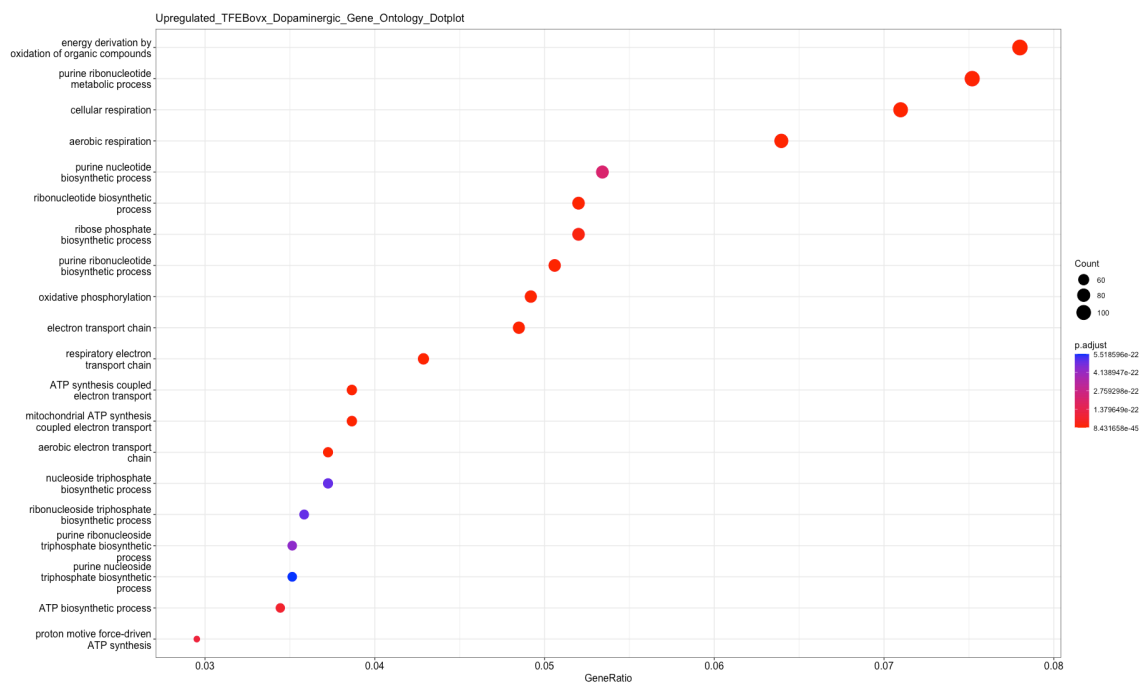
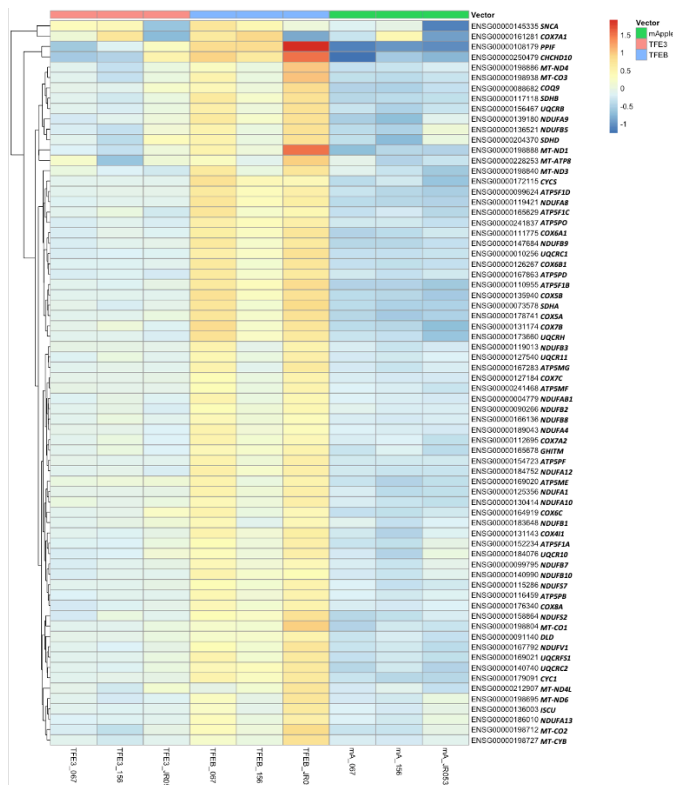


Figure 5.7 – Pathway analysis of TFEB and TFE3 overexpression in iPSC-DaNs reveals mitochondrial bioenergetic function for TFEB.

Top 20 Gene Ontology (GO) pathways from upregulated (LFC > 0.5) differentially expressed genes following (A) TFE3 overexpression or (B) TFEB overexpression in iPSC-DaNs.

(A) Aerobic respiration/OxPhos/Electron Transport Chain Heatmap



(B) TFEB-overexpression mitochondrial-related genes

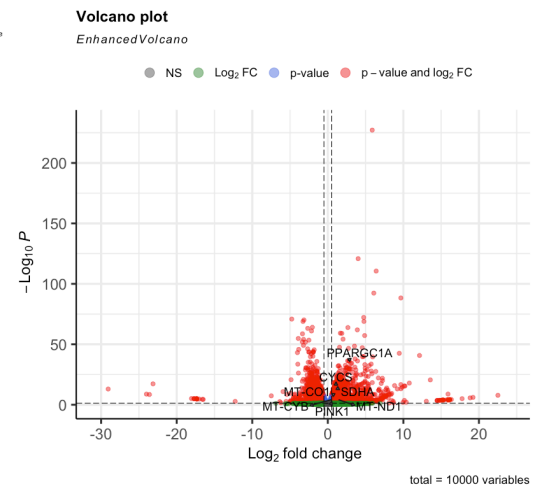


Figure 5.8 – Mitochondrial gene expression is upregulated by TFEB overexpression in iPSC-DaNs.

(A) rlog transformed heatmap of select genes relating to mitochondrial bioenergetics (ie. Oxidative phosphorylation, electron transport chain, respiration) corresponding to all samples. Red: TFE3 (left), Blue: TFEB (middle), Green: mApple (right). (B) TFEB overexpression volcano plot with exemplar differentially expressed mitochondria-related genes labelled. Origin of arrows indicate the location of the annotated gene on the volcano plot. Green boxes indicate upregulated genes.

5.7 Discussion

5.7.1 Genetic and pharmacological TFE3 activation upregulates lysosomal function

We demonstrated that TFE3 can be pharmacologically and genetically modulated in iPSC-derived neuronal models through TRPML1 activation, lentiviral overexpression, or CRISPRi knockdown. TFE3 activation upregulated CLEAR gene expression. TRPML1 agonist treatment increased CLEAR gene expression more broadly than TFE3 overexpression which did not increase genes such as *BECN1* or *ATP6V0A1*, suggesting lysosomal Ca^{2+} release may regulate multiple effectors contributing to enhanced lysosomal gene expression. TFE3 activation enhanced GCase in iPSC-DaNs, seen by qRT-PCR, RNA-Seq and functional assays. Thus, TFE3 activation may prove beneficial to overcoming GCase perturbation in PD (explored in Chapter 6).

Surprisingly, ML-SA5 alkalinised lysosomal pH. TRPML1 is thought to be partially permeable to protons and a model of the LSD, ML-IV (mucopolipidosis IV – TRPML1 dysfunction) displays over-acidified lysosomes (Soyombo et al., 2006). Activation of this channel for a prolonged period (48 hours here) may increase proton efflux from the lysosome through TRPML1 leading to alkalinisation of the lysosomal compartment. Lysosomal alkalinisation by TRPML1 activation must be further assessed to examine how viable TRPML1 agonists may be for PD treatment. Despite this finding, our data show no endolysosomal proteolysis perturbation and an enhancement of GCase. Previous publications have demonstrated benefits to using TRPML1 activation in PD models, thus beneficial effects of TRPML1 activation may offset any changes in lysosomal pH (Tsunemi et al., 2019; Pollmanns et al., 2022).

TFE3 overexpression increased lysosomal function in most assays, albeit many did not quite reach significance, likely through variability between iPSC-DaN lines. We assessed autophagic flux following TFE3 overexpression and found no significant changes but mild decreases in LC3-II flux and elevated p62 turnover. LC3-II changes point toward mildly reduced autophagosomal turnover in iPSC-DaNs following TFE3 overexpression. As p62 is a CLEAR gene, increased turnover may derive from increased synthesis by TFE3 (although no robust increases in *SQSTM1* mRNA was observed). p62 is a cargo-receptor for ubiquitinated substrates and has been

suggested to play cytoprotective role in aggregate clearance (Bjørkøy et al., 2005). Thus, while autophagosomal flux is unchanged (may be moderately reduced), selective autophagy of ubiquitinated substrates such as aggregates could be enhanced following TFE3 overexpression in iPSC-DaNs. Noteworthy, this assay is extremely variable. An alternative assay that may prove more useful is an LC3-GFP-RFP autophagic flux reporter. This assay requires exogenous expression of LC3 however, removing the possibility to assess autophagic responses resultant from transcriptional induction of LC3 (*MAP1LC3*), that has been shown to be regulated by TFE3 (Martina et al., 2014).

CRISPRi demonstrated excellent knockdown of *TFE3* in i³ neurons. Knockdown produced small reductions in expression of CLEAR genes examined. There may be compensatory responses accounting for the loss of TFE3 contributing to the minor reductions in CLEAR expression. This would be similar to our findings from RNA-Seq in which genes associated with TFEB/3 negative regulation were upregulated following TFE3 overexpression in iPSC-DaNs.

Despite TFE3 knockdown data not demonstrating robust alterations, particularly in lysosomal function, they do complement TFE3 overexpression data demonstrating a function for TFE3 activity upon CLEAR expression in neurons.

5.7.2 A dual role for TFEB and TFE3 upregulation of the v-ATPase

Both TFEB and TFE3 overexpression upregulated several v-ATPase subunits. Both have been shown to transcriptionally regulate this complex previously (Palmieri et al., 2011; Martina et al., 2014). V-ATPases has several functional roles such as pH sensing, luminal vesicle acidification, membrane fusion, nutrient sensing to regulate mTOR, endosome maturation and synaptic vesicle loading and transport (reviewed by Song et al., 2020). This myriad of functions has placed V-ATPases with significant relevance to neurodegeneration and PD. Further, *GBA*, *LRRK2*, and *SNCA* PD models with have all been shown to have alkalinised lysosomal compartments (Bourdenx et al., 2016; Wallings et al., 2019; Nascimento et al., 2020). Thus, upregulating v-ATPase expression by TFEB or TFE3 may be useful in combatting lysosomal perturbation in PD. While we did not observe ‘overacidification’ of lysosomes in healthy iPSC-DaNs, the next chapter will examine any lysosomal dysfunction including pH perturbation in patient-derived iPSC-DaNs carrying *GBA-N370S*, *LRRK2-R1441C* and *SNCA-Trp* mutations. Noteworthy, TFEB overexpression

downregulated *ATP6V0A1* by qRT-PCR, that interacts with LRRK2, but interaction is perturbed by *LRRK2-R1441C* mutation, thus TFEB overexpression may in fact exacerbate dysfunction (Wallings et al., 2019).

5.7.3 A more mitochondrial role for TFEB in iPSC-DaNs?

TFEB overexpression was less efficacious in CLEAR gene upregulation and lysosomal function than TFE3 overexpression. While some CLEAR genes were upregulated by TFEB overexpression in RNA-Seq, many were also downregulated, similar to our findings by qRT-PCR. Reduced lysosomal protein expression was not observed by western blotting however, suggesting a disconnect between the mRNA and protein levels. This may be resultant of elevated protein synthesis that has been reported following TFEB overexpression in mice (Torra et al., 2018). Nonetheless, the lack of CLEAR gene induction is contrary to previous observations (Decressac et al., 2013). The discrepancy here between our results and external findings may be a result of using different models, as CLEAR regulation was assessed through whole brain lysates in rodent models containing many cell-types beyond neurons. Alternatively, negative regulation upon the vector may be responsible as Rag GTPases and *FLCN* (data not shown), were both upregulated following overexpression of both TFEB and TFE3. A report previously assessing TFEB overexpression on an iPSC-derived neuronal model of Gaucher's disease found TFEB overexpression alone was insufficient to upregulate lysosome numbers in culture, even in control neurons, until recombinant GCase was co-expressed (Awad et al., 2015). Thus, basal overexpression in neurons without a stimulus or co-factor may not provide beneficial lysosomal effects until stimulated. These stimulatory factors may preside from non-neuronal cell-types *in vivo*, which may explain the lysosomal effects seen in mice but not in our iPSC-DaN cultures.

Our RNA-Seq data demonstrated an alternative primary function in DaNs for TFEB overexpression through enhancement of mitochondrial functional and bioenergetic pathways. Both transcription factors upregulated *Pgc1a*, a known CLEAR gene involved in mitochondrial biogenesis, however induction of mitochondrial genes were following TFEB overexpression rather than TFE3 overexpression, suggesting this as a more specific role for TFEB in neurons when re-introduced. Noteworthy, TFEB increased *PGC1 α* 2-fold more than TFE3 overexpression, which may contribute to this discrepancy.

TFEB and TFE3 are thought to have several redundant functions (Martina et al., 2014; Nezich et al., 2015; Oliveira et al., 2019). The transcription factors also carry their own individual functionality however and may work co-operatively or independently which dependent on context or cell-type (Pastore et al., 2016; Pastore et al., 2017; Oliveira et al., 2019). Our data display both differing and overlapping roles for TFE3 and TFEB in DaNs. Both transcription factors upregulated many lysosomal/CLEAR genes, but TFE3 activation enhanced the lysosomal network far superior to TFEB overexpression. Thus, while TFEB may play a menial role lysosomal gene expression showing some overlap with TFE3, the role of TFEB in mitochondrial function was far more robust and unique to that of TFE3.

This finding is intriguing given our previous observations of negligible TFEB expression in neurons. One may conclude that TFEB re-introduction into neurons do not aid lysosomal function in the co-operative manner first expected but will unmask a specific role for TFEB in enhancing mitochondrial capacity within neurons. Mitochondrial dysfunction is a major pathogenic mechanism in PD. Models that have assessed TFEB overexpression in PD have often used MPTP or SNCA overexpression, both of which have profound mitochondrial defects (Martin et al., 2006; Mustafa et al., 2021; Ryan et al., 2021). Thus, TFEB may confer neuroprotective effects through multiple mechanisms, one being the amelioration of mitochondrial deficits.

Regulation of mitochondrial gene transcription by TFEB is not novel. In fact, mitochondrial ontology was one of the most enriched in initial CLEAR network characterisation and displayed the highest number of genes regulated for all ontologies presented (Palmieri et al., 2011). Here, TFEB may be presenting a cell-type specific preferential upregulation of these mitochondrial genes to lysosomal genes.

TFEB overexpression was found to upregulate functions such as oxidative phosphorylation and ATP production in muscle, enhancing many genes overlapping with our dataset (Mansueto et al., 2017). Here, TFEB overexpression upregulated mitochondrial function independently of Pgc1 α , which may explain why mitochondrial gene expression was seen primarily in TFEB and not TFE3 despite both upregulating PGC1 α . Furthermore, TFEB overexpression did not enhance autophagic-lysosomal genes. Thus, TFEB overexpression in dopaminergic neurons replicates many of the findings seen in the muscle. A similar observation has recently been seen in Treg cells,

again upregulating mitochondrial bioenergetic and functional pathways rather than autophagic-lysosomal pathways (Xia et al., 2022). Thus, TFEB has a cell-type specific mitochondrial biogenesis function independent of lysosomal activation that appears in DaNs also.

This mitochondrial functional role for TFEB may not solely be due to the function of TFEB however, but through the ability to now form TFEB-TFE3 heterodimers in DaNs that would not be possible basally due to the lack of TFEB expression. The heterodimer may have an affinity for loci involved in the upregulation of mitochondrial biogenesis. Thus, TFEB alone may not solely be responsible for this finding, but unveiled co-operative interactions, such as that between TFEB and TFE3 may promote the expression of mitochondrial biogenesis genes. Further, endogenous TFE3 in DaNs may already occupy many available CLEAR sites in lysosomal gene promoters, preventing TFEB overexpression to effectively upregulate these genes. ChIP analysis of mitochondrial biogenesis target gene promoters would reveal whether the effect of TFEB overexpression upon these genes involved TFEB-TFE3 heterodimers or TFEB homodimers.

Nonetheless, re-introduction of TFEB appears to enhance mitochondrial gene expression. While this mitochondrial function independent of autophagy and lysosomal function for TFEB has been observed in other cell-types, it has not been placed in relevance to DaNs before and could provide a novel strategy for TFEB activation in neurodegeneration by overcoming bioenergetic deficits. Previous work from our lab have demonstrated bioenergetic failures via perturbed respiration concomitant with dysfunctional mitochondria (Zambon et al., 2019). Future work will assess how re-introduction of TFEB (genetically or via HDAC inhibition) may ameliorate mitochondrial phenotypes such as this.

5.7.4 TFEB and TFE3 overexpression regulates PD-associated gene expression.

Intriguingly, several PD-related genes were differentially expressed following TFEB or TFE3 overexpression. *SNCA* was upregulated by both. This may be a caveat to TFEB and TFE3 therapeutic potential in DaNs, given the dosage-dependent pattern of α Syn expression and PD severity (Ross et al., 2008). Fold change increases were ~1.4 and 1.9-fold following TFE3 and TFEB overexpression, respectively. Thus, TFEB and

TFE3 activation may affect pathology through α Syn transcriptional upregulation. Given previous studies have shown ameliorative effects against α Syn pathology, one would expect neuroprotective gene upregulation may off-set this (Decressac et al., 2013; Kilpatrick et al., 2015; Arotcantera et al., 2019).

2 other PD-related genes *GRN* and *GPNMB* were robustly upregulated by TFEB and TFE3 overexpression. Both genes have variants associated with PD risk (Nalls et al., 2019). *GRN* is associated with frontotemporal lobular dementia (FTLD) and the lysosomal storage disorder, neuronal ceroid lipofuscinosis (NCL) (Baker et al., 2006; Cruts et al., 2006; Smith et al., 2012). *GRN* plays roles in cathepsin maturation, GCase activity and lysosomal membrane integrity (Zhou et al., 2017; Valdez et al., 2020; Logan et al., 2021). In fact, *GRN* has been shown to be a CLEAR gene and is regulated by TFEB (Tanaka et al., 2013). Our results confirm *GRN* to be a target of TFEB and TFE3 in DaNs. *GRN* is intrinsically linked to lysosomal integrity and its dysfunction is associated with a broad number of neurodegenerative disorders, showing it could be a key factor for neuroprotection conferred by TFEB and TFE3 activation.

GPNMB has been also implicated in neurodegenerative diseases such as ALS and PD. *GPNMB* increases in SOD1-mutant ALS mice appear to be neuroprotective (Tanaka et al., 2012). In PD, *GPNMB* expression is increased in the SNc, possibly in response to lipid dysregulation by GCase perturbation (Moloney et al., 2019). Interestingly, *GPNMB* is a transcriptional target of MITF (Loftus et al., 2009; Gutknecht et al., 2015). Our results suggest this may extend to TFEB and TFE3.

Genes associated with PD predominantly through loss of function such as *GBA* and *VPS35* were upregulated by TFE3 overexpression, displaying potential neuroprotective benefits to TFE3 activation in PD. TFEB overexpression differentially expressed many more PD genes than TFE3 overexpression. TFEB overexpression upregulated *PINK1* corroborating the mitochondrial function seen with TFEB and not TFE3 in DaNs. Interestingly, TFEB overexpression downregulated *SCARB2* in DaNs. *SCARB2* encodes LIMP2 involved in GCase transport to the lysosome (Reczek et al., 2007). This may explain the reduced MDW-941 activity seen following TFEB overexpression. Interestingly, M6PR was also reduced with TFEB overexpression, suggesting broad abrogation of endolysosomal trafficking.

Overall, our data demonstrates enhanced lysosomal function following pharmacological (by TRPML1 activation) or genetic TFE3 activation. TFE3 overexpression was found to better upregulate lysosomal expression and function than TFEB overexpression. We demonstrated TFEB re-introduction to neurons by overexpression to largely upregulate genes associated with mitochondrial biogenesis and function in iPSC-DaNs, unveiling a cell-type specific role for the upregulation of TFEB in DaNs. Interestingly, TFEB and TFE3 overexpression in DaNs modulated numerous PD-associated genes, suggesting a close link with their activation and PD pathological mechanisms. Work in subsequent chapters aim to assess how modulation of these transcription factors may affect lysosomal phenotypes associated with iPSC-DaNs from PD-patients.

Chapter 6: Lysosomal dysfunction in PD-derived iPSC-DaNs, and rescue with TFE3 or TFEB

6.1 Introduction

We next examined the relationship between autophagic-lysosomal dysfunction and TFEB/TFE3 modulation in patient-derived iPSC-DaNs. iPSC-derived neuronal models, either patient-derived or genetically manipulated to carry PD-associated mutations, have demonstrated numerous autophagic/lysosomal phenotypes such as perturbed proteolysis, autophagosome maturation and transport, hydrolase activity (frequently GCase), lipid profiles and alterations in lysosomal number/morphology (Schondorf et al., 2014; Mazzuli et al., 2016; Fernandes et al., 2016; Lang et al., 2019; Ysselstein et al., 2019; Grigor'eva et al., 2023). We opted to study lysosomal dysfunction in iPSC-DaNs from patients carrying *SNCA-Trp*, *GBA-N370S*, or *LRRK2-R1441C* mutations. *SNCA-Trp* and *GBA-N370S* iPSC-DaNs have previously demonstrated numerous autophagic-lysosomal perturbations. With the exception of perturbed GCase activity, little has been done to examine lysosomal dysfunction in iPSC-DaNs carrying *LRRK2-R1441C* mutation, despite other models implicating this pathway with the mutation (Alegre-Abarrategui et al., 2009; Nguyen and Krainc, 2018; Ysselstein et al., 2019; Wallings et al., 2019).

Here, we profiled lysosomal function for each mutation in iPSC-DaNs, using the assays developed throughout this thesis, including, endolysosomal proteolysis, lysosomal GCase activity, lysosomal pH, lysosomal Ca^{2+} release and autophagic flux. This comprehensive analysis of the autophagic-lysosomal system in iPSC-DaNs should better classify which characteristics are perturbed in each mutation.

The significant functional overlap between TFEB and TFE3 with PD pathogenic mechanisms has led studies to identify TFEB and TFE3 to both be dysfunctional in post-mortem tissue or models of PD (Section 1.3.6.3). Interestingly, recently an iPSC-

DaN model carrying *GBA-N370S* mutations displayed mTORC1 hyperactivity to perturb TFEB expression and localisation leading to CLEAR gene dysregulation (Mubariz et al., 2023). While our results are contrary to a role for endogenous TFEB in this pathogenic process, TFE3 is also modulated by mTORC1, thus may be mislocalised in these neurons leading to lysosomal dysfunction.

In this chapter, we characterise lysosomal and TFE3-related phenotypes in iPSC-DaNs from patients carrying *LRRK2-R1441C*, *SNCA-Trp* and *GBA-N370S* mutations. We then use TRPML1 agonist, ML-SA5 to pharmacologically stimulate TFE3 activity, or TFEB and TFE3 overexpression to ameliorate phenotypes observed in patient-derived iPSC-DaNs to better understand the therapeutic potential of TFE3 and TFEB activation in restoring lysosomal dysfunction in PD.

Chapter aims:

- Characterise autophagic-lysosomal phenotypes relevant to proteolysis, lysosomal GCase, lysosomal pH, autophagic flux and lysosomal Ca^{2+} release in *LRRK2-R1441C*, *GBA-N370S* and *SNCA-Trp* iPSC-DaNs
- Characterise TFE3 expression and localisation in iPSC-DaNs from patients carrying *LRRK2-R1441C*, *GBA-N370S* and *SNCA-Trp* mutations.
- Correct lysosomal perturbations found in patient-derived iPSC-DaNs through ML-SA5 treatment, TFE3 overexpression or TFEB overexpression.

6.2 TFE3, CLEAR expression and lysosomal function in *LRRK2-R1441C* iPSC-DaNs

We first assessed iPSC-DaNs carrying *LRRK2-R1441C* mutation for lysosomal and TFE3 dysfunction, comparing them to control iPSC-DaNs.

To ensure phenotypic differences are not due to disparities in differentiation efficiency, and therefore the abundance of non-relevant cell types, we assessed the percentage of cells in culture positive for both MAP2 and TH. The differentiation efficiency of iPSC lines to DaNs was ~70-75% and no significant differences were seen in differentiation efficiency, although 870 appears slightly less efficient than others (Figure 6.1 A). This demonstrates *LRRK2-R1441C* iPSC-DaNs differentiates efficiently and similarly to control iPSC-DaNs.

We next assessed TFE3 activity in *LRRK2-R1441C* iPSC-DaNs in three ways: nuclear translocation, TFE3 expression, and CLEAR gene regulation. Basal TFE3 translocation was approximately 50%, and no significant differences between control and *LRRK2-R1441C* were observed (Figure 6.1 B). Further, no significant changes in TFE3 or CLEAR gene expression were seen between control and *LRRK2-R1441C* and iPSC-DaNs (Figure 6.1 C). Interestingly, *MCOLN1* demonstrated trending increases in expression in *LRRK2-R1441C* iPSC-DaNs, albeit this was not significant change. TFE3 and lysosomal protein expression between Control (lanes 1-3) and *LRRK2-R1441C* (lanes 13-15) iPSC-DaNs was carried out by western blot (Figure 6.1 D i). No significant differences in TFE3 or lysosomal protein expression were observed (Figure 6.1 D ii). These data indicate TFE3 function and downstream lysosomal protein expression are unperturbed in *LRRK2-R1441C* iPSC-DaNs.

We also examined autophagic flux in control and *LRRK2-R1441C* iPSC-DaNs through LC3-II and p62 turnover (Figure 6.1 E i). Bafilomycin treatment significantly increased LC3-II in control iPSC-DaNs and prompted near significant increases in *LRRK2-R1441C* iPSC-DaNs ($p = 0.0608$) (Figure 6.1 E ii). Bafilomycin also significantly increased p62 levels in both control and *LRRK2-R1441C* iPSC-DaNs (Figure 6.1 E iii). No significant differences in p62 or LC3-II flux between control and *LRRK2-R1441C* iPSC-DaNs were found (Figures 6.1 E iv-v).

We next assessed lysosomal function in *LRRK2-R1441C* iPSC-DaNs using the lysosomal functional toolbox developed throughout this thesis. We found no

differences lysosome number in *LRRK2-R1441C* measured by LAMP1 immunofluorescence (Figure 6.1 F). There was a trending decrease in MDW-941 spot number ($p = 0.086$), although the magnitude of decrease is minimal (Figure 6.1 G i). No changes were seen in MDW-941 spot intensity (Figure 6.1 G ii). Both lysosomal pH and DQ-BSA were unchanged in *LRRK2-R1441C* iPSC-DaNs compared to control (Figures 6.1 H-I).

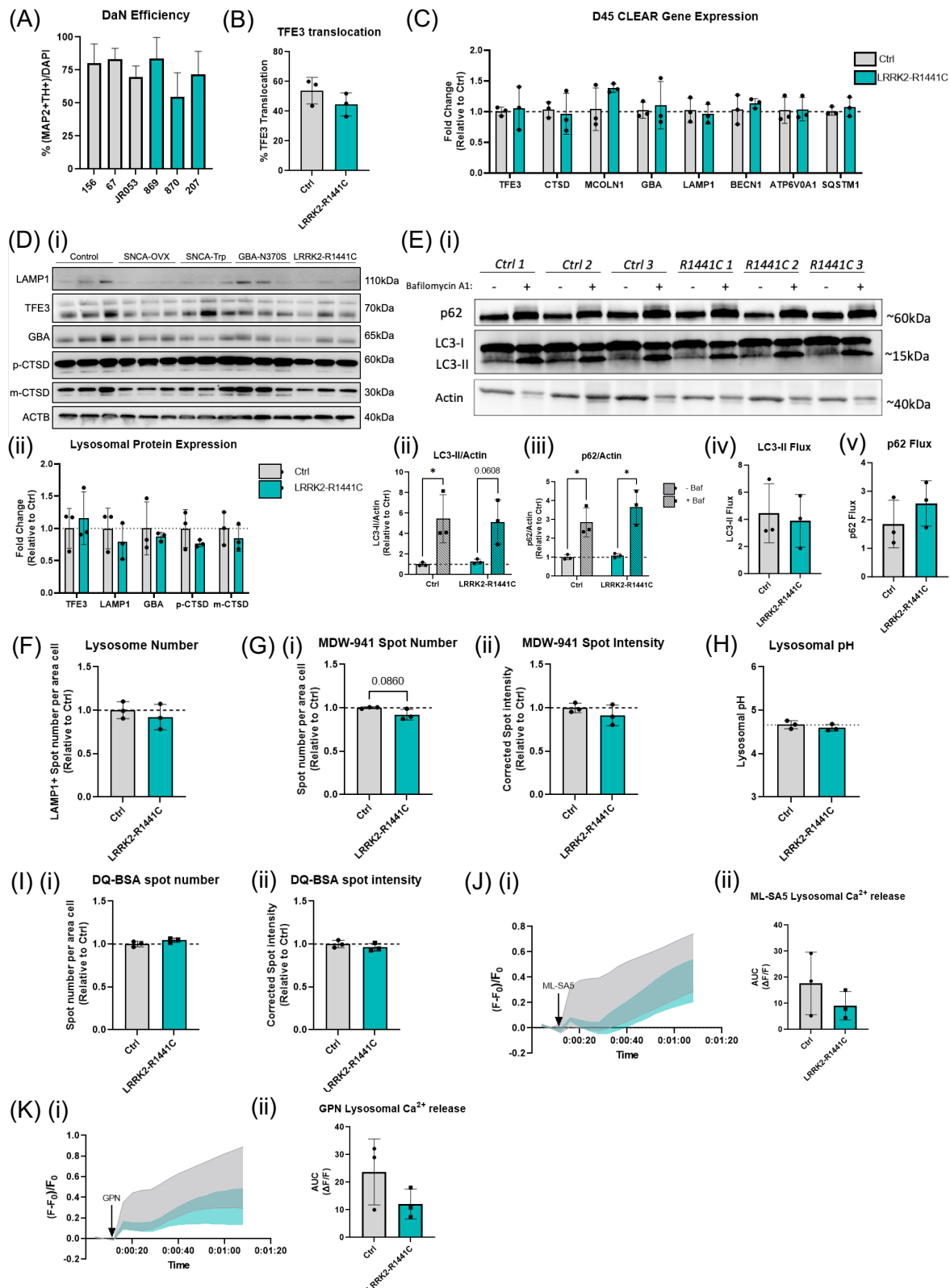


Figure 6.1 – TFE3 and lysosomal function/expression in *LRRK2-R1441C* iPSC-DaNs. (A) Dopaminergic differentiation efficiency in control lines (grey – 156, 067, JR) and *LRRK2-R1441C* lines (turquoise – 869, 870, 207), measured as % of TH and MAP2-positive cells. One-way ANOVA, with Tukey's multiple comparisons. N= 4 differentiations. (B) % cells displaying TFE3 nuclear translocation, seen by immunocytochemistry. (C) qRT-PCR mRNA

(Figure 6.1 continued...) expression of *TFE3* and *CLEAR* genes. (D) (i) Representative blot of lysosome-related protein expression in control (lanes 1-3) and *LRRK2-R1441C* (lanes 13-15) iPSC-DaNs. (ii) Quantification of lysosomal protein expression from western blotting. (E) (i) Representative blot and quantification of (ii) LC3-II and (iii) p62 protein levels $\pm$ Bafilomycin A treatment, (iv) LC3-II flux and (v) p62 flux. (F) Quantification of the number of LAMP1-positive puncta per area cell. (G) Quantification of MDW-941 (i) Spot number per area cell, and (ii) Corrected Spot intensity. (H) Quantification of lysosomal pH. (I) Quantification of DQ-BSA (i) spot number per area cell and (ii) corrected spot intensity. (J) Trace of LAMP1-GCaMP7 fluorescence in control (grey) and *LRRK2-R1441C* (turquoise) iPSC-DaNs over-time following addition of ML-SA5 (ii) Quantification of lysosomal Ca^{2+} release, measured by area under the curve from previous trace. (K) (i) Trace of changes in LAMP1-GCaMP7 fluorescence in control (grey) and *LRRK2-R1441C* (turquoise) iPSC-DaNs measured over-time following addition of GPN. (ii) Quantification of lysosomal Ca^{2+} release measured by area under the curve of previous trace. N= 3 control iPSC-DaNs, 3 *LRRK2-R1441C* iPSC-DaNs, 3-4 differentiations, except lysosomal Ca^{2+} release (N=2 differentiations). T-test carried out except for LC3-II and p62 $\pm$ Baf blots where Two-way ANOVA, with Šidák's multiple comparisons was carried out. * $p < 0.05$. Significance stated above bars if $p < 0.1$.

Lysosomal Ca^{2+} release was measured in patient-derived iPSC-DaNs by transducing cells with LAMP1-GCaMP7, used earlier (Figure 5.1 B). We stimulated lysosomal Ca^{2+} release through 2 different pharmacological means. We first used ML-SA5 to stimulate Ca^{2+} release by TRPML1. As seen before, treatment induced an initial peak in fluorescence, before a more pronounced secondary increase in fluorescence (Figure 6.1 J i). Quantification of Ca^{2+} release by calculation of the area under the curve, indicated no change in lysosomal Ca^{2+} release between control and *LRRK2-R1441C* iPSC-DaNs (Figure 6.1 J ii). We also used lysomotropic cathepsin substrate, glycyl-L-phenylalanine-2-naphthylamide (GPN), to evoke Ca^{2+} release from the lysosome (Yuan et al., 2021). Following GPN administration, a more robust initial increase in fluorescence was observed compared to ML-SA5 prior to a secondary increase (Figure 6.1 K i). Quantification of Ca^{2+} release shows no significant difference in GPN-induced Ca^{2+} release between *LRRK2-R1441C* and control iPSC-DaNs, albeit due to one control presenting much lower than the others (Figure 6.1 K ii). Overall, these data show no aberrant *TFE3* regulation and function, nor perturbed autophagic-lysosomal function in *LRRK2-R1441C* iPSC-DaNs.

6.3 TFE3, CLEAR expression and lysosomal function in *GBA-N370S* iPSC-DaNs

Next, *GBA-N370S* iPSC-DaNs were assessed. No differences in differentiation capacity were observed between *GBA-N370S* and control iPSC-DaNs, however two *GBA-N370S* lines (88-1 and 71-3) differentiated significantly worse than the other *GBA-N370S* iPSC-DaN line (82-26) (Figure 6.2 A). Thus, no obvious changes in differentiation capacity occur when comparing controls and *GBA-N370S*, however variability within *GBA-N370S* samples does occur.

No difference in TFE3 translocation was seen between *GBA-N370S* and control iPSC-DaNs (Figure 6.2 B). Similarly, there was no significant alteration in *TFE3* mRNA expression (Figure 6.2 C). A near-significant decrease in *LAMP1* expression was observed in *GBA-N370S* iPSC-DaNs, but no other changes in *CLEAR* gene expression (Figure 6.2 C). Further no changes in TFE3 and lysosomal protein expression was observed between *GBA-N370S* (Figure 6.2 D i – Lanes 1-3) and control (Figure 6.2 D i – Lanes 7-9) iPSC-DaNs (Figure 6.2 D)

Autophagic flux by LC3-II and p62 were assessed using western blot (Figure 6.2 E i - control: lanes 1-6; *GBA-N370S*: lanes 7-12). Bafilomycin increased LC3-II levels, significantly for *GBA-N370S* and near significantly for control iPSC-DaNs (Figure 6.2 E ii). P62 was also significantly increased in both genotypes by bafilomycin (Figure 6.2 iii). p62 and LC3-II flux were unchanged between *GBA-N370S* and control iPSC-DaNs (Figures 6.2 E iv-v). Collectively, no perturbation in TFE3 activity, lysosomal protein expression or autophagic flux was found in *GBA-N370S* iPSC-DaNs.

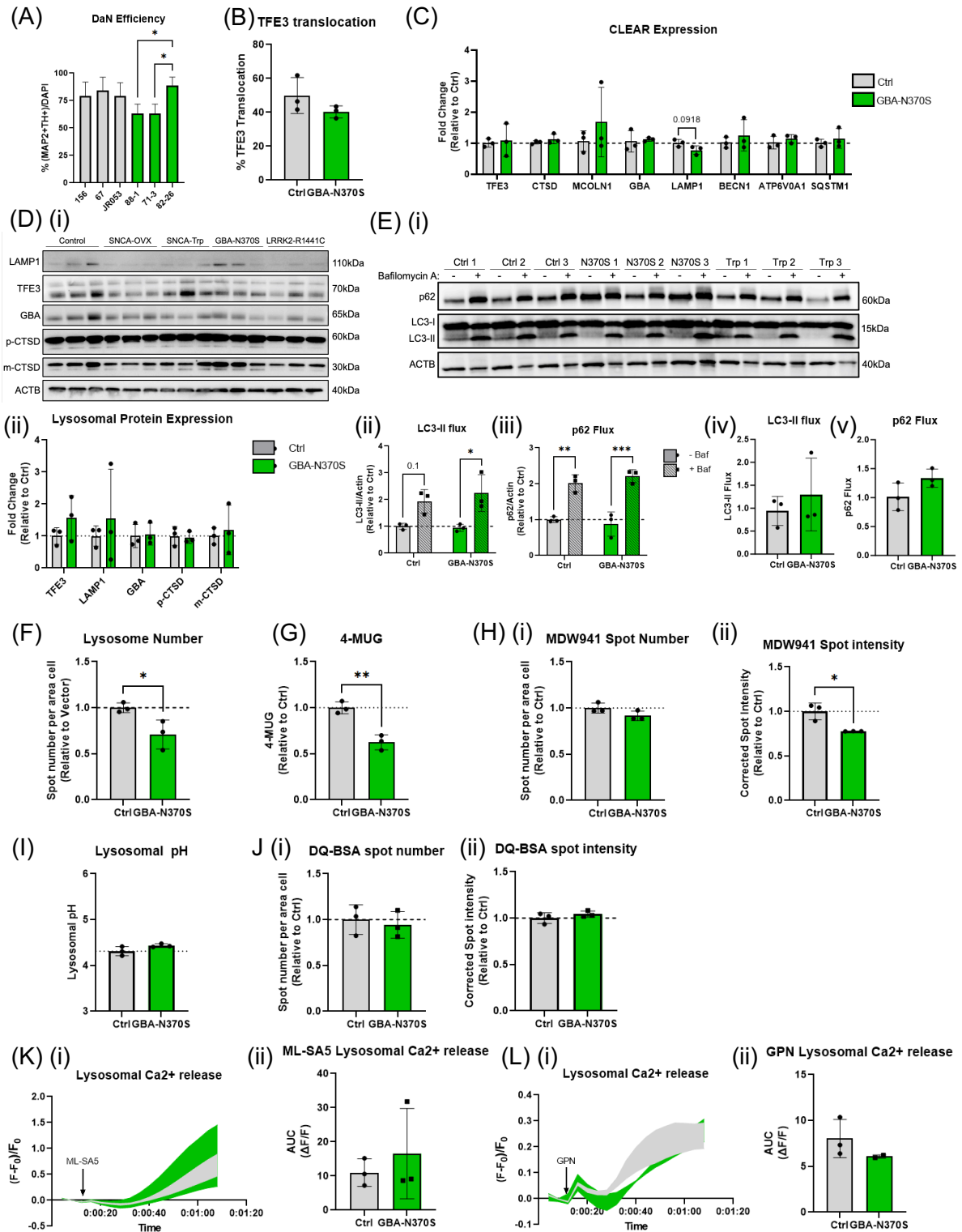


Figure 6.2 – TFE3 and lysosomal function/expression in *GBA-N370S* iPSC-DaNs.

(A) Dopaminergic differentiation efficiency in control lines (grey – 156, 067, JR) and *GBA-N370S* lines (green – 88-1, 71-3, 82-6), measured as % of TH and MAP2-positive cells. One-way ANOVA, with Tukey's multiple comparisons. N= 4 differentiations. (B) % cells displaying TFE3 nuclear translocation, seen by immunocytochemistry. (C) qRT-PCR mRNA expression of *TFE3* and CLEAR genes. (D) (i) Representative blot of lysosome-related protein expression in control (lanes 1-3) and *GBA-N370S* (lanes 10-12) iPSC-DaNs. (ii) Quantification of lysosomal protein expression from western blotting. (E) (i) Representative blot and quantification of (ii) LC3-II and (iii) p62 protein levels ± Bafilomycin A treatment, (iv) LC3-II flux

(Figure 6.2 continued...) and (v) p62 flux. (F) Quantification of the number of LAMP1-positive puncta per area cell. (G) Quantification of 4-MUG lysosomal GCase assay. (H) Quantification of MDW-941 (i) Spot number per area cell, and (ii) Corrected Spot intensity. (I) Quantification of lysosomal pH. (J) Quantification of DQ-BSA (i) spot number per area cell and (ii) corrected spot intensity. (K) Trace of changes in LAMP1-GCaMP7 fluorescence in control (grey) and *GBA-N370S* (green) iPSC-DaNs over-time following addition of ML-SA5. (ii) Quantification of lysosomal Ca^{2+} release, measured by area under the curve from previous trace. (L) (i) Trace of changes in LAMP1-GCaMP7 fluorescence in control (grey) and *GBA-N370S* (green) iPSC-DaNs measured over-time following addition of GPN. (ii) Quantification of lysosomal Ca^{2+} release measured by area under the curve of previous trace. N= 3 control iPSC-DaNs, 3 *GBA-N370S* iPSC-DaNs, 3-4 differentiations, except lysosomal Ca^{2+} release (N=1 differentiation). T-test carried out except for LC3-II and p62 $\pm$ Baf blots where Two-way ANOVA, with Šídák's multiple comparisons was carried out. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significance stated above bars if $p < 0.1$.

Interestingly, *GBA-N370S* iPSC-DaNs displayed significantly reduced LAMP1-positive puncta (Figure 6.2 F). As expected, lysosomal GCase was dysregulated in *GBA-N370S* iPSC-DaNs. 4-MUG activity was reduced by ~40% in *GBA-N370S* iPSC-DaNs and MDW-941 spot intensity was also significantly abrogated, although no change in spot number was seen (Figures 6.2 G-H).

No changes to lysosomal pH, nor to DQ-BSA could be observed between control and *GBA-N370S* iPSC-DaNs (Figures 6.2 I-J). We were only able to attempt lysosomal Ca^{2+} assays once with *GBA-N370S* iPSC-DaNs. Preliminary results indicated no significant changes in TRPML1-mediated or GPN-mediated Ca^{2+} release between control and *GBA-N370S* iPSC-DaNs (Figure 6.2 K-L).

Overall, these data display a perturbation in the endolysosomal number and GCase activity within *GBA-N370S* iPSC-DaNs compared to controls.

6.4 TFE3, CLEAR expression and lysosomal function in *SNCA-Trp* iPSC-DaNs

We applied the same assays to iPSC-DaNs carrying the *SNCA-Trp* mutation. Due to the rarity of this mutation, iPSC lines are 3 clones derived from the same patient. Control, *GBA-N370S*, and *LRRK2-R1441C* iPSC lines were from different individuals (Appendix Table S.3).

SNCA-Trp iPSCs differentiated into DaNs comparably to controls (Figure 6.3 A). TFE3 translocation was unchanged between *SNCA-Trp* and control iPSC-DaNs (Figure 6.3 B). TFE3 mRNA expression was also unchanged in *SNCA-Trp*, but a near significant increase in TFE3 protein expression was revealed by western blot ($p=0.0508$). CLEAR gene mRNA expression largely remained unchanged, except for a trending decrease in *GBA* expression (Figure 6.3 C). This reduction in *GBA* mRNA expression did not translate to protein expression (Figure 6.3 D). *SNCA-Trp* iPSC-DaNs displayed significantly reduced LAMP1 expression, and significantly increased p-CTSD, although m-CTSD remained unaffected (Figure 6.3 D). These results indicate a perturbation in endolysosomal compartment and impaired CTSD maturation, that may be off-set by a compensatory increase in total CTSD protein expression. Autophagic flux was assessed using LC3-II and p62 (Figure 6.3 E i – Control: lanes 1-3; *SNCA-Trp*: lanes 13-18). Bafilomycin significantly increased p62 and LC3-II in control and *SNCA-Trp* iPSC-DaNs (Figure 6.3 E ii-iii). LC3-II and p62 flux displayed no significant changes between the two genotypes (Figure 6.E iv-v).

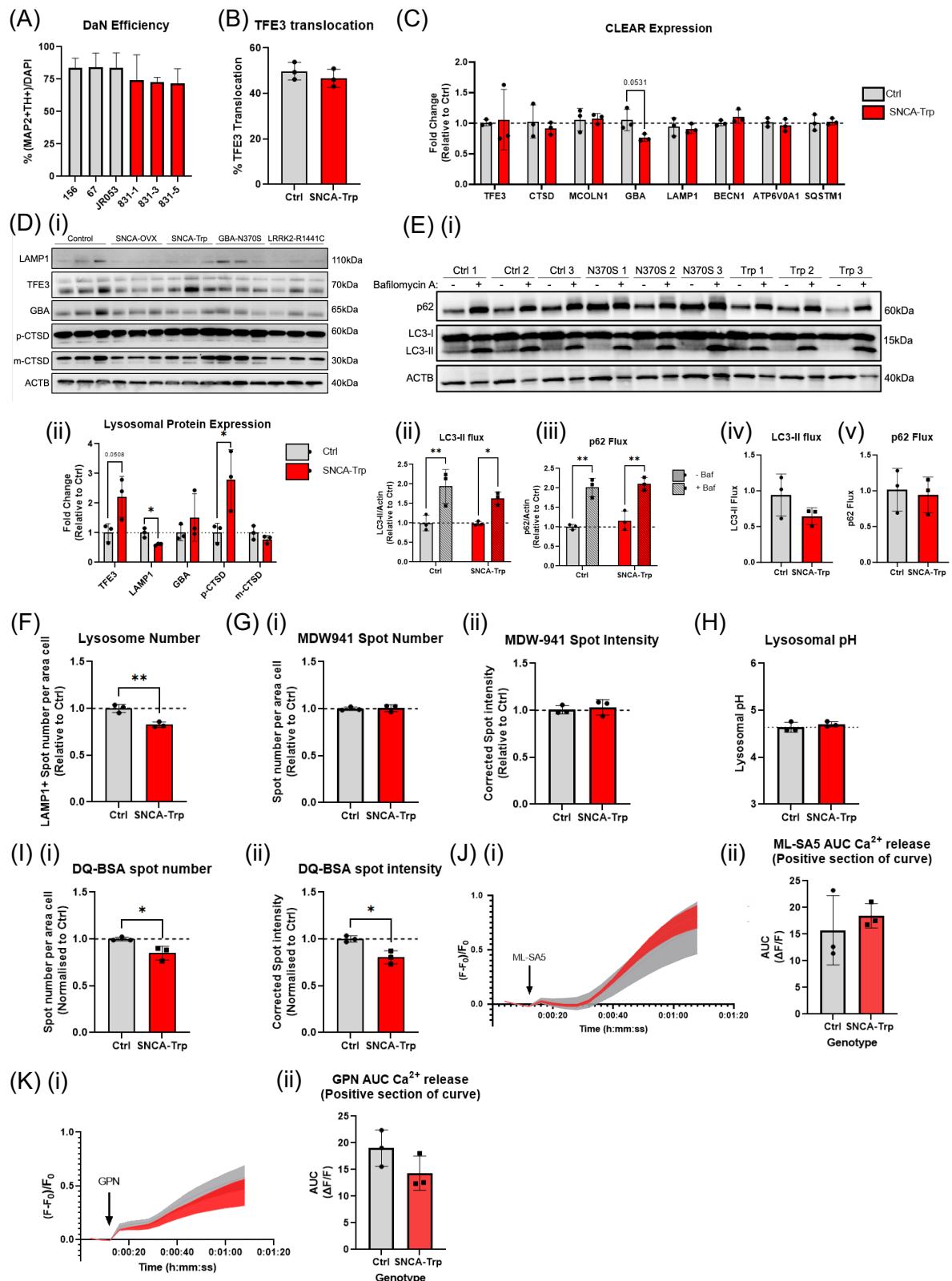


Figure 6.3 – TFE3 and lysosomal function/expression in *SNCA-Trp* iPSC-DaNs.

(A) Dopaminergic differentiation efficiency in control lines (grey – 156, 067, JR) and *SNCA-Trp* lines (red – 831-1, 831-3, 831-5), measured as % of TH and MAP2-positive cells. One-way ANOVA, with Tukey's multiple comparisons. N= 4 differentiations. (B) % cells displaying TFE3 nuclear translocation, seen by immunocytochemistry. (C) qRT-PCR mRNA expression

(Figure 6.3 continued...) of *TFE3* and *CLEAR* genes. (D) (i) Representative blot of lysosome-related protein expression in control (lanes 1-3) and *SNCA-Trp* (lanes 7-9) iPSC-DaNs. (ii) Quantification of lysosomal protein expression from western blotting. (E) (i) Representative blot and quantification of (ii) LC3-II and (iii) p62 protein levels $\pm$ Bafilomycin A treatment, (iv) LC3-II flux and (v) p62 flux. (F) Quantification of the number of LAMP1-positive puncta per area cell. (G) Quantification of MDW-941 (i) Spot number per area cell, and (ii) Corrected Spot intensity. (H) Quantification of lysosomal pH. (I) Quantification of DQ-BSA (i) spot number per area cell and (ii) corrected spot intensity. (J) Trace of changes in LAMP1-GCaMP7 fluorescence in control (grey) and *SNCA-Trp* (red) iPSC-DaNs over-time following addition of ML-SA5. (ii) Quantification of lysosomal Ca^{2+} release, measured by area under the curve from previous trace. (K) (i) Trace of changes in LAMP1-GCaMP7 fluorescence in control (grey) and *SNCA-Trp* (red) iPSC-DaNs measured over-time following addition of GPN. (ii) Quantification of lysosomal Ca^{2+} release measured by area under the curve of previous trace. N= 3 control iPSC-DaNs, 3 *SNCA-Trp* iPSC-DaNs, 3-4 differentiations, except lysosomal Ca^{2+} release (N=2 differentiations). T-test carried out except for LC3-II and p62 $\pm$ Baf blots where Two-way ANOVA, with Šidák's multiple comparisons was carried out. * $p < 0.05$; ** $p < 0.01$. Significance stated above bars if $p < 0.1$.

SNCA-Trp iPSC-DaNs had significantly reduced number of LAMP1-positive puncta, corroborating reduced LAMP1 expression (Figure 6.3 F). No changes in lysosomal GCaMP measured by MDW-941 were observed (Figure 6.3 G). Further, lysosomal pH was unaltered in *SNCA-Trp* iPSC-DaNs (Figure 6.3 H). Intriguingly, DQ-BSA found a significant reduction in the number and intensity of puncta in *SNCA-Trp* iPSC-DaNs, suggesting abrogated endolysosomal proteolysis (Figure 6.3 I). ML-SA5 and GPN both induced Ca^{2+} release in *SNCA-Trp* iPSC-DaNs to a similar magnitude seen in control iPSC-DaNs (Figures 6.3 J-K). Overall, data display an endolysosomal dysfunction in *SNCA-Trp* iPSC-DaNs through reduced endolysosomal number and proteolysis, possibly resultant from perturbed CTSD maturation.

6.5 Lysosomal function following *SNCA* overexpression in control iPSC-DaNs

Due to *SNCA-Trp* iPSC lines being clones from the same individual, we attempted to confirm the lysosomal dysfunction observed was due to increased *SNCA* expression and not resulting from one individual's variability compared to the control lines. To do this, we used an in-house mTagBFP2-T2A-*SNCA* overexpression (*SNCA-OVX*) vector (courtesy of Brent Ryan and Rachel Heon-Roberts). Control iPSC-DaNs were transduced with the *SNCA-OVX* vector at D20 and matured to D45. α Syn overexpression was validated in control iPSC-DaNs and displayed a 3-fold increase in expression (Figure 6.3 A i). We then assessed lysosomal protein expression, DQ-BSA, and LAMP1+ puncta to examine causality of α Syn overexpression upon lysosomal dysfunction.

Western blotting of non-transduced Control lines (Lanes 1-3) and *SNCA* overexpressing control lines (Lanes 4-6), reveal no significant change in lysosomal protein expression, although trending reductions in LAMP1 and CTSD are evident (Figure 6.3 A ii-iii). Noteworthy, this experiment was only carried out once, and control lines were not transduced with a control vector, hence future experimentation with appropriate controls will better address this.

Next, DQ-BSA was assessed in control lines transduced with a control mTagBFP2 vector, or the *SNCA-OVX* vector (Figure 6.4 B i). We observed significant reductions in DQ-BSA spot number and near-significant reductions in spot intensity ($p=0.0583$), recapitulating findings from *SNCA-Trp* iPSC-DaNs.

We also observed a significant reduction in LAMP1-positive puncta following overexpression of *SNCA*, similar to *SNCA-Trp* iPSC-DaNs (Figure 6.4 C ii).

These data demonstrate a similar endolysosomal perturbation following *SNCA* overexpression in control iPSC-DaNs to that seen in patient-derived *SNCA-Trp* iPSC-DaNs.

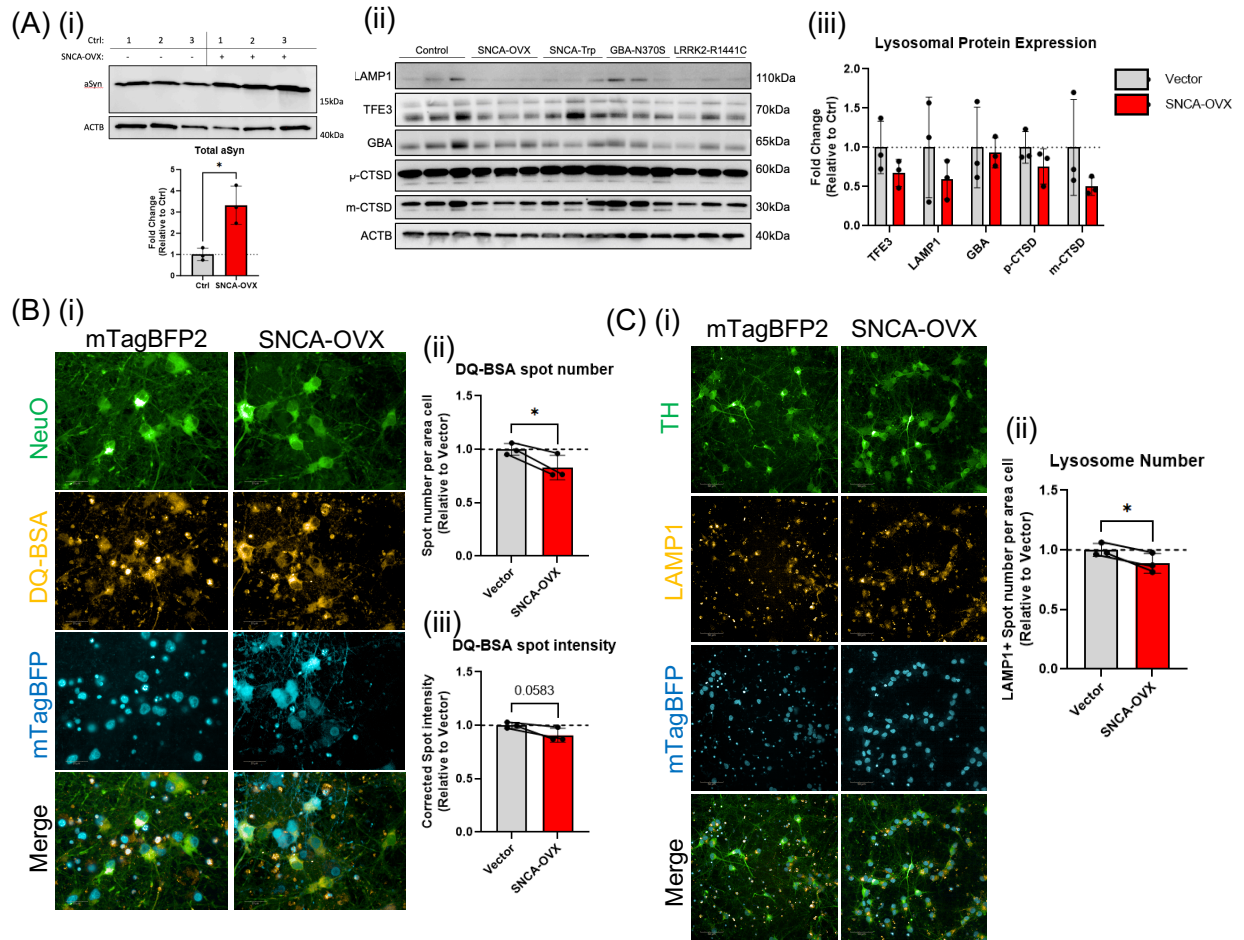


Figure 6.4 – Lysosomal protein expression and function in control iPSC-DaNs overexpressing SNCA.

(A) (i) Representative blot of α Syn overexpression in iPSC-DaNs transduced with mTagBFP2-T2A-SNCA lentivirus. (ii) Representative blot of lysosome-related protein expression in control (lanes 1-3) and SNCA overexpressing control iPSC-DaNs (lanes 4-7) iPSC-DaNs. (ii) Quantification of lysosomal protein expression from western blotting. N = 3 control iPSC-DaNs, 1 differentiation (B) (i) Representative images of DQ-BSA (orange) in live control iPSC-DaNs (stained with NeuO – green) transduced with control vector expressing nuclear mTagBFP2 (Left – blue) or SNCA overexpression lentivirus (Right – blue). (ii) Quantification of DQ-BSA (i) spot number per area cell and (ii) corrected spot intensity. N = 3 control iPSC-DaNs, 2 differentiations. (C) (i) Representative images and (ii) Quantification of the number of LAMP1-positive puncta per area cell in control iPSC-DaNs transduced with control nuclear mTagBFP2 vector or SNCA overexpression lentivirus. N = 3 control iPSC-DaNs, 3 differentiations. T-test carried out. * $p < 0.05$. Significance stated above bars if $p < 0.1$.

6.6 Correction of GCase and lysosomal deficits in *GBA-N370S* using ML-SA5, TFE3 overexpression and TFEB overexpression

The final sections of this chapter aim to correct lysosomal phenotypes observed in patient-derived iPSC-DaNs by ML-SA5 treatment, TFE3 overexpression, and TFEB overexpression.

We first attempted to correct the reductions in LAMP1-positive puncta and lysosomal GCase seen in *GBA-N370S* iPSC-DaNs (Figures 6.2 F-H).

Control and *GBA-N370S* iPSC-DaNs were treated with TRPML1 agonist, ML-SA5 for 48 hours as previously carried out. Contrary to findings in Chapter 5, ML-SA5 treatment significantly decreased MDW-941 spot number in control iPSC-DaNs, and reduced spot number in 2 of 3 *GBA-N370S* iPSC-DaNs (Figure 6.5 A). Thus, ML-SA5 treatment exacerbated a significant reduction in MDW-941 spot number between Control + DMSO and *GBA-N370S* + ML-SA5 (Figure 6.5 A). Conversely, MDW-941 spot intensity significantly increased in control iPSC-DaNs following ML-SA5 treatment, similar to previous findings (Figure 6.5 B). All 3 *GBA-N370S* iPSC-DaNs displayed increased spot intensity following ML-SA5 treatment, such that MDW-941 spot intensity was no longer significantly reduced compared to control iPSC-DaNs + DMSO (Figure 6.5 B). We also assessed GCase activity using 4-MUG. We did not replicate the significant perturbation in GCase activity here (Figure 6.5 C). ML-SA5 increased 4-MUG in all 3 controls although not reaching significance, however GCase activity did not change in *GBA-N370S* iPSC-DaNs (Figure 6.5 C).

No difference in LAMP1-positive puncta number between control and *GBA-N370S* could be observed, nor could an affect by ML-SA5 treatment upon this parameter in either genotype (Figure 6.5 D). Overall, data shows ML-SA5 treatment to increase in MDW-941 spot intensity indicative of increased lysosomal GCase abundance, however treatment also decreased MDW-941 spot number and did not alter GCase activity or endolysosomal number in *GBA-N370S* iPSC-DaNs.

ML-SA5 treatment

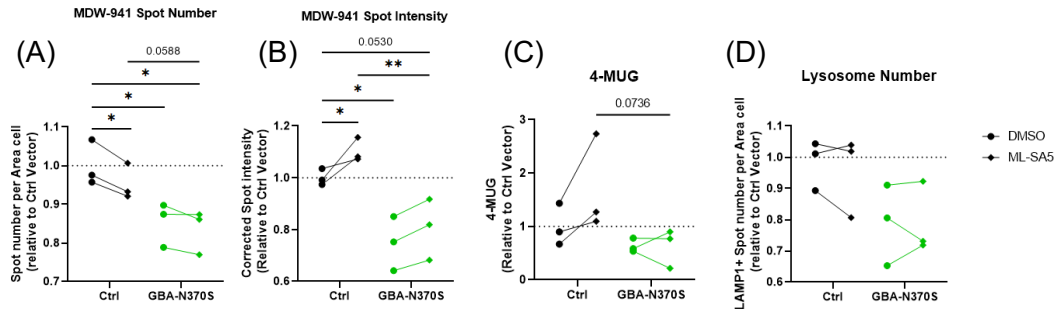


Figure 6.5 – Correction of lysosomal deficits in *GBA-N370S* iPSC-DaNs using ML-SA5. Quantification of effects of ML-SA5 (diamond) treatment compared to DMSO (circle) treatment in control (black) and *GBA-N370S* (green) iPSC-DaNs upon (A) MDW-941 spot number, (B) MDW-941 spot intensity, (C) 4-MUG and (D) LAMP1-positive puncta number. N = 3 control iPSC-DaN, 3 *GBA-N370S* iPSC-DaN, 3-4 differentiations. 2-way ANOVA, with Šídak's multiple comparisons was carried out. * $p < 0.05$; ** $p < 0.01$, p-value stated above the comparison if $p < 0.1$.

Next, we attempted to correct these phenotypes using genetic overexpression of TFE3. Here, we observed significant deficits in MDW-941 spot intensity, and near significant reductions in MDW-941 spot number between control and *GBA-N370S* iPSC-DaNs at baseline (Figure 6.6 A-B). There were also trending reductions in 4-MUG and number of LAMP1-positive puncta between control and *GBA-N370S* iPSC-DaNs at baseline (Figure 6.6 C-D). TFE3 overexpression increased MDW-941 spot number in all 3 control lines, such that significance was almost met ($p = 0.0869$) (Figure 6.6 A). 2 of 3 *GBA-N370S* iPSC-DaNs increased MDW-941 spot number to basal control levels, however the remaining *GBA-N370S* iPSC-DaN line displayed reduced MDW-941 spot number following TFE3 overexpression (Figure 6.6 A). Similarly, TFE3 overexpression significantly increased MDW-941 spot intensity in all 3 controls, and upregulated spot intensity in 2 of 3 *GBA-N370S* iPSC-DaNs (Figure 6.6 B). As such, a significant deficit in MDW-941 spot intensity *GBA-N370S* remained following TFE3 overexpression compared to control levels (Figure 6.6 B).

4-MUG revealed similar findings in that a significant increase in GCase activity was seen in control iPSC-DaNs, and 2 of 3 *GBA-N370S* increased, but 4-MUG remained unchanged in the remaining *GBA-N370S* iPSC-DaN line (Figure 6.6 C). TFE3 overexpression significantly upregulated the number of LAMP1-positive puncta in both control and *GBA-N370S* iPSC-DaNs (Figure 6.6 D). Noteworthy, the *GBA-N370S* iPSC-DaN line that did not respond to TFE3 overexpression in MDW-941 and 4-MUG was the same each time. Collectively, these data demonstrate TFE3 overexpression

to ameliorate deficits in GCase activity and endolysosomal numbers in *GBA-N370S* iPSC-DaNs, although individual variability may hinder beneficial responses conferred.

TFE3 overexpression

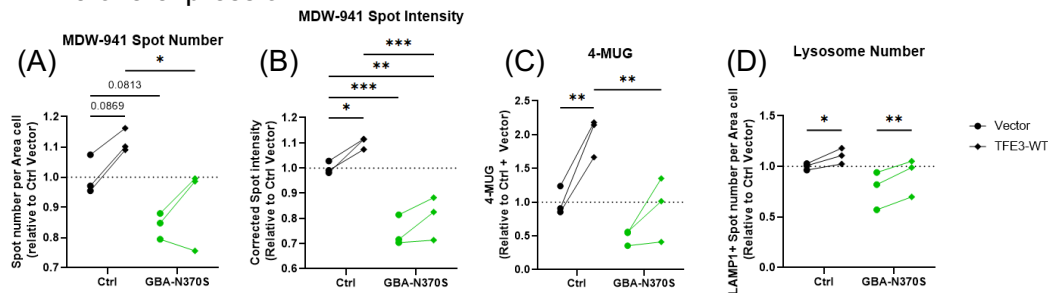


Figure 6.6 – Correction of lysosomal deficits in *GBA-N370S* iPSC-DaNs by TFE3 overexpression.

Quantification of effects of TFE3 (diamond) overexpression compared to control vector (circle) in control (black) and *GBA-N370S* (green) iPSC-DaNs upon (A) MDW-941 spot number, (B) MDW-941 spot intensity, (C) 4-MUG and (D) LAMP1-positive puncta number. N = 3 control iPSC-DaN, 3 *GBA-N370S* iPSC-DaN, 3-4 differentiations. 2-way ANOVA, with Šídak's multiple comparisons was carried out. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ p-value stated above the comparison if $p < 0.1$.

Finally, we assessed whether TFEB overexpression ameliorated deficits in *GBA-N370S* iPSC-DaNs. Again, significant reductions in MDW-941 spot intensity, and near significant deficits in spot number were observed at baseline in *GBA-N370S* iPSC-DaNs (Figure 6.7 A-B). *GBA-N370S* iPSC-DaNs also demonstrated trending decreases in 4-MUG and LAMP1-number (Figure 6.7 C-D). TFEB overexpression did not change MDW-941 spot number in either control or *GBA-N370S* iPSC-DaNs (Figure 6.7 A). TFEB overexpression reduced MDW-941 spot intensity in all control iPSC-DaNs, but less pronounced changes were seen in *GBA-N370S* iPSC-DaNs (Figure 6.7 B). Further, significant deficits in spot intensity remained relative to control baseline in *GBA-N370S* iPSC-DaNs following TFEB overexpression (Figure 6.7 B). 4-MUG GCase activity assay significantly increased in control iPSC-DaNs and near-significantly in *GBA-N370S* iPSC-DaNs ($p = 0.0885$), comparable to control baseline activity (Figure 6.7 C). TFEB overexpression increased LAMP1-puncta number significantly in control iPSC-DaNs, and in *GBA-N370S* iPSC-DaNs although not quite to significance ($p = 0.0693$) (Figure 6.7 D).

Overall, data suggests lysosomal GCase abundance not benefitted by TFEB overexpression, but activity is increased in both control and *GBA-N370S* iPSC-DaNs.

Data also demonstrate an ability for TFEB to upregulate endolysosomal number in both control and *GBA-N370S* iPSC-DaNs.

TFEB overexpression

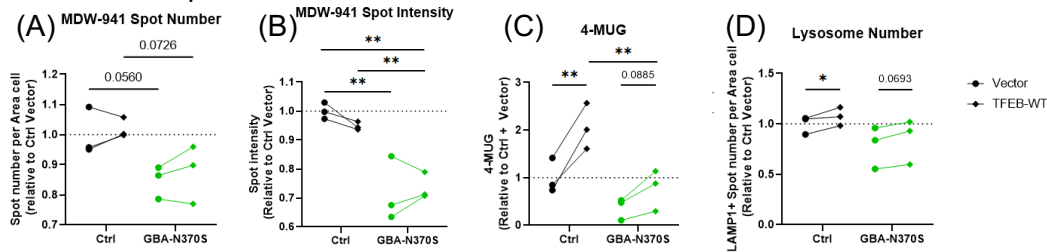


Figure 6.7 – Correction of lysosomal deficits in *GBA-N370S* iPSC-DaNs by TFEB overexpression.

Quantification of effects of TFEB (diamond) overexpression compared to control vector (circle) in control (black) and *GBA-N370S* (green) iPSC-DaNs upon (A) MDW-941 spot number, (B) MDW-941 spot intensity, (C) 4-MUG and (D) LAMP1-positive puncta number. N = 3 control iPSC-DaN, 3 *GBA-N370S* iPSC-DaN, 3-4 differentiations. 2-way ANOVA, with Šídak's multiple comparisons was carried out. * $p < 0.05$; ** $p < 0.01$, p-value stated above the comparison if $p < 0.1$.

6.7 Correction of endolysosomal deficits in *SNCA-Trp* iPSC-DaNs using ML-SA5, TFE3 overexpression and TFEB overexpression

We demonstrated that *SNCA-Trp* iPSC-DaNs displayed perturbations in endolysosomal number and proteolysis and confirmed this by *SNCA* overexpression in control iPSC-DaNs. We next attempted to correct these phenotypes in *SNCA-Trp* iPSC-DaNs using ML-SA5, TFE3 overexpression and TFEB overexpression.

Unexpectedly, all endolysosomal deficits could no longer be recapitulated (Figure 6.8-6.10). We postulated this may be from using different iPSC batches. Given we confirmed the phenotypes using α Syn overexpression in control iPSC-DaNs, we proceeded to assess whether TFEB/TFE3 activation could elevate endolysosomal function in these patient-derived iPSC-DaNs.

ML-SA5 treatment did not upregulate LAMP1-positive puncta number, DQ-BSA spot number or DQ-BSA spot intensity in either genotype (Figure 6.8 A-C). These data suggest TRPML1 agonists do not benefit endolysosomal function previously shown to be deficient in *SNCA-Trp* iPSC-DaNs.

ML-SA5 treatment

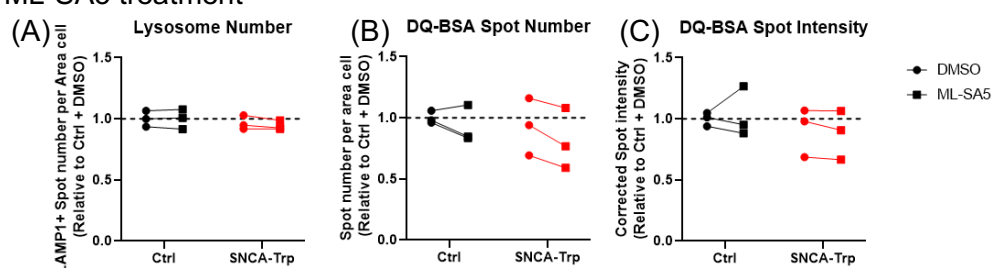


Figure 6.8 – Correction of lysosomal deficits in *SNCA-Trp* iPSC-DaNs using ML-SA5.

(A) Quantification of effects of ML-SA5 (diamond) treatment compared to DMSO (circle) treatment in control (black) and *SNCA-Trp* (red) iPSC-DaNs upon (i) LAMP1-positive puncta number, (ii) DQ-BSA spot number, and (iii) DQ-BSA spot intensity. N = 3 control iPSC-DaN, 3 *SNCA-Trp* iPSC-DaN, 3-4 differentiations. 2-way ANOVA, with Šídak's multiple comparisons was carried out.

TFE3 overexpression significantly upregulated LAMP1-positive puncta number in both control and *SNCA-Trp* iPSC-DaNs (Figure 6.9 A). TFE3 overexpression also increased DQ-BSA spot number in all lines examined, although we were unable to observe significance (Figure 6.9 B). Following TFE3 overexpression, DQ-BSA spot intensity was unchanged in control iPSC-DaNs (as expected from Chapter 5), but *SNCA-Trp*

iPSC-DaNs displayed a trending increase in DQ-BSA spot intensity (Figure 6.9 C). These data display TFE3 overexpression is able to enhance endolysosomal function in patient-derived iPSC-DaNs harbouring *SNCA-Trp* mutations.

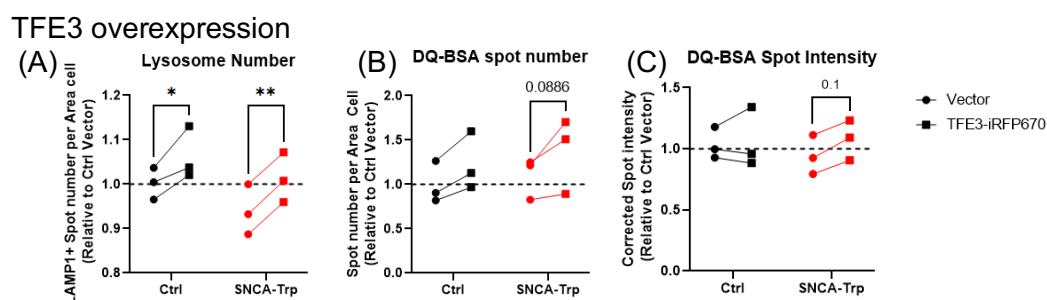


Figure 6.9 – Correction of lysosomal deficits in *SNCA-Trp* iPSC-DaNs by TFE3 overexpression.

Quantification of effects of TFE3 overexpression (diamond) compared to control vector (circle) in control (black) and *SNCA-Trp* (red) iPSC-DaNs upon (A) LAMP1-positive puncta number, (B) DQ-BSA spot number, and (C) DQ-BSA spot intensity. N = 3 control iPSC-DaNs, 3 *SNCA-Trp* iPSC-DaNs, 3-4 differentiations. 2-way ANOVA, with Šídak's multiple comparisons was carried out. * $p < 0.05$; ** $p < 0.01$, p-value stated above the comparison if $p < 0.1$.

Finally, we attempted to enhance endolysosomal function in *SNCA-Trp* iPSC-DaNs using TFEB overexpression. TFEB overexpression significantly increased the number of LAMP1-positive puncta in *SNCA-Trp* iPSC-DaNs and increased this close to significance in control iPSC-DaNs (Figure 6.10 A). Similar increases in DQ-BSA spot number in control and *SNCA-Trp* iPSC-DaNs were observed following TFEB overexpression (Figure 6.10 B). TFEB overexpression significantly upregulated DQ-BSA spot intensity specifically in *SNCA-Trp* iPSC-DaNs, whereas no changes were seen in control iPSC-DaNs (Figure 6.10 C). These data show TFEB overexpression to have beneficial effects upon endolysosomal number and function in *SNCA-Trp* iPSC-DaNs.

TFEB overexpression

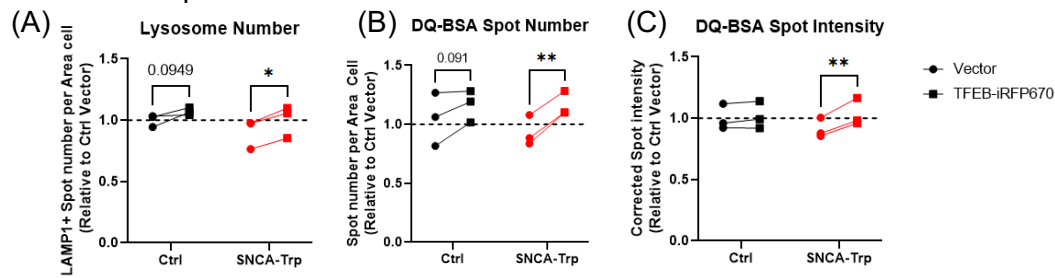


Figure 6.10 – Correction of lysosomal deficits in *SNCA-Trp* iPSC-DaNs by TFEB overexpression.

Quantification of effects of TFEB overexpression (diamond) compared to control vector (circle) in control (black) and *SNCA-Trp* (red) iPSC-DaNs upon (A) LAMP1-positive puncta number, (B) DQ-BSA spot number, and (C) DQ-BSA spot intensity. N = 3 control iPSC-DaNs, 3 *SNCA-Trp* iPSC-DaNs, 3-4 differentiations. 2-way ANOVA, with Šídák's multiple comparisons was carried out. **p<0.01, p-value stated above the comparison if p<0.1.

6.8 Discussion

This chapter comprehensively characterised TFE3 and lysosomal function using a toolbox of assays developed for autophagic flux, lysosomal number, Ca^{2+} release, pH, GCase activity and proteolysis in iPSC-DaNs from patients carrying *GBA-N370S*, *LRRK2-R1441C* and *SNCA-Trp* mutations.

6.8.1 TFE3 localisation and expression in patient-derived iPSC-DaNs

Interestingly, TFE3 localisation and expression was unaffected in all genotypes. Recently, *LRRK2-G2019S* iPSC-microglia were demonstrated to increase TFE3 expression, and kinase inhibition or LRRK2-KO induced nuclear localisation, but this did not translate to cortical neurons (Yadavalli and Ferguson, 2023). Thus, although we studied a mutation in the GTPase domain of LRRK2, TFE3 perturbation may be more susceptible to *LRRK2* mutations in non-neuronal cell-types, hence our findings. Surprisingly, TFE3 localisation and expression was not altered in *GBA-N370S* iPSC-DaNs, despite us and others demonstrating mTOR hyperactivity previously (Bogetoft et al., 2023; Mubariz et al., 2023). Mubariz and colleagues demonstrated TFEB mislocalisation as a result (2023). We could not replicate this finding with TFE3 and argue TFEB is unlikely to be expressed in these neurons, but the perturbation previously demonstrated should be noted. Recently, mTOR activity has been shown to differentially regulate substrates (such as TFEB/TFE3 or S6K/4EBP1) controlled by the activation of distinct Rag GTPase dimers through FLCN or Rheb activity (Napolitano et al., 2020; Gollwitzer et al., 2022). Thus, mTORC1 hyperactivity in *GBA-N370S* iPSC-DaNs may selectively phosphorylate S6K/4EBP1 and not TFE3 through aberrant activation of different Rag dimers, explaining this disparity between mTOR hyperactivity and TFE3 function in *GBA-N370S* iPSC-DaNs.

α Syn has been demonstrated to aberrantly bind TFEB and mislocalise the transcription factor by emulating 14-3-3 proteins (Decressac et al., 2013). TFE3 is also modulated by 14-3-3 proteins, thus we expected TFE3 mislocalisation in *SNCA-Trp* iPSC-DaNs but did not observe this (Martina et al., 2014). We did find an increase in TFE3 protein expression, independent of mRNA expression. α Syn overexpression has been shown to perturb the proteasome (Zondler et al., 2017), thus inhibition of TFE3 degradation may cause this phenotype. We were unable to replicate this by *SNCA* overexpression, however.

6.8.2 Lysosomal function in *LRRK2-R1441C* iPSC-DaNs

Few publications have examined lysosomal pathways in iPSC-derived neuronal models carrying *LRRK2-R1441C* mutation. Our data display little evidence for autophagic and lysosomal perturbation in *LRRK2-R1441C* iPSC-DaNs. We did observe a minor reduction in MDW-941 spot number close to significance. Interestingly, reduced GCase activity is one of the few lysosomal phenotypes demonstrated in *LRRK2-R1441C* iPSC-DaNs (Nguyen and Krainc, 2018; Ysselstein et al., 2019). The size of the effect in our data is small compared to previous reports, although a different assay is used. MDW-941 does not measure substrate cleavage thus, 4-MUG may provide better examination of GCase activity in our cells. Recently, the Vocadlo lab recently developed a GCase cleavage assay compatible with live-cell imaging that could incorporate advantages of both MDW-941 and 4-MUG into one assay (Ashmus et al., 2018).

We did not observe autophagic perturbation in *LRRK2-R1441C* iPSC-DaNs. This is in-line with a transgenic mouse model expressing *LRRK2-R1441C* and knock-in mice expressing *LRRK2-R1441G* (Tsika et al., 2014; Liu et al., 2014). Conversely, our lab demonstrated multiple autophagic and lysosomal perturbations in a BAC transgenic *LRRK2-R1441C* rat (Wallings et al., 2019). Wallings and colleagues revealed lysosomal alkalinisation, inhibited autophagosome maturation and proteolysis, as well as altered lysosomal Ca^{2+} dynamics, all of which we did not observe.

A caveat to our lysosomal Ca^{2+} release assay is the presence of extracellular Ca^{2+} (albeit at low concentrations). Physiologically, TRPML1 channels at the membrane (following exocytosis for example) are not permeable to Ca^{2+} due to the neutral pH, however treatment with an agonist can induce Ca^{2+} influx (Kilpatrick et al., 2016; Li et al., 2017). Work from colleagues in our lab demonstrate this to not greatly alter lysosomal Ca^{2+} dynamics in our system, however.

GPN effects have been shown independent of Ca^{2+} influx through the membrane however, and this was also unable to replicate perturbed calcium signalling seen in transgenic rats (Kilpatrick et al., 2013; Wallings et al., 2019). Discrepancies between our results and previous findings may result from the cultures used to assess Ca^{2+} release. We use a relatively pure population of iPSC-DaNs and due to our microscopy-based approach, we could select cells with neuronal morphology. The previous report

using *LRK2* transgenic rats used primary cultures contain astrocytes and an assay that can only measure whole-well Ca^{2+} .

Our lysosomal Ca^{2+} release data displayed a bi-phasic release curve, in which a smaller peak preceded a broader peak. Recent work by colleagues demonstrates this secondary release curve to be evoked from ER stores (not published). Thus, our experiments measure Ca^{2+} signalling kinetics proximal to lysosomes following an induction of lysosomal Ca^{2+} release that subsequently triggers a cell-wide response. This is interesting as it demonstrates how effectively lysosomes communicate with the ER. Nonetheless, to study lysosome-only Ca^{2+} release, one must clamp ER Ca^{2+} release using thapsigargin (or similar) pre-treatment (Kilpatrick et al., 2013). Future work in our lab is probing specific lysosomal Ca^{2+} release in patient-derived iPSC-DaNs by first clamping ER Ca^{2+} .

6.8.3 Lysosomal dysfunction in *GBA-N370S* iPSC-DaNs

As expected, we observed reduced GCase activity in *GBA-N370S* iPSC-DaNs. This was replicated by both MDW-941 and 4-MUG, indicating both a reduction in substrate cleavage and in the abundance of GCase within lysosomes. Reduced GCase activity was not related to altered GCase protein expression, consistent with previous reports (Fernandes et al., 2016).

Surprisingly, we found reductions in endolysosomal number in *GBA-N370S* iPSC-DaNs, contrary to previous reports demonstrating accumulation of swollen defective lysosomes (Schondorf et al., 2014; Fernandes et al., 2016; Lang et al., 2019). As we did not observe significant alterations in the number of DQ-BSA or MDW-941 puncta, our results may indicate a deficiency arising from a non-degradative LAMP1-positive subpopulation. Indeed, recent studies demonstrated the presence of non-degradative LAMP1-positive compartments devoid of GCase and cathepsins in neurons (Cheng et al., 2018). These may be proto-lysosomes derived during lysosomal reformation (Yu et al., 2010). Indeed, *Gba* deficient MEFs displayed reduced lysosomal reformation (Magalhaes et al., 2016). This was dependent upon inhibited mTORC1 activity however, a finding that opposes recent data in *GBA-N370S* iPSC-DaNs (Bogetoft et al., 2023; Mubariz et al., 2023). It is noteworthy upon assessing correction of *GBA-N370S* phenotypes, reductions in MDW-941 spot number could be observed, and LAMP1 spot number were reduced but not significant.

We were also unable to observe differences in lysosomal Ca^{2+} release in *GBA-N370S* iPSC-DaNs, unlike previous reports in human fibroblasts, however results were preliminary and future experimentation will more clearly elucidate any phenotypes associated with this genotype (Kilpatrick et al., 2016).

Collectively, we were able to compellingly reproduce deficient GCase activity in *GBA-N370S* iPSC-DaNs and data indicates a reduction in the endolysosomal compartment, but no perturbation in other autophagic-lysosomal functions or TFE3 activity was seen.

6.8.4 Lysosomal dysfunction in *SNCA-Trp* iPSC-DaNs

SNCA-Trp iPSC-DaNs displayed endolysosomal dysfunction in LAMP1 expression, lysosomal number and reduced proteolysis. Much of these findings were replicated in *SNCA* overexpressing iPSC-DaNs, reaffirming the association between endolysosomal deficiency and upregulation of αSyn expression.

Proteolytic dysfunction in *SNCA-Trp* iPSC-DaNs has been reported previously, along with aberrant hydrolase maturation, associated with perturbed trafficking to the lysosome (Mazzuli et al., 2016; Mazzuli et al., 2016). The significant increase in pro-CTSD in *SNCA-Trp* iPSC-DaNs corroborates these previous findings. As mature CTSD was unchanged, a compensatory mechanism may occur at this stage of maturation to overcome these deficits. Deficient lysosomal GCase activity in *SNCA-Trp* iPSC-DaNs has been frequently reported (Mazzuli et al., 2016; Mazzuli et al., 2016; Stojkovska et al., 2022). We did not observe this, however previous studies examined this dysfunction at later timepoints than in this thesis. Thus, GCase deficiency may develop as pathology develops in maturing *SNCA-Trp* iPSC-DaNs.

A reduction in LAMP1 protein was not previously observed in at *SNCA-Trp* iPSC-DaNs at DIV35, but reduced LAMP1-puncta could be seen at DIV62 in an alternate study, suggesting a progressive pathology concomitant with deficient hydrolase maturation that becomes apparent as early as DIV45 (used in this thesis) (Zambon et al., 2019; Viridi et al., 2022). Interestingly, reduced LAMP1 expression and puncta replicates knockdown of the SNARE protein, Ykt6 in iPSC-neurons (Cuddy et al., 2019). Interestingly here, patient-derived *SNCA-A53T* iPSC-DaNs displayed an aberrant interaction between αSyn and Ykt6 leading to dysfunctional hydrolase trafficking. Thus, our results greatly overlap with lysosomal perturbation and inhibition of Ykt6 relating to *SNCA* mutation. Further, Ykt6 knockdown induced LAMP1 secretion into the media explaining the reduced expression (Cuddy et al., 2019). Our lab has

previously demonstrated increased secretion of α Syn by *SNCA-Trp* iPSC-DaNs, demonstrating further overlap with a perturbation in Ykt6 (Zambon et al., 2019). Mechanistically, perturbed hydrolase trafficking by inhibiting Ykt6 function may impair lysosomal degradation (DQ-BSA) in *SNCA-Trp* iPSC-DaNs leading to lysosomal exocytosis of contents such as α Syn preventing cargo accumulation. This may remain homeostatic early in maturation (DIV35) but discrepancies develop as maturation proceeds (DIV45 onwards, failed hydrolases hydrolase maturation and reduced lysosomal puncta). As no obvious transcriptional changes were present in these neurons, activation of TFE3 or TFEB could benefit these neurons to enhance the system.

6.8.5 TFE3 or TFEB activation in patient-derived iPSC-DaNs

Both TFEB and TFE3 overexpression were able to increase endolysosomal number and proteolysis in *SNCA-Trp* iPSC-DaNs demonstrating an ability to increase lysosomal biogenesis and function in a PD context. Surprisingly, the phenotypes previously confirmed were no longer present when attempting to correct them. Future work will attempt to replicate the phenotypes in *SNCA-Trp* iPSC-DaNs, and *SNCA-OVX* control iPSC-DaNs, and observe how TFE3 or TFEB overexpression can upregulate lysosomal function in a context presenting lysosomal dysfunction.

TFE3 overexpression increased lysosomal number in *GBA-N370S* iPSC-DaNs, and lysosomal GCase in all lines examined, bar one *GBA-N370S* line. Overall, this demonstrates TFE3 to be therapeutically viable in correcting GCase deficits in PD but underlines the variability to treatment that may occur within a population. TFEB was also able to increase endolysosomal number in *GBA-N370S* and GCase activity but did not demonstrate robust elevation of MDW-941, suggesting activation of GCase may be prompted following TFEB overexpression, but trafficking of the hydrolase is not.

Collectively, we were able to reproduce lysosomal GCase deficits in *GBA-N370S* iPSC-DaNs and uncovered functional endolysosomal defects in *SNCA-Trp* iPSC-DaNs. TFEB and TFE3 overexpression in these neurons generally increased the function of these lysosomal parameters demonstrating the therapeutically viable role for activating these transcription factors in DaNs in a PD context.

Chapter 7: Discussion

7.1 Main findings and Discussion

7.1.1 TFE3 as the primary expressed therapeutic target in Parkinson's disease over TFEB

TFEB, and to a lesser extent TFE3, has drawn interest as a therapeutic target in PD due to conferring neuroprotection through upregulating genes involved in lysosomal biogenesis and cellular catabolism, but also via neurotrophic means (Decressac et al., 2013; Torra et al., 2018; Arotcantera et al., 2019; He et al., 2021). We sought to closely examine the biology of TFEB and TFE3 and how the transcription factors regulate lysosomal function in PD using a highly relevant model, iPSC-DaNs.

The initial and most striking findings were that TFEB was minimally expressed in neurons and TFE3 was the primary expressed transcription factor in this cell-type. Fascinatingly, this reproduced in multiple neuronal cell types, both human and rodent, using online datasets and in-house iPSC-derived models. These findings are in-line with Bordi and colleague's hypothesis that TFE3 is primarily associated with autophagy in neurons and TFEB activation is primarily glial (Bordi et al., 2016). Recently, Mubariz and colleagues demonstrated TFEB mislocalisation in *GBA-N370S* iPSC-DaNs (2023). As discussed earlier, antibody cross-reactivity may be in part causing the discrepancies in TFEB expression. Alternatively, Mubariz and colleagues use a different protocol for dopaminergic differentiation, deriving from embryoid bodies. This protocol derives DaNs from a PAX6+ neuronal precursor, a population that may not produce SNc DaNs (Claudia Caiazza et al., 2020). As there was a small neuronal population expressing TFEB from analysis of online RNA-Seq data, it would be of interest to examine if the DaNs modelled by Mubariz and colleagues represent this population or replicate our findings that TFE3 is indeed the primary transcription factor in these neurons.

Our findings present major implications in the study of TFEB and TFE3 in combatting neurodegeneration. TFE3 seems the best suited transcription factor for enhancing neuronal lysosomal biogenesis. Previous studies have demonstrated mTOR inhibition

and similar pharmacological activators to confer neuroprotection, under the assumption TFEB drove this effect (Decressac et al., 2013; Siddiqui et al., 2015; Zhuang et al., 2020). We pose that TFE3 may be driving neuroprotection through neuronal lysosomal biogenesis in these models, whereas TFEB and TFE3 both confer effects in non-neuronal cell-types. Indeed, we demonstrate by genetic overexpression and pharmacological stimulation, TFE3 activation to positively regulate CLEAR genes and lysosomal function in iPSC-DaNs. Interestingly, TFE3 activation produced a superior response for CLEAR activation and lysosomal function than TFEB, demonstrating the robust therapeutic potential of TFE3. We also confirmed TFE3 regulation upon CLEAR expression in neurons using CRISPRi in i³ neurons to knockdown *TFE3*, finding mild transcriptional dysregulation of CLEAR genes. The modest transcriptional effects following knockdown are similar to those observed previously (He et al., 2021). He and colleagues demonstrate a greater perturbation of protein levels of autophagic/lysosomal proteins; hence it would be interesting to assess protein expression following knockdown. As *TFE3* knockdown failed to significantly perturb functional assays, perhaps i³ neurons are more resilient to loss of TFE3 than DaNs. Colleagues in our lab have developed control and patient-derived iPSC-lines for DaN differentiation containing CRISPRi machinery. Using these, we aim to more directly assess how TFE3 perturbation affects DaNs directly.

Surprisingly, we found no pathological dysfunction of TFE3 localisation or expression in patient-derived iPSC-DaNs, despite TFEB activity frequently reported to be abrogated in different PD models (Decressac et al., 2013; Siddiqui et al., 2015; Wang et al., 2016; Mubariz et al., 2023). One discrepancy as to why TFE3 may be less susceptible than TFEB to mis-localisation could be due to TFEB containing a prion-like domain (PrLD) near the N-terminus that is absent in TFE3 (Yang et al., 2023). Due to this PrLD domain, TFEB, but not TFE3, has been shown to co-aggregate with pathological huntingtin. The same may be true regarding α Syn deposition, whereby TFEB is more prone to sequestration by aggregation than TFE3. This further highlights the therapeutic advantage for targeting TFE3 in neurodegenerative diseases as TFE3 function is less likely to be inhibited by pathological processes.

7.1.2 TFEB activation still could be a viable therapeutic strategy for PD through HDAC inhibition

Although our data presents an issue with pharmacologically stimulating TFEB translocation due to the low expression, one cannot discount a role for TFEB in neuroprotection. TFEB expression in non-neuronal cell-types render therapeutic activation a viable option for targeting these cell-types in neurodegeneration. A rat model of multiple systems atrophy (MSA) demonstrated TFEB overexpression only to confer neuroprotection when specifically overexpressed in oligodendrocytes (we find to physiologically express TFEB), whereas neuron-specific TFEB overexpression exacerbated pathology (Arotcantera et al., 2019). Thus, TFEB-specific activators may be a therapeutically viable strategy to combat MSA by non-neuronally enhancing catabolism. Li and colleagues demonstrated PKC activation by HEP14, preferentially activated TFEB and not TFE3 by GSK3 β inhibition (Li et al., 2016). Thus, HEP14 treatment may be used to specifically target lysosomal biogenesis in non-neuronal cells in MSA.

In PD however, TFEB overexpression (even neuron-specific) has demonstrated neuroprotective effects. Thus, we developed a high-throughput qRT-PCR screening strategy in iPSC-Ngn2 neurons to pharmacologically re-introduce *TFEB* into neurons. Overwhelmingly, HDAC inhibition robustly re-introduced TFEB expression in neurons. Concomitantly, HDAC inhibition greatly upregulated CLEAR gene expression and lysosomal GCase abundance, demonstrating this as a pharmacologically viable means of enhancing neuronal catabolism. As mentioned previously, HDAC2 has been shown to bind and repress *TFEB*, *TFE3* and CLEAR gene transcription (Annunziata et al., 2019). Indeed, our results point toward HDAC2 playing a role in *TFEB* regulation but in a redundant manner also with HDAC1 and HDAC3, such that inhibition of only one does not induce *TFEB* expression. This was re-iterated by CRISPRi screening that failed to stimulate *TFEB* expression when knocking down only one HDAC.

Our RNA-Seq data identified *GRN* as a target of TFEB overexpression in iPSC-DaNs. Strikingly, Class I HDAC inhibition has been shown to increase *GRN* expression by reducing *GRN* H3K27 acetylation, increasing TFEB expression and enhancing TFEB binding to the *GRN* locus (She et al., 2017). Furthermore, the authors could not delineate the Class I HDAC responsible from individual siRNA knockdown, concluding redundant regulation between them. The remarkable likeness of *GRN* regulation to

the regulation we observe upon *TFEB* in neurons reinforces our findings and demonstrates a functional role for increased *TFEB* expression following HDAC inhibition upon a target identified by our RNA-Seq data.

HDAC inhibition upregulated *CLEAR* expression more robustly than *TFEB* or *TFE3* overexpression. This is likely resultant from de-repression of lysosomal gene expression by HDAC inhibition (Annunziata et al., 2019). This de-repression then may facilitate more efficient binding of transcription factors such as *TFEB* or *TFE3* to *CLEAR* promoters further enhancing expression. Chromatin immunoprecipitation of lysosomal gene promoters will allow us to assess whether *TFEB/TFE3* bind more efficiently to downstream promoters in neurons following HDAC inhibition.

As HDAC inhibition robustly upregulated lysosomal gene expression and GCase activity, we will attempt to correct lysosomal phenotypes in PD-derived iPSC-DaNs using these compounds in the future.

7.1.3 *TFEB* activation in iPSC-DaNs upregulates mitochondrial gene expression

Interestingly, *TFEB* overexpression was predominantly associated with upregulation of mitochondria-related gene expression rather than autophagic-lysosomal regulation. It is plausible that Class I HDAC inhibition could enhance mitochondrial biogenesis at least partially through increased *TFEB* expression. Indeed, HDAC inhibition (particularly Class I HDACi) enhances mitochondrial biogenesis in cardiomyocytes, brown adipose tissue and skeletal muscle (Galmozzi et al., 2013; Lkhagva et al., 2018; Yang et al., 2019). These are the same tissues shown to display a cell-specific role for mitochondria/lipid biogenesis by *TFEB* independently of autophagy, mirroring our findings in DaNs (Mansueto et al., 2017; Evans et al., 2019; Trivedi et al., 2020). These cell-types have large mitochondrial burden and DaNs are no exception to this. Thus, *TFEB* function may be adapted depending upon the bioenergetic burden of different cell-types. In future experiments, we will probe further how HDAC inhibition may activate mitochondrial function in iPSC-DaNs and ameliorate bioenergetic dysfunction in *SNCA-Trp* iPSC-DaNs or confer neuroprotection in a *SNCA-OVX* mouse model of PD (Janezic et al., 2013; Zambon et al., 2019; Ryan et al., 2021).

HDAC inhibition upregulates both autophagic and mitochondrial function in cardiomyocytes, encompassing both functions observed by *TFE3* and *TFEB*

overexpression in iPSC-DaNs (Yang et al., 2019). As HDAC inhibition has been shown to activate TFE3 translocation (Li et al., 2022), treatment may derepress lysosomal gene expression, increase TFE3 nuclear localisation and upregulate TFEB expression to enhance both bioenergetic and catabolic processes within iPSC-DaNs. Collectively, evidence suggests HDAC inhibition to be a promising avenue for activating TFEB/TFE3 in neurons enhancing multiple PD-relevant pathways.

7.1.4 HDAC inhibition as a viable therapeutic target in PD?

One major question arises regarding the tractability of HDAC inhibition as a therapeutic target for PD. HDAC hyperactivity has long been associated with PD, and inhibition has been explored as a combative strategy (Shukla and Tekwani, 2020). Indeed, our lab demonstrated HDAC4 and Sirtuin1 hyperactivity in *GBA-N370S* and *SNCA-Trp* iPSC-DaNs, respectively, signifying the therapeutic potential of HDAC inhibition in iPSC-DaNs (Lang et al., 2019; Zambon et al., 2019).

Our data implicates Class I HDACs as regulators of neuronal TFEB expression. Recently, HDAC1/2 inhibition conferred neuroprotection against MPTP-induced neurotoxicity, suggesting this strategy may be useful in relation to PD (Choong et al., 2016). It is important to note that neurotoxic and neuroprotective effects of HDACs often depends upon context. For example, HDAC1 demonstrates neurotoxic effects when interacting with HDAC3; but is neuroprotective when interacting with other substrates such as HDRP (Bardai et al., 2012). Thus, for HDAC inhibition to be most therapeutically tractable, strategies must consider inhibition of specific complexes, gaining the desired effect without abrogating neuroprotective functions. In this case, specific HDAC1/3 inhibition should provoke TFEB induction in neurons and abolish neurotoxic effects of the complex. Further, more selective inhibitors of HDACs will lead to reduced unwanted side effects associated with inhibiting such broad regulators.

Several HDAC inhibitors are FDA approved making them enticing compounds for repurposing in neurodegeneration (Ho et al., 2020). Vorinostat, a pan-HDACi, underwent a phase I/II clinical trial for Niemann Pick Type C (NCT02124083), but results found a high % serious adverse effects. Alternatively, RG2833, a selective HDAC1/3 inhibitor in phase I clinical trials for Friedrich's ataxia demonstrated good tolerance by patients (Soagni et al., 2014). This reiterates the need for selective compounds targeting specific HDACs to improve the likelihood of successful

therapeutics when targeting HDACs. As RG2833 activated *TFEB* expression in our screen, this is a good candidate to take for further study.

Another stand-out compound from our data was ACY-738, a HDAC6, HDAC1, HDAC2 and HDAC3 inhibitor (preferential to HDAC6). This compound increased *TFEB* expression and lysosomal function in our screening. Interestingly, ACY-738 has high brain bioavailability, although the half-life is short (Jochems et al., 2014). This may make ACY-738 a good compound for structural activity relationship (SAR) development, aiming to reduce turnover while retaining bioavailability in the brain. ACY-738 recently was used to combat Alzheimer's disease models, but at concentrations selective for HDAC6 inhibition (Majid et al., 2015). It would be interesting to observe how increasing the compound concentration may further aid neuroprotective function at levels relevant to neuronal *TFEB* induction.

Overall, HDAC inhibition is a promising therapeutic strategy in neurodegeneration. Developing selective compounds targeting specific HDAC functions such as regulation of *TFEB* expression will improve the tractability and success of this approach.

7.1.5 Lysosomal dysfunction and *TFEB/TFE3* in patient-derived iPSC-DaNs

This thesis rigorously assessed lysosomal function in patient-derived iPSC-DaNs carrying *GBA-N370S*, *LRRK2-R1441C* and *SNCA-Trp* iPSC-DaNs. We benefited from previous studies assessing these mutations through the use of an array of functional high-content lysosomal assays enabling us to dissect lysosomal pH, GCCase activity, Ca^{2+} release, endolysosomal number and proteolysis.

We found minor lysosomal GCCase deficits in *LRRK2-R1441C* iPSC-DaNs by MDW-941 but few other lysosomal perturbations were found associated with this genotype. A large body of literature have demonstrated lysosomal defects resultant from the most common *LRRK2* mutation, *LRRK2-G2019S*, and even *LRRK2* kinase activity to regulate GCCase activity (Madureira et al., 2020). Thus, functional analysis of lysosomal function in iPSC-DaNs carrying the *LRRK2-G2019S* mutation using the assays laid out here, may be useful for further comprehensively identifying lysosomal perturbations associated with *LRRK2* kinase hyperactivity in iPSC-DaNs.

GBA-N370S iPSC-DaNs replicated dysfunction in GCCase activity, and we also found perturbation in endolysosomal number. Both were increased by *TFE3* overexpression, albeit one *GBA-N370S* line did not respond to this. *TFEB* overexpression increased

the activity of GCase (by 4-MUG) within neurons but not the abundance within the lysosome (MDW-941). The absence of increased functional lysosomal GCase levels, corroborates with our findings that TFEB does not increase *GBA* expression in neurons. Further, RNA-Seq found TFEB overexpression downregulated *SCARB2* (encoding LIMP2), which may result in reduced GCase trafficking to the lysosome, prompting the reduction in MDW-941 spot number observed. As to how GCase activity is *increased* following TFEB overexpression, GRN upregulation by TFEB may enhance the activation of GCase within the cell itself. Indeed, GRN interacts with GCase and facilitates its activity (Zhou et al., 2019). Thus, GCase activity may be promoted by TFEB overexpression through transcriptional induction of GRN, without a robust increase in GCase expression or trafficking to the lysosome.

HDAC and HSP90 inhibitors increased lysosomal GCase from our screening. Interestingly, both are involved in the degradation of nascent GCase peptides (Lu et al., 2011; Yang et al., 2013). Thus, HDAC and HSP90 inhibitors may upregulate GCase in the neuron by prolonging the half-life of the protein and increasing *GBA* expression. We will assess how these inhibitors modulate GCase activity in patient-derived *GBA-N370S* iPSC-DaNs.

We also found and validated a role for *SNCA* overexpression upon modulating endolysosomal number and proteolysis. Both parameters were increased by TFEB and TFE3 overexpression. Few compounds from our TFEB qRT-PCR screening increased DQ-BSA (likely associated with toxicity at high concentrations). However, ACY-738 demonstrated an enhancement of both GCase and DQ-BSA signal in iPSC-Ngn2 neurons. This further underlines the potential of this compound to evoke positive catabolic responses within neurons and could be used for combatting phenotypes associated with *SNCA* in PD.

7.2 Conclusions

Overall, this thesis demonstrated a significant role for TFE3 in lysosomal biogenesis in iPSC-DaNs. TFEB expression is extremely low in most neuronal populations and is primarily a non-neuronal transcription factor, whereas TFE3 expression is robust throughout neuronal and non-neuronal cell-types. Genetic modulation of TFE3 (by overexpression or CRISPRi-mediated knockdown) or pharmacological activation by

TRPML1 agonist ML-SA5, demonstrated a positive-regulatory function for TFE3 upon CLEAR genes and lysosomal biogenesis in iPSC-derived neuronal models.

qRT-PCR screening for *TFEB* transcriptional activators in neurons revealed HDACs and HSP90 to regulate *TFEB* expression. HDAC inhibition robustly upregulated TFEB protein expression and downstream lysosomal gene expression in cortical and dopaminergic neurons. The most likely candidates from pharmacological data insinuate redundant functions of HDAC1, HDAC2 and HDAC3 on the *TFEB* locus. TFEB overexpression was primarily found to upregulate mitochondrial genes and did little to increase lysosomal function or gene expression compared to TFE3 in control iPSC-DaNs. This may allude to a novel mechanistic role for TFEB in DaNs in regulating mitochondrial biogenesis and function, an interesting PD-relevant avenue to follow-up in the future.

We applied several lysosomal assays to study lysosomal function in patient-derived iPSC-DaNs. Lysosomal GCase activity was perturbed in *GBA-N370S* iPSC-DaNs, and to a lesser extent *LRRK2-R1441C* iPSC-DaNs. Ablation of the endolysosomal compartment was observed in both *GBA-N370S* and *SNCA-Trp* iPSC-DaNs, and dysfunctional endolysosomal proteolysis displayed following overexpression of *SNCA* either in patient-derived iPSC-DaNs, or by lentiviral overexpression in control iPSC-DaNs.

The positive regulation upon lysosomal function seen by TFE3 activation also replicated in patient-derived iPSC-DaNs carrying *SNCA-Trp* and *GBA-N370S* mutations, demonstrating the therapeutic potential of TFE3 in PD. Furthermore, despite TFEB overexpression having a less lysosome-based function, this overexpression was still amenable to upregulate endolysosomal function and GCase substrate cleavage in patient-derived iPSC-DaNs.

Future work aims to demonstrate TFE3 activation to confer neuroprotection *in vivo*. We will also further characterise and implicate HDAC inhibition in the upregulation of lysosomal function in patient-derived iPSC-DaNs, through de-repression of TFEB and downstream lysosomal genes. The major goal will be to develop this novel strategy of pharmacologically targeting neuronal TFEB expression and lysosomal biogenesis in a therapeutically viable, neurodegeneration-specific context.

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Appendix

S.1 iPSC-derived Cell Culture

S.1.1 Day-by-Day differentiation of iPSCs into DaNs by mFPP expansion protocol

iPSCs (Table S.3) were thawed and expanded (Section 2.1.1 and 2.1.2.1). Here, Day 0 refers to the start of differentiation (not the day of passage/thaw).

Day -3: Culture wells were coated with Geltrex and incubated overnight at 37°C.

Day -2: Cells were passaged with Accutase (Section 2.1.2.1). Upon resuspension of cells, 20 μ L cells were combined with 1.5 μ L Solution 18 and counted on Chemometec NucleoCounter NC-250. Cells seeded at 1.25×10^5 cells/cm² density.

Day -1: Full media exchange with mTESR⁺⁺.

Day 0: Cells were observed for even confluency (~90%) and good morphology. If this was not the case, cells were re-passaged to rectify the issue before differentiation. Here onwards, media was doubled (ie. 2 mL $\rightarrow$ 4 mL in 6-well culture). A full media change using the corresponding Day 0 constituents (Table S.2) was carried out. Base media (KO-DMEM-KSR here) (Table S.1) was warmed before growth factors (Table S.2) were added. Growth factors were added directly prior to filtering/feeding.

Day 1-10: Cells were fed with media constituents displayed in Table S.2. Odd numbered days were full media changes, even numbered days were $\frac{1}{2}$ media changes. As with Day 0, base media is warmed, then growth factors were added prior to filtering and media change.

Day10^{exp1}-10^{exp18}: Here, midbrain floor plate progenitors are expanded 4 times over the space of 19 days. Expansion occurs on Day10^{exp1}, Day10^{exp7}, Day10^{exp13} and Day10^{exp18}. All other days follow the intermittent full-feed / half-feed alternation pattern, with reflecting constituents in Table S.2. On expansion day, enzymatic single cell passage is carried out with Accutase similarly to Section 2.1.2.1, except cells were passaged at a 1:2 ratio, resuspended in single cell suspension using Day10^{exp} media, supplemented with 10 μ M Rocki, and wells were seeded with double media (ie. 4 mL in 6 wells). The day proceeding a day of expansion, a full media exchange was carried out with Day10^{exp} (no Rocki) and the interchanging full-media/half-media rotation

followed again. Day10^{exp17}, the appropriate number of culture wells were coated with Geltrex overnight at 37°C.

Day10^{exp18}: Here, passaging of cells onto culture plates took place in the same manner as previous Day10^{exp} passages. This time however, two spinning falcons containing KO-DMEM were prepared for each line. One falcon was designated for further differentiation of mFPPs, and the other was designated for cryopreservation of surplus mFPPs (See Section S.1.2 for mFPP cryopreservation). Prior to centrifugation, once the surplus mFPPs have been placed in the spinning falcon, cell concentration is assessed by combining 20 µL cell suspension and 1.5 µL Solution 18 and counting on the Chemometec Nucleocounter NC-250. This is required to know the number of mFPPs are present for aliquoting and cryopreservation. The mFPPs taken forward for further differentiation were resuspended in Day10^{exp18} media and seeded into the pre-coated culture wells prepared on Day10^{exp17}.

Day10^{exp19}: Remaining wells received a full media change with pre-warmed and filtered Day10^{exp} media (no ROCKi).

Day11-20: Cells were fed with prepared, warmed and filtered media containing the constituents of the corresponding media from Table S.2. Cells underwent a full media change on odd numbered days and a 50% media change on even numbered days.

Day 18: 96-well Phenoplate (Perkin Elmer, 6055302) and 96-well half area) (Corning, 4517) were used for high-content imaging were coated with Poly-L-ornithine (PLO) overnight at 37°C. Biolaminin(LN521) was thawed overnight on ice, in 4°C fridge.

Day 19: Biolaminin(LN521) was diluted 1:10 using KO-DMEM. PLO was aspirated from the precoated 96-well plates and the diluted Biolaminin was added to the plates. Non-imaging plates (24-well, 12-well or 6-well) were coated with Geltrex. Plates were incubated at 37°C overnight.

Day 20 (terminal plating): Cells were observed for culture health. Accutase was let warm to ambient temperature. Day 20 (contains ROCKi) Media was prepared (Table S.2), warmed and filtered. Spinning falcons containing KO-DMEM were prepared and warmed. Eppendorfs for each cell line containing 490 µL PBS were prepared for later cell dilution when counting. Prior to passaging, Biolaminin(LN521) and Geltrex was aspirated from the wells and half volume of Day 20 media was replaced. Culture plates were then placed back in the incubator at 37°C until cells were ready to be seeded. Passaging took place approximately 4 lines at a time (too many would leave cells in

suspension for too long). Media was removed from the cells, wells were washed once with PBS, before Accutase was added to the cells and incubated for 5 mins, 37°C. Following, cells were washed off the plate and gently resuspended into the corresponding spinning falcon. Cells were then centrifuged at 350 r.c.f for 5 mins. Supernatant was aspirated and cells were resuspended into a single cell solution with Day 20 media. Extra care was made to ensure single cell suspension of the cells (cell clumps make them harder to image). 10 µL cell suspension was taken and diluted in the 490 µL PBS Eppendorf. 20 µL of the diluted cell suspension then was combined with 1.5 µL solution 18 and counted on a Chemometec NucleoCounter NC250. Cells were seeded at 1.87×10^5 cells/cm² in 96-well imaging plates, and 4.47×10^5 cells/cm² in 6-, 12-, or 24-well format. Once seeded, cells were incubated at 37°C, 5% CO₂ until Day 22.

Day 21: No feed was done on this day. Cells were checked under the microscope for culture health and seeding density.

Day 22 (mitomycin C): Cells were observed for culture health. Day 22+ media, Neurobasal media (for washing cells), and mitomycin C media (Neurobasal + mitomycin C) (Table S.2) were all prepared, warmed and filtered. Mitomycin C was thawed/added and media was filtered, just prior to addition to the cells. Media was aspirated from the cells and replaced with Mitomycin C media. Cells were incubated with Mitomycin C at 37°C, for 1 hour. Following incubation, mitomycin C media is aspirated, cells were washed once with neurobasal media. The neurobasal media was then aspirated and replaced with Day22+ media (Table S.2).

Day 24+: A 50% feed with Day 22+ media was administered to the cells bidaily for the remainder of the maturation process. iPSC-derived dopaminergic neurons were cultivated until either day 45 or day 65.

Table S.1 - Base Media constituents

Media (Constituents)	Concentration
KO-DMEM-KSR	
KO-DMEM media	85%
Knockout serum replacement (KSR)	15%
Beta-mercaptoethanol	10 µM
L-glutamine	2 mM
Non-Essential Amino Acids (NEAA)	1X

NNB		
Neurobasal Medium		100%
N2 Supplement		0.5X
B27 Supplement (without Vitamin A)		0.5X
L-Glutamine		2 mM
NB		
Neurobasal Medium		100%
B27 Supplement (Without Vitamin A)		1X
L-Glutamine		2 mM

Table S.2 - Growth Factor Constituents for iPSC-DaN Differentiation Days

Day	Constituents	Concentration
0	<i>KO-DMEM-KSR</i>	<i>100%</i>
	<i>(Table S.1)</i>	
	<i>LDN-193189</i>	<i>100 nM</i>
1+2	<i>SB-431542</i>	<i>10 μM</i>
	<i>KO-DMEM-KSR</i>	<i>100%</i>
	<i>LDN-193189</i>	<i>100 nM</i>
	<i>SB-431542</i>	<i>10 μM</i>
	<i>SHH C24II</i>	<i>100 ng/mL</i>
	<i>Purmorphamine</i>	<i>2 μM</i>
	<i>FGF8a</i>	<i>100 ng/mL</i>
3+4	<i>KO-DMEM-KSR</i>	<i>100%</i>
	<i>LDN-193189</i>	<i>100 nM</i>
	<i>SB-431542</i>	<i>10.84 μM</i>
	<i>SHH C24II</i>	<i>100 ng/mL</i>
	<i>Purmorphamine</i>	<i>2 μM</i>
	<i>FGF8a</i>	<i>100 ng/mL</i>
	<i>CHIR-99021</i>	<i>3 μM</i>

5+6	<i>KO-DMEM-KSR</i>	75%
	<i>NNB (Table S.1)</i>	25%
	<i>LDN-193189</i>	100 nM
	<i>SHH C24II</i>	100 ng/mL
	<i>Purmorphamine</i>	2 μ M
	<i>FGF8a</i>	100 ng/mL
	<i>CHIR-99021</i>	3 mM
7+8	<i>KO-DMEM-KSR</i>	50%
	<i>NNB</i>	50%
	<i>LDN-193189</i>	100 nM
	<i>CHIR-99021</i>	3 μ M
9+10/10^{exp1}-10^{exp19}	<i>KO-DMEM-KSR</i>	25%
	<i>NNB</i>	75%
	<i>LDN-193189</i>	100 nM
	<i>CHIR-99021</i>	3 μ M
10^{exp1}, 10^{exp7}, 10^{exp13}, + Rock inhibitor (Y27632)		10 μ M
10^{exp18}		
11+12	<i>NB (Table S.1)</i>	100%
	<i>BDNF</i>	20 ng/mL
	<i>GDNF</i>	20 ng/mL
	<i>TGFβ3</i>	1 ng/mL
	<i>DAPT</i>	10 μ M
	<i>Ascorbic Acid</i>	200 μ M
	<i>Dibutyl cAMP</i>	500 μ M
	<i>CHIR-99021</i>	3 μ M CHIR-99021
D13-D19	<i>NB</i>	100%
	<i>BDNF</i>	20 ng/mL

	<i>GDNF</i>	20 ng/mL
	<i>TGFβ3</i>	1 ng/mL
	<i>DAPT</i>	10 μM
	<i>Ascorbic Acid</i>	200 μM
	<i>Dibutyl cAMP</i>	500 μM
D20	+ <i>Rock inhibitor (Y27632)</i>	10 μM
Day 22 (Mitomycin C)	<i>Neurobasal medium</i>	100%
	<i>Mitomycin C</i>	1 μg/mL
D22+ (Maturation Media)	<i>NB</i>	100%
	<i>BDNF</i>	20 ng/mL
	<i>GDNF</i>	20 ng/mL
	<i>TGFβ3</i>	1 ng/mL
	<i>DAPT</i>	10 μM
	<i>Ascorbic Acid</i>	200 μM
	<i>Dibutyl cAMP</i>	500 μM

S.1.2 Cryopreservation of mFPPs

On Day10^{exp18}, mFPPs were passaged. mFPP freezing medium (90% Day 10^{exp} medium (Table S.2 - no Rocki), 10% DMSO) was prepared. Upon centrifugation, pelleted mFPPs designated for cryostorage were resuspended in mFPP freezing medium to 6x10⁶ cells/mL. Resuspension was aliquoted into cryovials, placed into a CoolCell Freezing container and stored at 80°C. Frozen cryovials were transported to liquid nitrogen until future use.

Table S.3 - Information on iPSC lines used for dopaminergic neuron differentiation

Donor ID	Clone	Genotype	Thesis Abbreviation	Characterisation
SFC156-03	01	Control	156	Lang et al., 2019
SFC067-03	01	Control	067	Lang et al., 2019

JR053	01	Control	JR053	Lang et al., 2019
MK088	01	GBA- N370AS/Wt	88-1	Fernandes et al., 2016
MK071	03	GBA- N370S/Wt	71-3	Fernandes et al., 2016
MK082	26	GBA- N370S/Wt	82-26	Fernandes et al., 2016
SFC869-03	04	LRRK2- R1441C	869	Connor-Robson et al., 2019
SFC870-03	05	LRRK2- R1441C	870	Connor-Robson et al., 2019
JR207	03	LRRK2- R1441C	207	Connor-Robson et al., 2019
SFC-831-03	01	SNCA-Trp	831-1	Zambon et al., 2019
SFC-831-03	03	SNCA-Trp	831-3	Zambon et al., 2019
SFC-831-03	05	SNCA-Trp	831-5	Zambon et al., 2019

S.1.3 i³ Neuron differentiation from iPSCs

Days leading up to Day 0: G3dCas9 iPSCs were thawed and maintained as described in Section 2.1.1.

Day -1: 6-well plates were coated overnight with Geltrex at 37°C.

Day 0: Day 0 (Tables S.5-S.6) was prepared, filtered and warmed in a bead bath. Geltrex was aspirated from the coated culture wells and replaced with media. G3dCas9 iPSCs were then passaged as per Section 2.1.2. iPSCs were resuspended in Day 0 media and counted. Cells were then seeded at 1.56×10^5 cells/cm² and incubated at 37°C and 5% CO₂.

Assay plates were coated with PLO coating buffer (Table S.4) on Day 0.

Days 1-2: Day 1-2 media (Tables S.5-S.6) was prepared, filtered and warmed. iPSC progression toward neural precursor morphology was assessed as well as health of the culture. Full media changes were carried out each day.

PLO was aspirated from assay plates on **Day 1**, washed with water 3 times and allowed to dry overnight.

Once the assay plates had dried overnight, on **Day 2** Laminin coating was added to the assay plates and incubated overnight at 37°C.

Day 3: Day 3 media (Tables S.5-S.6) was prepared, filtered and warmed. Accutase and spinning falcons containing KO-DMEM were allowed to reach ambient temperature. Laminin coating was removed from the assay plates and replaced with half volume of media. Media was removed from induced cells, PBS was then used to wash the wells before cells were incubated with Accutase for 5 minutes, 37°C, 5% CO₂. Cells were washed off the plate and added to the spinning falcon. Cells were centrifuged at 350 r.c.f for 5 mins. Supernatant was aspirated and cells were resuspended into a single cell suspension with Day 3 media. Cells were counted and seeded at a density of 9.2×10^4 cells/cm² in 96 or 384-well plates for imaging, 1.84×10^5 cells/cm² for qRT-PCR in 384-well plates and 2.85×10^5 cells/cm² in 12- or 24-well plates. Addition of lentiviral vectors were added after seeding. Cells (and virus if applicable) were incubated overnight at 37°C, 5% CO₂.

Day 4+: A full media change was carried out on Day 4 using prepared, warmed and filtered Day 4+ media. Post Day 4, a half-media feed was carried out twice a week with Day 4+ media until assaying (14 days later).

Table S.4 - Poly-L-Ornithine Coating Buffer

Constituents	Concentration
<i>Boric Acid</i>	<i>100 mM</i>
<i>Sodium Tetraborate</i>	<i>25 mM</i>
<i>Sodium Chloride</i>	<i>75 mM</i>
<i>MilliQ H₂O</i>	<i>To 10 mL</i>
<i>Sodium Hydroxide</i>	<i>1 M (Adjust pH to 8.4)</i>
<i>PLO (Dissolve once buffer above is made)</i>	<i>1 mg/mL</i>

Table S.5 - i3 Neuron Base media

Media (Constituents)	Concentration
Basal Induction Media	
DMEM/F12	100%
N2 Supplement	1X
Non-Essential Amino Acids	1X
L-Glutamine	2 mM
Maturation Base Media	
BrainPhys	100%
B27 Supplement with Vitamin A	

Table S.6 - Media constituents of i3 Neuron differentiation by day

Day	Constituents	Concentration
0	Base Induction Media (Table 2.3)	100%
	Doxycycline Hyclate	2 µg/mL
	Rocki (Y27632)	10 µM
1-2	Base Induction Media	100%
	Doxycycline Hyclate	2 µg/mL
3+	Maturation Base Media (Table 2.3)	100%
	BDNF	10 ng/mL
	NT-3	10 ng/mL
	Laminin	1 µg/mL
	Doxycycline	2 µg/mL
Day 3 only	Rocki	10 µM

S.1.4 Materials

Table S.7 – iPSC-derived Culture + Differentiation

Product Name	Supplier	Cat. No.
mTESR1 + Supplement	<i>STEMCELL Technologies</i>	85850
<i>Penicillin/Streptomycin</i>	<i>Life Technologies</i>	15140122
<i>Matrigel hESC-qualified matrix</i>	<i>SLS</i>	354277
<i>Rock inhibitor (Y-27632 dihydrochloride)</i>	<i>Srtratech/ApexBio</i>	A3008-APE-10mg
<i>STEMPro Accutase</i>	<i>Life Technologies</i>	15420062
<i>TrypLE</i>	<i>Life Technologies</i>	15604013
<i>ES-grade FBS</i>	<i>SAFC</i>	F7524-500mL
<i>Solution 18</i>	<i>Chemometec</i>	910-3018
<i>Geltrex</i>	<i>Life Technologies</i>	A1413302
<i>Poly-L-Ornithine</i>	<i>Sigma</i>	P4957
<i>Biolaminin(LN521)</i>	<i>Biolamina AB</i>	LN521-05
<i>KO-DMEM Media</i>	<i>Life Technologies</i>	10829018
<i>Knockout Serum Replacement</i>	<i>Life Technologies</i>	10828010
<i>Beta-mercaptoethanol</i>	<i>Life Technologies</i>	21985023
<i>L-Glutamine</i>	<i>Life Technologies</i>	25030024

<i>Non-Essential Amino Acids</i>	<i>Life Technologies</i>	<i>11140035</i>
<i>Neurobasal Medium</i>	<i>Life Technologies</i>	<i>21103049</i>
<i>N2 Supplement</i>	<i>Life Technologies</i>	<i>17502048</i>
<i>B27 Supplement (w/o Vit A)</i>	<i>Life Technologies</i>	<i>12587010</i>
<i>LDN-193189</i>	<i>Sigma</i>	<i>SML0559-5MG</i>
<i>SB-431542</i>	<i>Strattech/ApexBio</i>	<i>A8249-APE-10mg</i>
<i>SHH C42II</i>	<i>Biotechne (Tocris/R&D)</i>	<i>1845-SH-500</i>
<i>Purmorphamine</i>	<i>Biotechne (Tocris/R&D)</i>	<i>4551/10</i>
<i>FGF8a</i>	<i>Cambridge Biosciences</i>	<i>Z03172-1</i>
<i>CHIR-99021</i>	<i>Biotechne (Tocris/R&D)</i>	<i>4423/10</i>
<i>BDNF</i>	<i>Peprotech</i>	<i>450-02</i>
<i>GDNF</i>	<i>Peprotech</i>	<i>450-10</i>
<i>TGFβ3</i>	<i>Peprotech</i>	<i>100-36E</i>
<i>DAPT</i>	<i>Abcam</i>	<i>Ab120633-50mg</i>
<i>Ascorbic Acid</i>	<i>Sigma</i>	<i>A4544-25G</i>
<i>Dibutyl cAMP</i>	<i>Sigma</i>	<i>D00627-5X1G</i>
<i>Mitomycin C</i>	<i>Abcam</i>	<i>Ab120797-2mg</i>
<i>BrainPhys</i>	<i>STEMCELL Technologies</i>	<i>05790</i>
<i>Doxycycline hyclate</i>	<i>Merck</i>	<i>D9891-1G</i>
<i>B27 supplement with Vit A</i>	<i>Life Technologies</i>	<i>17504044</i>
<i>Neurotrophin 3 (NT-3)</i>	<i>Peprotech</i>	<i>450*03-50UG</i>
<i>Laminin Mouse Protein</i>	<i>Life Technologies</i>	<i>23017015</i>
<i>DMEM/F12</i>	<i>Life Technologies</i>	<i>11320033</i>

S.2 Compound addition

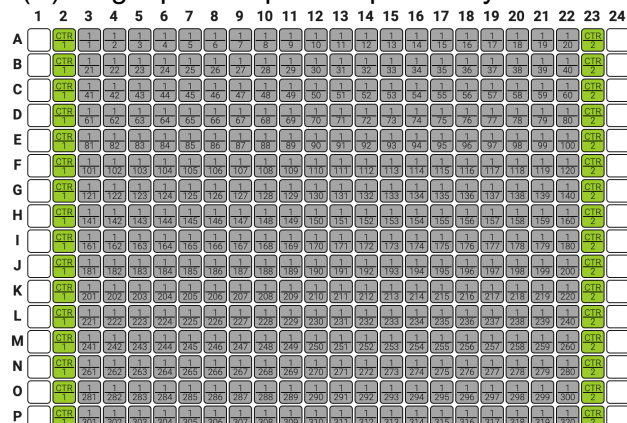
160 nL Compound was dispensed into a 384-well polypropylene v-bottom plate using a Labcyte Echo 555. 40 μ L of pre-warmed and filtered Feeding media was then added to each well using a Viaflo-384 and mixed to create a 4X solution. 16.7 μ L of the 4X compound solution was then added to the remaining 50 μ L media in the cell culture wells for a final volume of 66.7 μ L. Compound treatment duration was 48 hours by incubation at 37°C, 5% CO₂, 95% humidity, before cells were assayed (primarily by qRT-PCR, but also by MDW-941 and DQ-BSA).

Compound layout was dependent upon the assay. Single dose experiments were treated as per Figure S.1 A at 10 μ M concentrations. Dose-response assays were laid

out as per Figure S.1 B, in which compounds were added at 3-fold dilutions from 10 μ M in duplicate across the plate.

DMSO negative controls were placed in column 2 of each 384-well plate, and positive controls (usually ACY-738 or LAQ824) were placed in column 23 of each 384-well plate. Columns 1 and 24 were not treated with any compound or vehicle.

(A) Single-point 10 μ M compound layout



(B) 10-point dose response compound layout

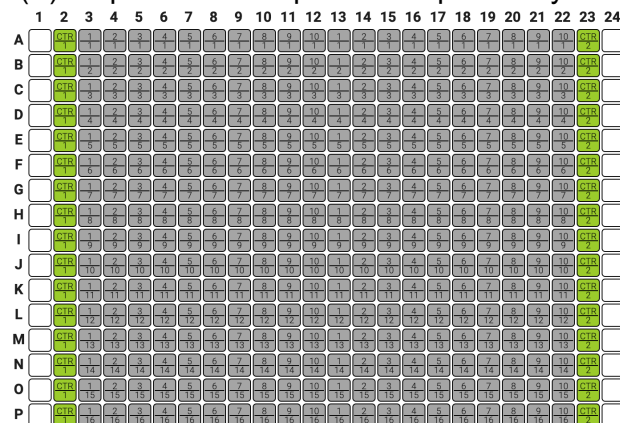


Figure S.1 – Compound plate layouts.

(A) Single-point compound treatment. (B) 10-point compound response curve treatment. Top ordinal = 3-fold dilution of 10 μ M (1 = 10 μ M, 2 = 3.33 μ M ...). Bottom Ordinal = Compound number. CTR1 = Negative control: DMSO. CTR2 = Positive control: ACY-738.

S.3 Biogen Bioactive Compound Deck Target Classes

Table S.8 - Biogen Bioactive Compound Library Target Class Compound numbers

Compound Class	Number of Compounds
Acyltransferase	9
Apoptosis	5
ATPase	8
Catalytic receptor	8
Cell Adhesion	1
Chaperone	14
CYP	11
Cytokine	2
Cytoskeleton	1
Dye	2
Epigenetic	120
Exchange Factor	0
Glycosyltransferase	10
GPCR	774
Growth Factor Receptor	2
GTPase	16
GTP Activating Protein	1

Hydrolase	47
IAP Family	2
Imaging	7
Immunology	21
Inorganic	0
Ion Channel	162
Isomerase	3
Kinase	607
Kinetochore	1
Lectin	1
Ligase	1
Lipase	4
Lipid metabolism	37
Lyase	9
Membrane Receptor	2
Membrane Transport Protein	3
miRNA	1
Mitochondria	2
Motor Protein	6
Natural Product	139
Natural Product (Human)	28
Neuroscience	16
Neurotransmitter Metabolism	11
NSAID	24
Nuclear Hormone Receptor	66
Nucleotide metabolism	1
Oncology	28
Oxidase	2
Oxidoreductase	29
Pattern Recognition receptor	1
PDE	23
Phosphatase	8
Protease	92
Serine Hydrolase	2
Signalling pathway	2
Splicing	1
Steroid	39
Steroid biosynthesis	8
Transcription	22
Transcription Factor	1
Transferase	19
Transmembrane protein	1
Transporter	71
Ubiquitin	35
UPR	1
Viral/Parasitic/Antibiotic	177
Wnt Pathway	8
N/A	182

S.4 Generation of ultracompetent *StbI3 Escherichia coli* (*E.coli*)

Stocks

Stocks of chemically competent *StbI3 E.coli* were prepared using Mix & Go *E. coli* transformation kit and buffer set (Zymo, T3002), Zymobroth (Zymo, M3015-500) and chemically competent *StbI3 E.coli*.

Day 0: LB agar plates **without** antibiotic selection (Section 2.3.2 for LB broth components) were prepared and *StbI3 E.coli* were streaked on. Bacteria were then incubated overnight at 37°C in a Thermo Scientific Heraeus oven.

Day 1: In the morning, the plate was removed, parafilm and stored in 4°C until the evening. 30 minutes prior to colony selection, the agar plate was placed back in the incubator to warm to 37°C. A colony was then picked using a pipette tip and the bacterial colony was placed in a starter culture (5.5 mL LB selection medium in a U-bottom centrifuge tube). The bacterial culture was incubated overnight at 37°C in a New Brunswick Innova 44 incubator shaker, at 250rpm.

Day 2: 5 mL 1X Wash buffer and 1X Competent buffer (from Mix & Go *E.coli* transformation kit and buffer set) were prepared and placed on ice. 500 µL of the bacterial starter culture was taken and added to 50mL ZymoBroth in a 500 mL culture flask. The culture is further incubated at 37°C, 250 rpm. The OD_{600nm} of the bacterial culture was measured every hour using a spectrophotometer. Once OD_{600nm} reached between 0.4-0.6, the bacterial culture was placed on ice for 10 mins. The cells were centrifuged between 1,600-2,500 r.c.f for 10 mins at 4°C. The clarified supernatant was aspirated and the bacterial pellet was resuspended in 5 mL cold 1X Wash buffer using a rocker at 4°C. The suspension was once again centrifuged. The pellet was resuspended in 1X Competent buffer using a rocker, before the bacterial suspension was aliquoted into 25 µL aliquots and stored at -80°C.

S.5 Sanger Sequencing

S.5.1 BigDye sequencing

BigDye sequencing was carried out for EF1 α -TFEB-iRFP670 and EF1 α -TFE3-iRFP670 constructs. Designed primers were ordered via IDT, reconstituted into a 100 μ M stock. Primers were diluted 1:10 (10 μ M). BigDye sequencing reactions were carried out in a skirted 96-well plate (4titude, 4ti-0750). A master mix containing 5X sequencing buffer, BigDye (Applied Biosystems, 4458687) and the construct to be sequenced was dispensed into each well (2 wells for every primer). Each sequencing primer was added to 2 wells containing the master mix. Multiple primers were not placed in the same well. Reaction reagents are presented in Table S.9. Once all primers had been dispensed into BigDye master mix, the PCR reaction displayed in Table S.10 was carried out in a thermocycler. Following, the PCR product was precipitated by adding 4 μ L Precipitation master mix (125 mM EDTA, 3 M NaAc) and the addition of 50 μ L 100% ethanol. The reaction was then incubated for 15 mins, room temperature before centrifugation at 3000rcf, 4°C, 30 mins. Supernatant was removed from sequencing wells to leave the pelleted genetic material. The plate was pulse-centrifuged before the pellet was washed with 70 μ L 70% ethanol and centrifuged at 1650 rcf, 4°C, 15 mins. Again, the supernatant was discarded the plate was pulse-centrifuged and the pellets were left to dry for 5 mins. Sanger sequencing was then carried out.

Table S.9 - BigDye Sequencing Reaction Components

Component	Amount
BigDye	0.5 μ L
5X Sequencing Buffer	1.7 μ L
Ligated DNA product (100ng/ μ L)	1 μ L
MilliQ H ₂ O	6.75 μ L
Sequencing Primer (10 μ M)	1 μ L

Table S.10 - BigDye PCR reaction conditions

Step	Temp	Time
1	95°C	1 minute
2 (30 cycles)	95°C	10 seconds
	50°C	5 seconds

3	60°C	4 minutes
4	4°C	Indefinite

S.5.2 Source Bioscience sequencing

5 µL sgRNA construct was provided to Source Biosciences with the appropriate primer.

S.5.3 Sequencing analysis

Sequencing files were aligned by importing sequencing data into Snapgene using the 'Align Multiple DNA Sequences' Function onto the designated backbone. Alignments were examined for correct insertion and absence of mutations in the construct.