Molecular mechanisms of OXR1 function

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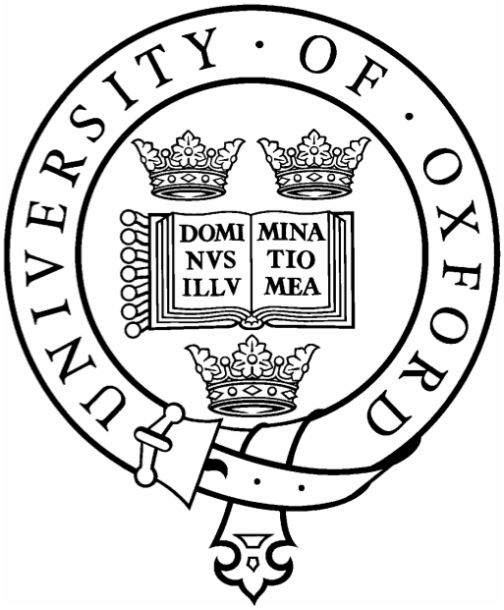

Supervisor: Professor Dame Kay E. Davies

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Abstract

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By 2040, the World Health Organization expects neurodegenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and Parkinson's disease, to surpass cancer as the second most common cause of death worldwide. Currently, only treatments for symptoms of these diseases are available. Thus, research is critical to alleviate this public health burden by elucidating the pathogenic processes and developing novel therapies. While exact mechanisms by which these heterogeneous neuropathological conditions become manifest in patients remain unclear, growing evidence suggests that oxidative stress (OS) makes a significant contribution to neuronal dysfunction and apoptosis in all major neurodegenerative diseases. Recently, the gene *oxidation resistance 1 (Oxr1)* has emerged as a critical regulator of neuronal survival in response to OS. Oxr1 is expressed throughout the central nervous system, and its highly conserved TLDc domain protects neurons from oxidative damage through an unknown mechanism.

This thesis aimed to define mechanisms by which *Oxr1* confers neuronal sensitivity to OS, and to determine its role in neurodegenerative diseases. I found that Oxr1 mediates cytoplasmic localization of ALS-associated proteins Fused in Sarcoma (FUS) and transactive response DNA binding protein 43 kDa (TDP-43) through a TLDc domain- and arginine methylation-dependent pathway. Next, I investigated *in vivo* neuroprotective functions of Oxr1, and demonstrated that neuronal Oxr1 over-expression extends survival and ameliorates behavioural dysfunction and pathology of an ALS mouse model. In particular, neuronal Oxr1 over-expression strikingly delays neuroinflammation during ALS pathogenesis. Finally, I characterised a mouse model that specifically deletes *Oxr1* from motor neurons. While loss of *Oxr1* in ChAT-positive motor neurons does not cause overt neurodegeneration in the spinal cord, constitutive loss of *Oxr1* leads to neuroinflammation in the cerebellum and spinal cord. Taken together, these studies illuminate functions of *Oxr1* in the complex antioxidant defence network and present implications for future therapeutic strategies.

Declaration

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List of abbreviations

Abca1: ATP-Binding Cassette Sub-Family A Member 1
Abra: Muscle Activator of Rho Signalling
AD: Alzheimer's disease
ALS: amyotrophic lateral sclerosis
ApoE: Apolipoprotein E
ARE: antioxidant-response element
ARF6: ADP-ribosylation factor 6
ASK1: Apoptosis signal-regulating kinase 1
ATF3: Activating transcription factor 3
ATP2C2: ATPase, Ca^{2+} transporting, type 2C, member 2
BAC: bacterial artificial chromosome
BACE1: β-site amyloid precursor protein cleaving enzyme 1
BAG-1: BCL2-associated athanogene
Bax: Bcl-2-associated X protein
BBB: Blood brain barrier
Bcl-2: B-cell lymphoma 2
Bcl-xl: B-cell lymphoma-extra large
Bel: bella mice
BSA: bovine serum albumin
C1q: complement component 1, q subcomponent
C1qa: complement component 1, q subcomponent, α polypeptide
C1qb: complement component 1, q subcomponent, β polypeptide
C1qc: complement component 1, q subcomponent, γ polypeptide
C9ORF72: chromosome 9 open reading frame 72
C20ORF118: Chromosome 20 open reading frame 118
Ccdc25: Coiled-coil domain-containing protein 25
CCL3: chemokine (C-C motif) ligand 3
Ccnd1: Cyclin D1
Cd40: Cluster of differentiation 40
Cd52: Cluster of differentiation 52
Cd68: Cluster of differentiation 68
CD40L: CD40 ligand
ChAT: Choline acetyltransferase
ChIP: chromatin immunoprecipitation
CNS: central nervous system
CNTFR: ciliary neurotrophic factor receptor
Cox2: Cyclooxygenase-2
Cra1: Cramping 1
CSMN: corticospinal motor neurons
Ctgf: Connective tissue growth factor
Ctss: Cathepsin S
Ctsz: Cathepsin Z
Cul3: Cullin 3
DCP1A: mRNA-decapping enzyme 1A
DIG: Digoxigenin
DIV: day *in vitro*
DJ-1: parkinson protein 7
Dpysl2: Dihydropyrimidinase-related protein 2
DUSP1: Dual specificity protein phosphatase 1
DCTN1: dynactin 1
E: embryonic day
EDL: Extensor digitorum longus
eEF1A2: eukaryotic translation elongation factor 1 alpha 2
Eif4a2: eukaryotic translation initiation factor 4A2
Eif4enif1: Eukaryotic translation initiation factor 4E transporter
eIF4E: Eukaryotic translation initiation factor 4E
ELP3: elongator acetyltransferase complex subunit 3
ENU: *N*-ethyl-*N*-nitrosourea
Epb41l2: Band 4.1-like protein 2
Epb41l3: Band 4.1-like protein 3
ER: endoplasmic reticulum
ERα: estrogen receptor α

ERK: Extracellular signal-regulated kinase
ERCC-1: excision repair cross-complementing rodent repair deficiency, complementation group 1
EXOSC3: exosome component 3
fALS: familial amyotrophic lateral sclerosis
FKBP13: FK506 binding protein 13 kDa
FRT: flippase recombinase target
FTH: Ferritin heavy subunit
FTL: Ferritin light subunit
FTLD: frontotemporal lobar degeneration
FUS: fused in sarcoma
G6PD: glucose-6-phosphate dehydrogenase
Gapdh: glyceraldehyde-3-phosphate dehydrogenase
GARS: glycyl-tRNA synthetase
GATA3: GATA binding protein 3
GC: granule cell
GFAP: Glial fibrillary acidic protein
GluR1: Glutamate receptor 1
GPx1: glutathione peroxidase 1
GPx4: glutathione peroxidase 4
GSTs: Glutathione S-transferases
H&E: haematoxylin and eosin
HD: Huntington's disease
HDAC6: Histone Deacetylase 6
HexB: Hexosaminidase B
Hip: Hsc/Hsp70-interacting protein
hnRNPs: heterogeneous ribonucleoproteins
HO-1: heme-oxygenase 1
Hop: Hsp70/Hsp90 organizing protein
Hspa1b: Heat shock protein 1B
HSP27: Heat shock protein 27
HSP70: Heat shock protein 70
HSPB8: heat shock 22kDa protein 8
HtrA2: High Temperature Requirement Protein A2
Htt: huntingtin
IFNB-1b: interferon beta-1b
IGHMBP2: immunoglobulin mu binding protein 2
IP: immunoprecipitation
JNK: Jun N-terminal kinase
Keap1: Kelch-like ECH-associated protein 1
LC: liquid-chromatography
Limma: moderated t-test with a Benjamini and Hochberg multiple testing correction
Loa: legs at odd angles
LRP1B: Low-density lipoprotein receptor-related protein 1b
Lyz2: Lysozyme 2
MAP: mitogen-activated protein
MAPK: Mitogen-activated protein kinase
MHC: major histocompatibility complex
miR: microRNA
Mpeg1: Macrophage-expressing gene 1
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS: mass spectrometry
MT-I: Metallothionein-I
MT-III: Metallothionein-III
N2A: Neuro2A
NCOA7: nuclear receptor coactivator 7
NdufA10: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa
NF-κB: nuclear factor-κ B
Nkx2-5: NK2 homeobox 5
Nmd: neuromuscular degeneration
NMJ: neuromuscular junction
NOX: NADPH oxidase
NOX1: NADPH oxidase 1
NOX2: NADPH oxidase 2
NQO1: NAD(P)H dehydrogenase, quinone 1
NQO2: NAD(P)H dehydrogenase, quinone 2
Nrf2: nuclear erythroid 2-related factor 2
OS: oxidative stress
OXR1: oxidation resistance 1

Oxr1-FL: full length isoform of Oxr1
Oxr1-C: short isoform of Oxr1
P: postnatal day
PBX: pre-B-cell leukemia homeobox
PD: Parkinson's disease
PFA: paraformaldehyde
PIN1: peptidylprolyl cis/trans isomerase, NIMA-interacting 1
PINK1: PTEN induced putative kinase 1
piRNA: Piwi-interacting RNA
pmn: progressive motor neuronopathy
PPARγ: peroxisome proliferator-activated receptor gamma
PRMT1: Protein arginine N-methyltransferase 1
PRMT3: Protein arginine N-methyltransferase
Psd2: PH and SEC7 domain-containing protein 2
Psmb10: Proteasome subunit beta type-10
PTEN: Phosphatase and tensin homolog
qRT-PCR: quantitative real-time polymerase chain reaction
R26RlacZ: Rosa26 locus lacZ reporter
Rac1: Ras-related C3 botulinum toxin substrate 1
RCC: renal cell carcinoma
RNS: reactive nitrogen species
RMA: Robust Multi-array Average
ROS: reactive oxygen species
RT: room temperature
sALS: sporadic amyotrophic lateral sclerosis
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
Serpina3n: Serine (Or Cysteine) Proteinase Inhibitor, Clade A, Member 3
SHP-1: Src homology region 2 domain-containing phosphatase-1
SMN: survival motor neuron
SOD1: Cu,Zn-superoxide dismutase
SOD2: Manganese superoxide dismutase
Sprr1a: Small proline-rich protein 1A
STAT1: signal transducer and activator of transcription 1
STUB1: STIP1 homology and U-box containing protein 1
TA: tibialis anterior
TAF15: TATA-binding protein-associated factor 2N
TBC: Tre-2/Bub2/Cdc16
TBC1D24: TBC1 domain family, member 24
TBCE: tubulin folding cofactor E
TDP-43: transactive response DNA binding protein 43 kDa
TEC: tumor endothelial cell
TIA-1: T-cell-restricted intracellular antigen-1
TLDc: TBC and LysM domain containing proteins
tm1a: knockout-first conditional allele
tm1c: conditional allele
TRAP1: TNF receptor-associated protein 1
TRIM37: tripartite motif containing 37
Trx1: thioredoxin 1
Ube3a: Ubiquitin-protein ligase E3A
UBQLN2: Ubiquilin 2
UNC13A: Unc-13 Homolog A (C. Elegans)
VAPB: Vesicle-Associated Membrane Protein-Associated Protein B And C
VCP: Valosin-containing protein
Vdac1: Voltage-dependent anion-selective channel protein 1
VEGF: Vascular endothelial growth factor
VEGFR-2: vascular endothelial growth factor receptor 2
VSP54: vacuolar protein sorting 54
Vim: Vimentin
Wst: wasted
XBP-1: X-box binding protein 1
XIAP: X-linked inhibitor of apoptosis protein

1 Introduction

1.1 Oxidative Stress and Neurodegeneration

Neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and Parkinson's disease (PD), are characterised by a progressive dysfunction and death of specific neuronal populations. Without successful therapeutic strategies to prevent or reverse the course of disease, these neurological disorders are becoming an increasing public health burden, particularly as the aging population grows (Chong *et al.*, 2005). While the etiology of different neurodegenerative diseases remains unclear, research efforts have gained tremendous understanding about molecular pathways underlying the pathogenesis of these heterogeneous neurological disorders, identifying a number of common themes, including protein aggregation, oxidative stress (OS), mitochondrial dysfunction, and neuroinflammation (Bossy-Wetzel *et al.*, 2004; Jellinger, 2009).

OS is the condition characterised by an insufficient response from antioxidant defences to balance the production of reactive oxygen species (ROS). ROS are produced from normal cellular functions, such as mitochondrial respiration, proteolytic pathways, and phospholipid metabolism; however, elevated levels of ROS can lead to harmful consequences, including DNA damage, protein modification, membrane degradation, and cell death. To protect against ROS-induced damage, eukaryotic organisms have developed an elaborate network of antioxidants, from enzymatic scavengers, such as superoxide dismutase, glutathione peroxidase, and peroxyredoxins, to non-enzymatic molecules, such as glutathione, flavonoids and vitamins (Ott *et al.*, 2007; Calabrese *et al.*, 2008). Under ROS-induced stress, when cells are unable to repair the additional oxidative damage and restore redox homeostasis, apoptotic pathways become activated (Ott *et al.*, 2007).

The brain is particularly susceptible to oxidative damage because it has a high rate of metabolic activity and relatively limited antioxidant defence mechanisms when compared to other organs (Halliwell and Gutteridge, 2007). Unsurprisingly, OS has been shown to be a central component of the pathophysiology of many neurological diseases, including AD, ALS, HD, and PD (Behl, 1999; Browne *et al.*, 1999; Jenner, 2003; Lin and Beal, 2006; Barber and Shaw, 2010). Increased ROS-induced damage is observed in areas of the brain that undergo region-specific neurodegeneration in these different pathological conditions, such as the neocortex and hippocampus in AD, the substantia nigra in PD, the neocortex and spinal cord in ALS, and the basal ganglia in HD (Dexter *et al.*, 1989; Beal *et al.*, 1997; Browne *et al.*, 1997; Hensley *et al.*, 1998; Pedersen *et al.*, 1998; Butterfield *et al.*, 2002). Despite these data, whether OS is a primary causative event or a secondary consequence of neurodegenerative processes remains unclear.

Nevertheless, emerging lines of evidence suggest that OS plays an integral role in both intra- and extracellular processes governing cell death in neurological disorders (Figure 1.1) (Andersen, 2004; Fernandez-Checa *et al.*, 2010). Mutations in proteins that are central to antioxidant defence have been found to cause ALS and PD (Andersen, 2004; Fernandez-Checa *et al.*, 2010). Moreover, activation of inflammatory responses in the central nervous system and mitochondrial dysfunction in neurons can lead to additional production of ROS, while increased levels of ROS can lead to protein misfolding and aggregation, thereby perpetuating a cyclical process that ultimately leads to neuronal death (Andersen, 2004; Lin and Beal, 2006; Ott *et al.*, 2007; Winklhofer *et al.*, 2008; Fernandez-Checa *et al.*, 2010).

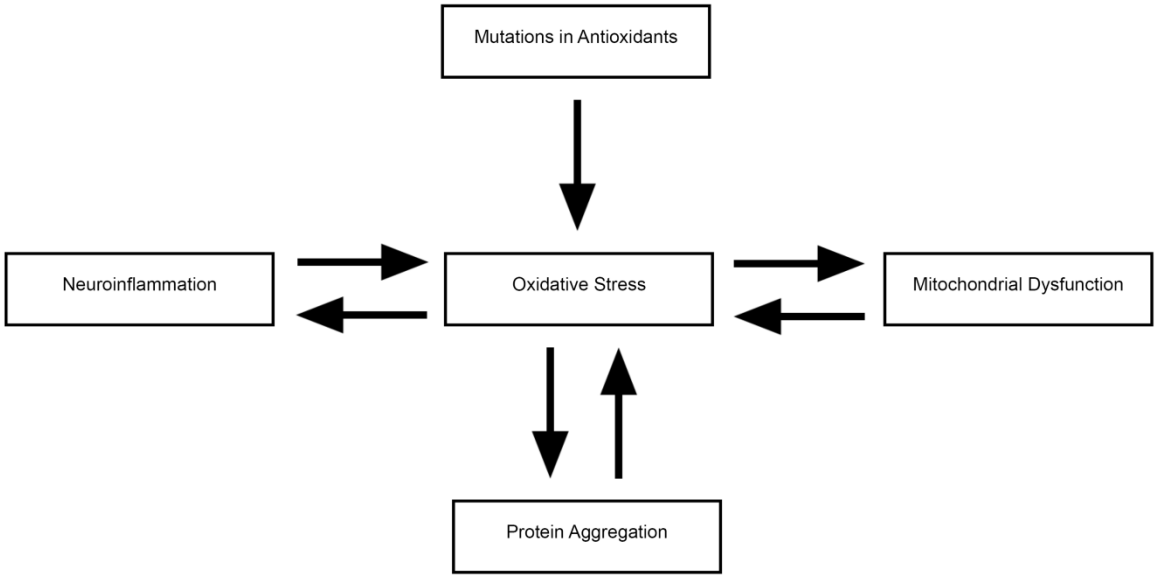

Figure 1.1. Integral role of oxidative stress in neurodegenerative diseases
Recent studies demonstrate the central role of oxidative stress in these neurological disorders. Importantly, a number of mutations in antioxidants have been discovered, which lead to enhanced generation of reactive oxygen species or renders neurons more susceptible to oxidative damage. Furthermore, mutations in proteins regulating protein folding have been found to underlie neurodegenerative diseases, and oxidative stress causes protein misfolding and aggregation, which can subsequently further dysregulate important cellular processes leading to greater oxidative stress. Oxidative injury to neurons activates the inflammatory response, but activated glial cells and astrocytes in neurodegenerative diseases cause excitotoxicity from reduced glutamate uptake and further oxidative damage from release of reactive oxygen species. Finally, oxidative stress in neurodegenerative disorders also affects mitochondrial function, and mitochondrial dysfunction has been centrally linked to oxidative stress through reduced detoxification or increased generation of reactive oxygen species. (Reviewed in Andersen, 2004 and Fernandez-Checa *et al.*, 2010). Adapted with permission from Macmillan Publishers Ltd: [Nature Medicine] Andersen JK (2004) Oxidative stress in neurodegeneration: cause or consequence? Nature Medicine 10 Suppl:S18-25, copyright (2004).

1.1.1 Mutations in antioxidants

Mutations in a number of antioxidant proteins have been found to be responsible for various neurodegenerative diseases, which indicate that inability to detoxify ROS and repair oxidative damage can lead to pathogenic outcomes. Cu,Zn-superoxide dismutase (SOD1) is an important cytoplasmic enzyme that converts superoxide radicals into oxygen and hydrogen peroxide, and mutations in *SOD1* are involved in approximately 20% of familial ALS (Fridovich, 1975; Andersen, 2004; Fernandez-Checa *et al.*, 2010). Moreover, transgenic mouse models that express various familial ALS-linked mutant SOD1 recapitulate many clinical features of ALS patients, including elevated levels of ROS and motor neuron death (Gurney *et al.*, 1994; Wong *et al.*, 1995; Hall *et al.*, 1998; Warita *et al.*, 2001; Panov *et al.*, 2011; McGoldrick *et al.*, 2013). While a number of mechanisms have been proposed, evidence increasingly suggests that mutations in SOD1 cause an unknown toxic gain-of-function that enhances the generation of free radicals, ultimately leading to motor neuron degeneration (Yim *et al.*, 1996; Bruijn *et al.*, 1998; Andersen, 2004; Barber and Shaw, 2010).

Different gain-of-function mechanisms have been proposed for mutant SOD1, though none of these proposed mechanisms sufficiently explain the etiology of ALS. Some studies have reported that mutant SOD1 has increased peroxidase activity that catalyse the reverse of the dismutase reaction, or generate hydroxyl radicals from dismutation-produced hydrogen peroxide, but another study found no difference in peroxidase activity between wild-type SOD1 and mutant SOD1 (Yim *et al.*, 1990, 1993; Wiedau-Pazos *et al.*, 1996; Singh *et al.*, 1998; Roe *et al.*, 2002; Yonashiro *et al.*, 2009; Barber and Shaw, 2010). Mutant SOD1 may lead to altered zinc binding, which can also produce superoxide in a reverse dismutase reaction, and the superoxide produced can subsequently react with nitric oxide to produce peroxynitrite (Lyons *et al.*, 1996; Crow *et al.*, 1997; Estevez *et al.*, 1999;

Roberts *et al.*, 2007; Smith and Lee, 2007; Barber and Shaw, 2010; Sahawneh *et al.*, 2010). While modulation of zinc levels affects disease progression and survival in $SOD1^{G93A}$ ALS mice, deletion of neuronal or inducible nitric oxide synthase does not alter survival of $SOD1^{G93A}$ ALS mice (Facchinetti *et al.*, 1999; Son *et al.*, 2001; Ermilova *et al.*, 2005; Barber and Shaw, 2010). Moreover, given that copper is required for increased ROS-producing activity of mutant SOD1, and enzymatically inactive SOD1 either by deletion of copper chaperone for SOD1 or mutating copper-binding sites of SOD1 (including two ALS-associated mutations H46R and H48Q), still leads to motor neuron degeneration, mutant SOD1 likely substantially contributes to OS through an important mechanism independent of its catalytic function (Subramaniam *et al.*, 2002; Wang *et al.*, 2003; Barber and Shaw, 2010). Interestingly, mutant SOD1 in microglia causes Ras-related C3 botulinum toxin substrate 1 (Rac1) to be constitutively active, leading to NADPH oxidase (NOX) 2 (NOX2) activation and enhanced microglial ROS production, which results in motor neuron damage and apoptosis (Harraz *et al.*, 2008). Finally, recent studies have found that mutant SOD1 causes down-regulation of transcription factor nuclear erythroid 2-related factor 2 (Nrf2), which under OS, translocates from the cytoplasm to the nucleus to increase expression of antioxidant defence pathway genes with an upstream antioxidant-response element (ARE) enhance sequence (Kirby *et al.*, 2005; Sarlette *et al.*, 2008).

Mutations in two antioxidant proteins, parkinson protein 7 (DJ-1) and Phosphatase and tensin homolog (PTEN) induced putative kinase 1 (PINK1), cause autosomal recessive PD, in which dopaminergic neurons of the substantia nigra degenerate (Bonifati *et al.*, 2003; Li *et al.*, 2005; Wood-Kaczmar *et al.*, 2006). DJ-1 and PINK1, both of which are localised to the mitochondria, are important regulators of cellular survival under OS, and loss of DJ-1 and PINK1 results in elevated levels of ROS production and greater neuronal apoptosis (Yokota *et al.*, 2003; Taira *et al.*, 2004; Kim *et al.*, 2005; Yang *et al.*, 2006;

Pridgeon *et al.*, 2007; Gautier *et al.*, 2008; Guzman *et al.*, 2010). DJ-1 is preferentially localised to mitochondria under OS to protect cells from ROS-induced damage; and oxidation of Cys106 of DJ-1 is necessary for relocalisation to mitochondria and its antioxidant function, possibly through the Apoptosis signal-regulating kinase 1 (ASK1)/p38/mitogen-activated protein (MAP) kinase pathway (Canet-Aviles *et al.*, 2004; Junn *et al.*, 2005; Andres-Mateos *et al.*, 2007; Blackinton *et al.*, 2009; Junn *et al.*, 2009; Waak *et al.*, 2009; Wilson, 2011). Furthermore, oxidation of Cys106 regulates binding of DJ-1 to p53, and subsequent expression of p53 target genes; in particular, oxidized DJ-1 down-regulates Dual specificity protein phosphatase 1 (DUSP1), which activates protective effects of extracellular signal-regulated kinase (ERK) (Kato *et al.*, 2013). Interestingly, DJ-1 also binds metal ions, potentially as a copper chaperone through a binding site involving Cys106, and confers protection against metal ion toxicity, which is ablated by PD-associated mutations A104T and D149A (Bjorkblom *et al.*, 2013; Girotto *et al.*, 2014). Importantly, DJ-1 stabilizes Nrf2 by preventing Nrf2 from binding with its inhibitor protein, Keap1, and subsequent ubiquitination of Nrf2 (Clements *et al.*, 2006). In addition, DJ-1 increases expression of thioredoxin 1 (Trx1), through Nrf2-activated ARE-mediated induction, and PD-associated DJ-1^{L166P} And DJ-1^{M26I} impair function of this particular pathway (Clements *et al.*, 2006; Im *et al.*, 2012). Due to PINK1's important role in mitochondrial function (Li *et al.*, 2005; Lin and Beal, 2006; Trempe and Fon, 2013), PINK1 function will be described in Section 1.1.4.

1.1.2 Protein misfolding and aggregation

Mutations in a number of ubiquitin-proteasome-related proteins have been found to cause various neurodegenerative diseases, which indicate that impairment of protein clearance may lead to aberrant protein misfolding or clearance, and subsequently, OS.

Mutations in Ubiquilin 2 (UBQLN2) and Valosin-containing protein (VCP), two proteins involved in ubiquitin-proteasome degradation, are responsible for some forms of ALS (Johnson *et al.*, 2010; Deng *et al.*, 2011). UBQLN2 also participates in protein clearance pathways, including proteasome degradation and autophagy, and ALS-associated mutations in UBQLN2 decreases UBQLN2 binding with UBXD8, an endoplasmic reticulum (ER) membrane protein that mediates translocation of ubiquitinated ER-associated protein degradation substrates, and leads to accumulation of defective proteins (Xia *et al.*, 2013). VCP, a type II member of AAA+-ATPase family, directly mediates ER-associated degradation of ubiquitinated proteins, and plays an important role in autophagy (Dai and Li, 2001; Rabinovich *et al.*, 2002; Ritson *et al.*, 2010). Furthermore, a mouse model over-expressing ALS mutant $hVCP^{R155H}$ exhibits motor neuron degeneration, accumulation of TDP-43-positive aggregates, oxidative damage, and astrogliosis, all of which are reminiscent of ALS pathology (Johnson *et al.*, 2010; Yin *et al.*, 2012). Parkin encodes an ubiquitin E3 ligase, and PD-associated mutations in parkin leads to alterations in the ubiquitin-proteasome pathway (Lin and Beal, 2006). In particular, parkin is located on the outer mitochondrial membrane, and loss of parkin leads to mitochondrial swelling, increased OS levels, cytochrome c release, and caspase activation (Darios *et al.*, 2003; Goldberg *et al.*, 2003; Palacino *et al.*, 2004). Furthermore, recent evidence suggests that parkin regulates the clearance of proteins that are involved in dopamine metabolism and damaged from dopamine oxidation (Jiang *et al.*, 2004; Jiang *et al.*, 2012).

In addition, mutations in proteins can render the proteins more prone to misfolding, particularly under OS, and these mutant proteins have also been associated with elevated OS, thereby further linking OS and protein aggregation in neurodegeneration. For example, fused in sarcoma (FUS) and transactive response DNA-binding protein 43kda (TDP-43) primarily function as regulators of gene transcription, and mRNA splicing and processing,

and mutations in FUS and TDP-43 are associated with ALS (Zinszner *et al.*, 1997; Kabashi *et al.*, 2008; Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008; Kwiatkowski *et al.*, 2009; Tan and Manley, 2009; Vance *et al.*, 2009; Lagier-Tourenne *et al.*, 2010; Hoell *et al.*, 2011; Kino *et al.*, 2011; Polymenidou *et al.*, 2011; Sephton *et al.*, 2011). Importantly, mutations in FUS and TDP-43 cause them to translocate from the nucleus and form cytosolic aggregates, particularly under OS (Arai *et al.*, 2006; Neumann *et al.*, 2006; Mackenzie *et al.*, 2007; Colombrita *et al.*, 2009; Johnson *et al.*, 2009; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Bosco *et al.*, 2010a; Deng *et al.*, 2010; Dormann *et al.*, 2010; Dewey *et al.*, 2011; Parker *et al.*, 2012). Interestingly, cytoplasmic localization of ALS mutant FUS and TDP-43 has also been linked to increased cellular toxicity and OS-damage (Johnson *et al.*, 2009; Barmada *et al.*, 2010; Kabashi *et al.*, 2010; Huang *et al.*, 2011a; Vaccaro *et al.*, 2012; Wu *et al.*, 2013; Qiu *et al.*, 2014). The mechanisms that propagate mutant FUS and TDP-43 to cytoplasmic inclusions and underlie mutant FUS and TDP-43-mediated OS damage remain unclear.

Furthermore, oxidative damage to proteins with integral cellular functions may cause increased protein misfolding, leading to disruption of important regulatory pathways or a toxic gain-of-function (Andersen, 2004; Winklhofer *et al.*, 2008). Accumulation of β-amyloid aggregates is neurotoxic and forms a central component of AD pathogenesis (Yankner, 1996). Interestingly, OS induces accumulation of insoluble β-amyloid, and β-amyloid induces cell death through elevated levels of H_2O_2 and lipid peroxidation through flavin oxidase activity, suggesting that OS may lead to aggregation of β-amyloid, which increases OS, creating a toxic cycle leading to neuronal apoptosis (Behl *et al.*, 1994; Frederikse *et al.*, 1996; Misonou *et al.*, 2000; Murray *et al.*, 2007; Tamagno *et al.*, 2012). Responsible for catalysing changes in protein conformation, peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (PIN1) is oxidized and down-regulated in the AD

hippocampus (Sultana *et al.*, 2006). Interestingly, oxidative modification of PIN1 inhibits its protein activity, and loss of PIN1 function leads to abnormal protein folding of amyloid precursor protein and tau, resulting in elevated levels of toxic insoluble β-amyloid peptides and hyperphosphorylation of tau, both of which contribute to AD neurodegeneration (Liou *et al.*, 2003; Pastorino *et al.*, 2006; Sultana *et al.*, 2006).

In a similar fashion, ROS-induced damage to parkin has been proposed to also contribute to neurodegeneration in sporadic PD (Winklhofer *et al.*, 2008). Under OS, misfolding and aggregation of wild-type parkin increase, leading to inactivation of parkin and failure of parkin to activate proper pro-survival pathways (Winklhofer *et al.*, 2003; Wang *et al.*, 2005; Winklhofer *et al.*, 2008). In particular, oxidative modification S-nitrosylation of parkin reduces its ubiquitination activity and protective function (Chung *et al.*, 2004; Yao *et al.*, 2004). These findings are further corroborated by detection of insoluble aggregates of parkin that have been modified by oxidative products in the substantia nigra of sporadic PD patients (LaVoie *et al.*, 2005). Oxidative insults also induce aggregation of α-synuclein, a protein mutated in autosomal dominant PD, and over-expression of α-synuclein leads to aggregate formation and elevated ROS levels, further suggesting that misfolding of proteins and OS are inextricably linked (Hashimoto *et al.*, 1999; Hsu *et al.*, 2000; Paxinou *et al.*, 2001; Takahashi *et al.*, 2007).

1.1.3 Neuroinflammatory response

Activation of microglia and astrocytes in response to central nervous system (CNS) injury or neurodegeneration may lead to additional production of ROS or other cytotoxic agents, which ultimately cause neuronal death (Andersen, 2004; Vijitruth *et al.*, 2006). For example, it has been hypothesized that damaged neurons in neurodegenerative diseases release ROS into the extracellular space, which induces ROS-mediated damage and

reduces glutamate uptake in neighbouring astrocytes; this in turn generates a cyclical process through which increased extracellular glutamate levels cause excitotoxicity, further promoting neuronal degeneration (Rao *et al.*, 2003). Furthermore, ROS from damaged neurons activate glial cells, producing additional ROS, reactive nitrogen species (RNS), and proinflammatory trophic factors, which also promote a cyclical process through which more glia cells are activated, and these toxic factors continue injuring neurons (Banati *et al.*, 1993; Koutsilieri *et al.*, 2002; Zhao *et al.*, 2004b).

Growing evidence demonstrates that activated microglia and astrocytes substantially contribute to ALS motor neuron degeneration (Ilieva *et al.*, 2009; Ferraiuolo *et al.*, 2011; Bowerman *et al.*, 2013). Interestingly, loss of ALS-associated mutant SOD1 in either astrocytes or microglia significantly delays disease progression (Boillee *et al.*, 2006; Yamanaka *et al.*, 2008). Furthermore, a recent study demonstrated that transplantation of $SOD1^{G93A}$ glial progenitors into spinal cord of wild-type rats causes astrogliosis and microgliosis, aberrant motor function, and eventually, motor neuron degeneration (Papadeas *et al.*, 2011). The mechanism of astrocyte-mediated motor neuron degeneration remains unknown, but microglia-mediated motor neuron apoptosis depends on constitutive activation of nuclear factor-κ B (NF-κB) during ALS pathogenesis (Frakes *et al.*, 2014). In addition, co-cultures of primary mutant SOD1 astrocytes or glia with primary motor neurons, or embryonic mouse and human stem-cell-derived motor neurons decrease motor neuron survival due to astrocyte-derived trophic factors, such as NOX2-associated OS and Prostaglandin D2 (Di Giorgio *et al.*, 2007; Nagai *et al.*, 2007; Di Giorgio *et al.*, 2008; Marchetto *et al.*, 2008). Mutant SOD1 in microglia also constitutively activates Rac1, which prolongs NOX2 production of ROS, while deletion of *NOX2* extends survival of mutant SOD1 mice (Wu *et al.*, 2006; Marden *et al.*, 2007; Harraz *et al.*, 2008).

Neuroinflammation is a central feature of PD pathogenesis in patients and in mouse models (McGeer *et al.*, 1988; Czlonkowska *et al.*, 1996; Kohutnicka *et al.*, 1998; Gao *et al.*, 2008; Hirsch and Hunot, 2009; Tansey and Goldberg, 2010; Akundi *et al.*, 2011). The presence of activated astrocytes render dopaminergic neurons, neurons that degenerate in PD, significantly more susceptible to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity, which functions through blocking mitochondrial complex I function (McNaught and Jenner, 1999). Conversely, inhibition of microglial production of NOX2 protects dopaminergic neurons in a PD mouse model (Wu *et al.*, 2003). Furthermore, recent research demonstrated that loss of DJ-1 in microglia renders cells more sensitivity to dopamine, resulting in increased pro-inflammatory cytokines, ROS, and nitric oxide, which reduce survival of dopaminergic neurons *in vitro* (Trudler *et al.*, 2013). Furthermore, DJ-1 directly modifies the interaction between signal transducer and activator of transcription 1 (STAT1) and its phosphatase, Src homology region 2 domain-containing phosphatase-1 (SHP-1), and reduces activation of STAT1, and loss of DJ-1 in astrocytes and microglia increase inflammatory response and phosphorylation of STAT1 (Kim *et al.*, 2013). In addition, aggregation of α-synuclein induces activation of microglia *in vitro*, which further increases α-synuclein inclusion-induced dopaminergic neuron apoptosis in neuron-glia co-cultures in a manner dependent on phagocytosis and NOX-mediated ROS (Zhang *et al.*, 2005).

Studies have also identified neuroinflammation in AD and HD patients, and mouse models of AD and HD (McGeer *et al.*, 1988; Griffin *et al.*, 1989; Singhrao *et al.*, 1999; Sapp *et al.*, 2001; Maeda *et al.*, 2007; Simmons *et al.*, 2007; Moller, 2010; Rozemuller *et al.*, 2012; Ghosh *et al.*, 2013). β-amyloid peptides, major components of amyloid plaques that are widespread in regions of neurodegeneration in AD patients, activate microglia, and subsequently activated microglia produce ROS and RNS, which cause DNA damage and

neuronal death (Goodwin *et al.*, 1995; Meda *et al.*, 1995; Ii *et al.*, 1996; Rossi and Bianchini, 1996; Akama *et al.*, 1998; Hu *et al.*, 1998; Coraci *et al.*, 2002; Qin *et al.*, 2002; Xie *et al.*, 2002). The mechanisms by which β-amyloid peptides induce microglial production of ROS or RNS are complex, though increasing evidence suggests that NOX plays a central role (McDonald *et al.*, 1997; Bianca *et al.*, 1999; Shimohama *et al.*, 2000; Park *et al.*, 2005; Wilkinson and Landreth, 2006; Milton *et al.*, 2008). Moreover, mutant huntingtin (htt), the genetic cause of HD, is expressed in microglia and forms aggregates, and mutant htt-expressing microglia have accumulation of ferritin, evidence of OS (Simmons *et al.*, 2007). A recent study found that mutant htt in microglia promotes pro-inflammatory response by increasing the expression and transcriptional activity of PU.1 and C/EBPs, two transcription factors important for myeloid fate determination (Crotti *et al.*, 2014). Interestingly, mutant htt-expression in microglia induces neuronal death in glial and neuronal co-cultures, as well as *in vivo* under lipopolysaccharide-induced sterile inflammation (Crotti *et al.*, 2014).

1.1.4 Mitochondrial dysfunction

Because mitochondria are responsible for producing the majority of ROS, mitochondrial dysfunction significantly contributes to oxidative damage and subsequent neuronal death in neurodegenerative disease (Lin and Beal, 2006; Ott *et al.*, 2007; Fernandez-Checa *et al.*, 2010). Many mutations that cause neurodegenerative diseases are found in proteins that localise to mitochondria. In particular, mutations in DJ-1, parkin, and PINK1 are associated with autosomal recessive PD, and modulate mitochondrial function potentially through a common pathway (Bonifati *et al.*, 2003; Li *et al.*, 2005; Lin and Beal, 2006; Xiong *et al.*, 2009; Trempe and Fon, 2013). DJ-1 and parkin contributions to PD have been discussed earlier, but PINK1 is a mitochondrial kinase protein central to

mitochondrial function. Genetic deletion of *Pink1 in vivo* leads to increased dopaminergic neuron apoptosis after treatment with MPTP (Przedborski *et al.*, 2004; Haque *et al.*, 2012). Loss of PINK1 function causes dysregulation of mitochondrial calcium homeostasis, resulting in calcium-induced decrease of mitochondrial membrane potential, impaired respiration, and increased OS (Gandhi *et al.*, 2009). Furthermore, mutant PINK1 inhibits phosphorylation and reduces activity of High Temperature Requirement Protein A2 (HtrA2), an important protease, and loss of HtrA2 causes an accumulation of unfolded proteins in mitochondria, elevated levels of ROS, and ultimately, neurodegeneration (Plun-Favreau *et al.*, 2007; Moisoi *et al.*, 2009; Tain *et al.*, 2009). In addition, recent studies have identified two proteins important for mitochondrial function as phosphorylation substrates of PINK1: TNF receptor-associated protein 1 (TRAP1) and Miro (Pridgeon *et al.*, 2007; Wang *et al.*, 2011c; Liu *et al.*, 2012). TRAP1 function is not well-understood, but it actss as a mitochondrial molecular chaperone, and PINK1 protects against OS-induced apoptosis through TRAP1-dependent suppression of cytochrome c release from mitochondria (Pridgeon *et al.*, 2007). Interestingly, over-expression of TRAP1 rescues PINK1-associated mitochondrial pathology and dysfunction in *Drosophila* (Costa *et al.*, 2013; Zhang *et al.*, 2013). Miro participates in the motor/adaptor complex that regulates axonal transport of mitochondria, and PINK1 phosphorylates Miro, and promotes degradation of Miro through a Parkin-dependent pathway, which is important for clearance of damaged mitochondria (Wang *et al.*, 2011c; Liu *et al.*, 2012). Finally, a recent study showed that PD mutations in PINK1 leads to loss of mitochondrial complex I activity, and subsequent decrease in mitochondrial membrane potential and ubiquinone reducing capacity through loss of NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa (NdufA10) phosphorylation (Morais *et al.*, 2009; Morais *et al.*, 2014).

In addition, mutations in proteins responsible for neurodegenerative diseases can also cause aberrant localisation or interactions, resulting in mitochondrial dysfunction. For example, ALS mutations in SOD1 cause abnormal localisation of SOD1 in the outer membrane and inside the intermembrane space of mitochondria (Vijayvergiya *et al.*, 2005; Ferri *et al.*, 2006; Kawamata and Manfredi, 2008; Vande Velde *et al.*, 2008; Israelson *et al.*, 2010). Interestingly, accumulation of mutant SOD1 in the mitochondrial intermembrane space increases generation of ROS and cytochrome c-catalysed oxidation, and is sufficient to cause motor neuron degeneration and decreased cytochrome oxidase activity *in vivo* (Goldsteins *et al.*, 2008; Igoudjil *et al.*, 2011). Similarly, mutant htt interacts with transcription factor p53 and the mitochondrial membrane to disrupt mitochondrial function, causing reduced Ca^{2+} retention capacity, dissipation of membrane potential, and increased levels of ROS production when mitochondria are subject to stress insult (Panov *et al.*, 2002; Bae *et al.*, 2005; Lim *et al.*, 2008). Furthermore, polyglutamine expansions, which are pathogenic in mutant htt, inhibit ADP-stimulated respiration (State III respiration) through increased production of ROS in mitochondria, and this specific impairment is rescued by treatment with antioxidant N-acetyl-l-cysteine or cytochrome c (Puranam *et al.*, 2006). Taken together, growing evidence suggests that OS, while not always the primary causative event, plays an important role in promoting cellular dysregulation that result in widespread neurodegeneration observed in various disorders.

1.2 Molecular Controls of Antioxidant Defence Networks

The exact mechanisms underlying pathogenic processes caused by oxidative damage are still not well understood, but tremendous progress has been made in understanding molecular controls of antioxidant defence pathways, many of which have also been implicated in neurological disorders. Furthermore, over-expression of different

proteins central to antioxidant defence has been found to ameliorate pathology and extend lifespan of many neurodegenerative disease models.

1.2.1 Antioxidant enzymes

A number of antioxidant enzymes counter ROS-induced damage by catalysing reactions that convert ROS into less harmful products. Located in the mitochondria, superoxide dismutase is responsible for converting superoxide into hydrogen peroxide and oxygen (Fridovich, 1975). As mentioned earlier, mutations in SOD1 are responsible for cases of familial ALS, and altered function in mutant SOD1 results in abnormal generation of ROS (Yim *et al.*, 1996; Bruijn *et al.*, 1998; Yonashiro *et al.*, 2009; Panov *et al.*, 2011). Loss of SOD2 function in mice causes early postnatal lethality, neurodegeneration, and is responsible for elevated levels of oxidative damage, while over-expression of SOD2 improves memory deficits in an AD mice model (Li *et al.*, 1995; Lebovitz *et al.*, 1996; Williams *et al.*, 1998; Melov *et al.*, 1999; Lynn *et al.*, 2005; Massaad *et al.*, 2009). Two other important antioxidant enzymes glutathione peroxidase (Gpx) and peroxiredoxin (Prdx) subsequently reduce hydrogen peroxide by-products from SOD dismutation of superoxide to water (Ott *et al.*, 2007). Loss of GPx1 *in vivo* renders neuronal populations in selective regions of the brain more susceptible to OS-induced apoptosis (Zhang *et al.*, 2000; Boonplueang *et al.*, 2005). *Glutathione peroxidase 4* ($GPx4)^{-/-}$ mice die *in utero* at embryonic day 7.5 (E7.5), and $GPx4^{+/-}$ mice and cell lines heterozygous for *GPx4* are more sensitive to OS-induced apoptosis; however, over-expression of *GPx4* confers cellular protection against ROS-related damage (Yant *et al.*, 2003; Ran *et al.*, 2004). Interestingly, down-regulation of *GPx4* has also been detected in ALS models, including pre-symptomatic $SOD1^{G93A}$ ALS mice (Kirby *et al.*, 2005; Guipponi *et al.*, 2010). Similarly, *Prdx3* null mice show increased susceptibility to oxidative insult, while *Prdx3*

over-expression in hippocampal neurons is neuroprotective (Hattori *et al.*, 2003; Li *et al.*, 2007). Furthermore, PRDX3 expression is significantly reduced in brain tissue of AD patients (Kim *et al.*, 2001). Finally, catalase is another antioxidant enzyme that degrades the ROS intermediate H_2O_2. Interestingly, activity of catalase has been found to be reduced in brains of AD patients (Gsell *et al.*, 1995; Marcus *et al.*, 1998). Moreover, β-amyloid interacts with catalase and inhibits catalase activity, and inhibiting this interaction *in vitro* rescues β-amyloid-induced ROS generation and cell toxicity (Milton, 1999; Inbar *et al.*, 2006; Habib *et al.*, 2010). Over-expression of mitochondria-targeted catalase in an AD mouse model extends life span and ameliorates many phenotypes, including abnormal processing of amyloid precursor protein, β-amyloid production, aberrant β-site amyloid precursor protein cleaving enzyme 1 (BACE1) enzymatic activity, oxidative damage, and cognitive behaviour deficits (Mao *et al.*, 2012).

1.2.2 Transcriptional controls over antioxidant defence

In response to increasing levels of ROS, cells also depend on a number of transcription factors to up-regulate expression of different members of the antioxidant defence network. Nrf2 is a well-studied transcription factor that binds to the ARE in the promoter region and induces expression of diverse target genes under OS (Venugopal and Jaiswal, 1998; Moinova and Mulcahy, 1999; Kensler *et al.*, 2007; Baird and Dinkova-Kostova, 2011; Ma, 2013; Shelton and Jaiswal, 2013). Nrf2 target genes encode antioxidant proteins, such as NAD(P)H dehydrogenase, quinone 1 (NQO1) and NAD(P)H dehydrogenase, quinone 2 (NQO2); detoxifying proteins, such as Glutathione S-transferases (GSTs); metabolic proteins, such as glucose-6-phosphate dehydrogenase (G6PD) and peroxisome proliferator-activated receptor γ (PPARγ); and antiapoptotic proteins, such as B-cell lymphoma 2 (Bcl-2) (Itoh *et al.*, 1997; Chanas *et al.*, 2002;

Thimmulappa *et al.*, 2002; Nioi *et al.*, 2003; Cho *et al.*, 2005; Wang and Jaiswal, 2006; Kensler *et al.*, 2007; Baird and Dinkova-Kostova, 2011; Niture and Jaiswal, 2012; Ma, 2013; Shelton and Jaiswal, 2013). The mechanisms regulating Nrf2 function are still not well understood, but Kelch-like ECH-associated protein 1 (Keap1) plays an important role by modulating nuclear localisation of Nrf2 (Itoh *et al.*, 1999; Kensler *et al.*, 2007; Baird and Dinkova-Kostova, 2011; Ma, 2013; Shelton and Jaiswal, 2013). In particular, Keap1 targets Nrf2 for degradation by binding to Cullin 3 (Cul3), an E3 ubiquitin ligase, and chemopreventive agents and OS react with cysteines in Keap1, releasing Nrf2 to be transported into the nucleus to activate ARE-associated genes (Zhang and Hannink, 2003; Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004; Wakabayashi *et al.*, 2004; Zhang *et al.*, 2004; Furukawa and Xiong, 2005). Over-expression of Nrf2, particularly in astrocytes, has beneficial effects on a number of *in vitro* and *in vivo* models of neurodegenerative diseases (Calkins *et al.*, 2005; Shih *et al.*, 2005; Vargas *et al.*, 2006; Jakel *et al.*, 2007; Vargas *et al.*, 2008; Chen *et al.*, 2009; Kanninen *et al.*, 2009; Yang *et al.*, 2009; Calkins *et al.*, 2010; Stack *et al.*, 2010; Barone *et al.*, 2011; Neymotin *et al.*, 2011).

NF-κB is a nuclear factor that is sensitive to ROS and plays a central role in cell survival by controlling transcription of many downstream targets (Sen and Packer, 1996; Piva *et al.*, 2006; Qin *et al.*, 2007; Morgan and Liu, 2011). NF-κB is composed of several DNA-binding family members, which form homodimers or heterodimers with the predominant form being the p50-RelA heterodimer (Sen and Packer, 1996; Harhaj and Sun, 1999; Piva *et al.*, 2006; Qin *et al.*, 2007). Normally, NF-κB is sequestered in the cytoplasm by inhibitory IκB family proteins, such as IκB-α and IκB-β (Henkel *et al.*, 1993; Sen and Packer, 1996; Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Piva *et al.*, 2006; Qin *et al.*, 2007; Morgan and Liu, 2011). In general, stimuli ranging from viral and bacterial infection to stress-inducing agents activate the NF-κB complex by phosphorylating IκB and

targeting IκB for degradation; however, the mechanisms by which OS activates NF-κB are still not well-understood (Henkel *et al.*, 1993; Sen and Packer, 1996; Piva *et al.*, 2006; Qin *et al.*, 2007; Morgan and Liu, 2011). ROS can activate NF-κB by inducing phosphorylation of RelA and promoting RelA binding with DNA, or by phosphorylation of IκBα at alternative tyrosine residues, which may unmask NF-κB without degradation of IκBα (Schieven *et al.*, 1993; Zhong *et al.*, 1998; Beraud *et al.*, 1999; Canty *et al.*, 1999; Schoonbroodt *et al.*, 2000; Zhong *et al.*, 2002; Takada *et al.*, 2003; Jamaluddin *et al.*, 2007; Nowak *et al.*, 2008; Gloire and Piette, 2009; Morgan and Liu, 2011). After translocating to the nucleus, NF-κB binds to κB DNA-binding motifs and induces expression of immunity-related genes, such as *Cyclooxygenase-2* (*Cox2*), cell proliferation genes, such as *Cyclin D1* (*Ccnd1*), and cell survival genes, such as *B-cell lymphoma-extra large (Bcl-xl)* (Guttridge *et al.*, 1999; Chen *et al.*, 2000; Kaltschmidt *et al.*, 2002; Piva *et al.*, 2006; Qin *et al.*, 2007). Interestingly, activation of NFκB is linked to many human diseases, including neurological disorders, and inhibition of NF-κB has been proposed as a neuroprotective therapy (Piva *et al.*, 2006; Qin *et al.*, 2007; Flood *et al.*, 2011). NF-κB inhibitors reduce β–amyloid production *in vitro*, and selective inhibition of NF-κB protects dopaminergic neurons from MPTP-induced toxicity in the substantia nigra (Ghosh *et al.*, 2007; Paris *et al.*, 2007). While inhibition of NF-κB in astrocytes does not affect ALS pathogenesis *in vivo*, inhibition of NF-κB in glia delays disease progression and prolongs survival by 15% in $SOD1^{G93A}$ ALS mice (Crosio *et al.*, 2011; Frakes *et al.*, 2014).

1.2.3 Non-enzymatic antioxidants

In addition, recent work has identified additional molecular controls that confer protection against OS, but do not act as antioxidant enzymes. As described previously, mutations in DJ-1 and PINK1 are responsible for a subset of autosomal recessive PD, and

DJ-1 and PINK1 are important regulators of cellular sensitivity to OS. Interestingly, studies have shown that over-expression of DJ-1 and PINK1 has neuroprotective effects. Increased expression of DJ-1 significantly reduces protein aggregation of mutant A53T α-synuclein, a protein associated with autosomal dominant PD, and mutant α-synuclein-mediated apoptosis (Zhou and Freed, 2005). DJ-1 interacts with mutant SOD1, and up-regulation of DJ-1 decreases mutant SOD1-mediated oxidative damage and apoptosis (Yamashita *et al.*, 2010). Similarly, DJ-1 interacts with mutant htt, and over-expression of DJ-1 protects against mutant htt aggregation and toxicity in yeast, and decreases neurodegeneration in a Drosophila model of HD (Sajjad *et al.*, 2014). Over-expression of PINK1 rescues α-synuclein-associated PD phenotypes *in vitro* and *in vivo* (Todd and Staveley, 2008; Kamp *et al.*, 2010).

Another non-enzymatic antioxidant, neuroglobin has high binding affinity for oxygen and is highly expressed in neurons throughout the CNS (Burmester *et al.*, 2000; Reuss *et al.*, 2002; Burmester and Hankeln, 2009). Overexpression of neuroglobin confers protection against OS *in vitro* and *in vivo* though the mechanism of neuroglobin function is still unclear (Fordel *et al.*, 2006; Burmester and Hankeln, 2009; Liu *et al.*, 2009; Antao *et al.*, 2010; Li *et al.*, 2010; Singh *et al.*, 2013). Neuroglobin acts as a sensor of OS with a ferrous oxygen-bound form under normal conditions and becomes converted to a ferric bis-His conformation under OS, a conformational change required for neuroglobin function (Watanabe *et al.*, 2012). In addition, neuroglobin interacts with redox protein cytochrome c to maintain cytochrome c in its reduced state, and prevents cytochrome c release from mitochondria *in vitro* (Fago *et al.*, 2006; Raychaudhuri *et al.*, 2010; Hota *et al.*, 2012; De Marinis *et al.*, 2013). Neuroglobin can also function as a nitrite reductase under OS, converting nitrite into NO, which subsequently binds to cytochrome c oxidase, and the nitrite reductase function of neuroglobin is modulated by 14-3-3 binding and

phosphorylation (Jayaraman *et al.*, 2011; Tiso *et al.*, 2011). Interestingly, hippocampal expression of neuroglobin is up-regulated in early and moderately advanced AD patients, and down-regulated in late-stage AD patients (Sun *et al.*, 2013). Moreover, over-expression of neuroglobin reduces levels of insoluble amyloid plaques and reverses spatial learning deficits in a transgenic AD mice model, and attenuates tau hyperphosphorylation through an Akt-dependent pathway *in vivo* (Khan *et al.*, 2007; Chen *et al.*, 2012b).

1.2.4 Chaperone proteins with neuroprotective properties

Protein misfolding and aggregation are linked to both neurodegeneration and OS as described earlier (Meriin and Sherman, 2005), thus unsurprisingly, chaperone proteins, which are involved in regulation of protein folding, can also be activated by OS and possess neuroprotective properties (Muchowski and Wacker, 2005). For example, misfolding of proteins caused by OS induces expression of Heat Shock Protein 70 (HSP70), which has neuroprotective properties and acts as a major mitochondrial chaperone by regulating transportation and folding of precursor proteins (Kang *et al.*, 1990; McDuffee *et al.*, 1997; Russo *et al.*, 2001; Winklhofer *et al.*, 2003). HSP70 functions through an ATP-controlled manner by interacting with hydrophobic regions of proteins, and with different co-chaperones, such as DnaJ proteins, BCL2-associated athanogene (BAG-1), Hsc/Hsp70-interacting protein (Hip), Hsp70/Hsp90 organizing protein (Hop), and STIP1 homology and U-box containing protein 1 (STUB1), to regulate particular target proteins of interest (Cyr *et al.*, 1994; Hohfeld *et al.*, 1995; Takayama *et al.*, 1997; Chen and Smith, 1998; Ballinger *et al.*, 1999; Laufen *et al.*, 1999; McDonough and Patterson, 2003; Mayer and Bukau, 2005). Interestingly, over-expression of HSP70 reduces abnormal cytoplasmic or nuclear aggregation caused by protein misfolding and subsequent cell death in many models of neurodegenerative disorders (Bruening *et al.*,

1999; Warrick *et al.*, 1999; Cummings *et al.*, 2001; Auluck *et al.*, 2002; Winklhofer *et al.*, 2003; Dong *et al.*, 2005; McLear *et al.*, 2008; Nagel *et al.*, 2008; Hoshino *et al.*, 2011). Unfortunately, up-regulation of HSP70 does not affect the life-span or disease progression in ALS mouse models, and recruitment of HSP70 in mutant SOD1-positive aggregates suggests that HSP70 function is dysregulated in ALS and over-expression of HSP70 is unable to overcome this dysfunction (Liu *et al.*, 2005).

Another heat shock protein, Heat Shock Protein 27 (HSP27), is activated by phosphorylation through the p38/mitogen-activated protein (MAP) kinase and MAP kinase-activated protein kinase-2/3 pathway under elevated ROS, and provides cellular protection against oxidative damage (Huot *et al.*, 1995; Mehlen *et al.*, 1995; Huot *et al.*, 1996; Rogalla *et al.*, 1999; Merendino *et al.*, 2002; Liao *et al.*, 2008). Interestingly, Hsp27 appears to confer cellular sensitivity to OS by regulating glutathione levels and maintaining glutathione in its reduced form (Mehlen *et al.*, 1995; Preville *et al.*, 1999; Baek *et al.*, 2000; Paul and Arrigo, 2000; Arrigo, 2001). However, the role of HSP27 in neurodegenerative disease remains unclear. Although HSP27 reduces aggregation of misfolded proteins and protects against neurodegeneration, up-regulation of HSP27 has had mixed results in alleviating ROS-induced damage in different neurodegenerative disease models (Wyttenbach *et al.*, 2002; Ito *et al.*, 2003; Krishnan *et al.*, 2006; Perrin *et al.*, 2007; Zourlidou *et al.*, 2007; Liao *et al.*, 2008; Abisambra *et al.*, 2010; Toth *et al.*, 2013). Impressively, HSP27 modulates AD disease progression and symptoms in mouse models, including improving spatial learning, electrophysiological abnormalities in neurons, neuronal plasticity deficits, and β-amyloid accumulation (Abisambra *et al.*, 2010; Toth *et al.*, 2013).

1.2.5 Protein regulators of apoptosis

Since OS causes neuronal death during pathogenesis of neurodegenerative disease, recent studies have also shed light on apoptotic pathways activated by oxidative damage (Ott *et al.*, 2007). p53, an important transcription factor in cell cycle regulation, plays an important role in one such OS-induced apoptotic pathway (Ott *et al.*, 2007). In neurons, ROS-related DNA damage activates p53 and subsequently, p53 activity can lead to expression of downstream proteins in apoptotic machinery, including Bcl-2 family proteins, such as Bcl-2-associated X protein (Bax) (Renzing *et al.*, 1996; Morrison *et al.*, 2003; Ott *et al.*, 2007). Translocation of Bax from the cytosol to the mitochondria is a critical step in the p53-activated cell death pathway, and inhibiting Bax translocation can prevent apoptosis (Wolter *et al.*, 1997; Deng and Wu, 2000; Ott *et al.*, 2007). Oligomeric Bax forms a component of the mitochondrial apoptosis-induced channel, which controls the release of pro-apoptotic factors, such as cytochrome c, into the cytosol (Liu *et al.*, 1996b; Guo *et al.*, 2004; Dejean *et al.*, 2005; Ott *et al.*, 2007; Li *et al.*, 2008). The release of cytochrome c from mitochondria is essential for activation of the caspase cascade, leading to apoptosis (Li *et al.*, 1997; Ott *et al.*, 2007).

Furthermore, proteins involved in the apoptotic pathways have been associated with disease pathogenesis of various neurodegenerative disorders. Interestingly, mutant htt interacts with p53, up-regulates nuclear p53, and increases p53 transcriptional activity (Bae *et al.*, 2005). Deletion of p53 rescues mitochondrial membrane depolarization and cytotoxicity in a HD cell line, and ameliorates respiratory complex IV activity and behavioural abnormalities in a HD mouse model (Bae *et al.*, 2005). The PD-associated L166P mutation in DJ-1 results in DJ-1 localisation in mitochondria and stronger interaction with Bcl-xl, leading to dissociation of Bax from Bcl-xl, and accumulation of Bax on the outer mitochondrial membrane, which ultimately activates the Bax-mediated

apoptosis pathway (Ren *et al.*, 2012). Similarly, ALS-associated mutations in SOD1 cause mutant SOD1-binding to anti-apoptotic protein Bcl-2 in spinal cord mitochondria, thus altering Bcl-2 conformation, and through exposure of the toxic BH3 domain of Bcl-2, inducing mitochondrial dysfunction and releasing of cytochrome c from mitochondria (Pasinelli *et al.*, 2004; Pedrini *et al.*, 2010). While intraspinal injection of adeno-associated virus encoding Bcl2 is unable to extend survival of $SOD1^{G93A}$ ALS mice, gene transfer of *Bcl2* improves motor neuron survival and neuromuscular function (Azzouz *et al.*, 2000). Moreover, small peptides that specifically inhibit mutant SOD1 interaction with Bcl-2 ameliorate mitochondrial dysfunction and increase cell survival *in vitro* (Tan *et al.*, 2013). Deletion of *Bax* rescues motor neuron apoptosis, ameliorates motor dysfunction, and extends lifespan in $SOD1^{G93A}$ ALS mice (Gould *et al.*, 2006). While many molecular controls of various functions within antioxidant defence networks have been identified, mechanisms by which they confer protection against OS both alone and in concert with other important regulatory proteins are still under rigorous investigation.

1.3 Oxr1 is an important regulator of neuronal sensitivity to oxidative stress

Identified in a screen for human genes with oxidative protective properties, *Oxidation Resistance 1* (*OXR1*) is found in all eukaryote genomes (Volkert *et al.*, 2000; Shkolnik *et al.*, 2008). Induced by OS, Oxr1 is localised to the mitochondria under oxidative stress conditions, and to the cytoplasm after extended stress conditions (Elliott and Volkert, 2004; Finelli, 2010; Oliver *et al.*, 2011; Murphy and Volkert, 2012). OXR1 reduces spontaneous DNA mutations in *Escherichia coli* oxidative repair-defective mutant strains, and exon 8 is necessary for this particular oxidative antimutator function (Volkert *et al.*, 2000; Murphy and Volkert, 2012). Furthermore, *Saccharomyces cerevisiae* mutants that lack sc*OXR1* demonstrate increased sensitivity to OS, a phenotype that is rescued by

expression of mitochondria-targeted human OXR1 protein (Volkert *et al.*, 2000; Elliott and Volkert, 2004). Over-expression of Oxr1 also protects against OS-damage induced by 4-hydroxynonenal in a retinal cell line (Murray *et al.*, 2013). Interestingly, Oxr1 appears to participate in antioxidant pathways, acting downstream of Jun N-terminal kinase (JNK), an important protein in the stress-induced JNK signalling cascade, and upstream of catalase and glutathione peroxidase in the mosquito *Anopheles gambiae* (Jaramillo-Gutierrez *et al.*, 2010). Finally, the Drosophila homolog, *mustard*, has been shown to regulate the immune response through the Drosophila-specific immune deficiency pathway (Wang *et al.*, 2012).

Recent work in the Davies laboratory on a recessive *N*-ethyl-*N*-nitrosourea (ENU) mutant, named bella (*bel)* mouse, has uncovered a novel and centrally important role for Oxr1 in protecting neurons against OS-induced apoptosis (Oliver *et al.*, 2011). Homozygous *bel* mice develop a severe ataxic gait and display growth retardation after postnatal day 14 (P14), and fail to survive beyond P26, experiencing significant oxidative damage and neuronal death in the granule cell (GC) layer of the cerebellum (Oliver *et al.*, 2011). The *bel* mutation contains a 190kb deletion, which ablates expression of *Oxr1* and *Muscle Activator of Rho Signalling* (*Abra*); however, only *Oxr1* is expressed within the CNS. Furthermore, genetic rescue experiments showed that an *Oxr1* transgene alleviates the *bel* phenotype for at least 8 months, demonstrating that deletion of *Oxr1* alone is responsible for the neuropathology observed in *bel* mutants (Oliver *et al.*, 2011).

In vitro studies have further corroborated the role of *Oxr1* in conferring neuronal sensitivity to OS (Oliver *et al.*, 2011). *Bel* mutant primary GCs that are exposed to OS experience significantly more cell death than wild-type primary GCs, a phenotype that is rescued by over-expression of Oxr1 and recapitulated by knock-down of *Oxr1*. Moreover, over-expression of Oxr1 in primary GC cultures significantly decreases exogenous peroxide-induced apoptosis, suggesting that Oxr1 has neuroprotective properties against

OS (Oliver *et al.*, 2011). Interestingly, the short Oxr1 (Oxr1-C) isoform, which predominantly consists of the highly conserved C-terminal TBC (Tre-2/Bub2/Cdc16) and LysM domain containing proteins (TLDc) domain, is not only fully functional in reducing susceptibility of wild-type and *bel* GC cultures to OS-induced cell death, but also successfully rescues the *bel* phenotype *in vivo* (Figure 1.2) (Oliver *et al.*, 2011). Furthermore, biochemical experiments suggested that while Oxr1 does not possess antioxidant catalytic activity, Oxr1 directly reacts with H_2O_2 through oxidation of reactive cysteine residues in the TLDc domain (Oliver *et al.*, 2011). Thus, these results suggest that Oxr1 may be sensitive to cellular ROS levels similar to DJ-1, and oxidation of Oxr1 in the presence of OS may play a significant regulatory role within the cell for proper function of antioxidant pathways to counteract elevated ROS levels (Oliver *et al.*, 2011; Wilson, 2011).

Oxr1 contains a Lysin motif (LysM), a Glucosyltransferases, Rab-like GTPase activators and Myotubularins (GRAM) domain, and a highly conserved C-terminal TLDc domain (Figure 1.2) (Volkert *et al.*, 2000; Shkolnik *et al.*, 2008). The LysM participates in bacterial wall degradation and peptidoglycan interactions, and the function of the GRAM domain has been linked to membrane-associated processes (Buist *et al.*, 2008; Jiang *et al.*, 2008). The precise function of the TLDc domain remains uncharacterised, but this highly conserved domain is found in OXR1 and four other proteins, Chromosome 20 open reading frame 118 (C20ORF118), KIAA1609, nuclear receptor coactivator 7 (NCOA7), and TBC1D24 (Figure 1.2) (Durand *et al.*, 2007; Shkolnik *et al.*, 2008; Falace *et al.*, 2010; Oliver *et al.*, 2011). While the structure of TLDc domain has been recently crystallized, the structure does not resemble that of any other known protein families (Blaise *et al.*, 2012). In *C. elegans*, KIAA1609 ortholog EAK-7 inhibits nuclear DAF-16/FoxO function through an indirect mechanism, and *eak-7* single mutants survive 25% longer than wild-type *C. elegans* through a DAF-16/FoxO dependent manner; however, it remains unknown

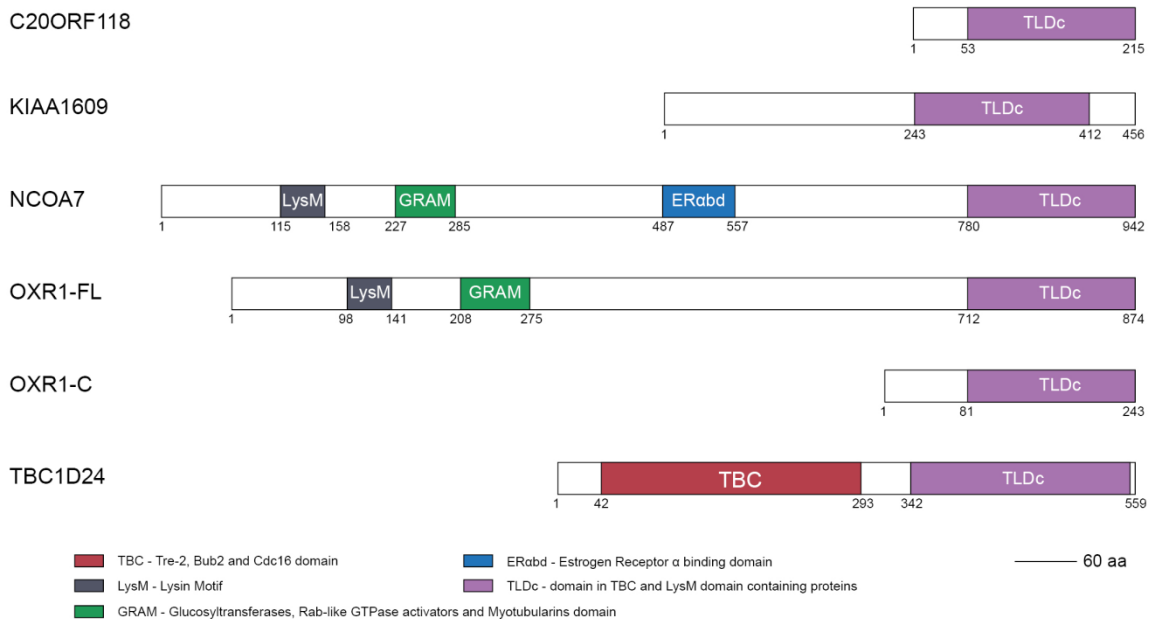

Figure 1.2. Schematic representation of TLDc domain family proteins
Schematic diagram of proteins containing the highly conserved domain in TBC and LysM domain containing proteins (TLDc) domain: C20ORF118, KIAA1609, NCOA7, OXR1, and TBC1D24, and demonstrating difference in structure of the full length OXR1 isoform (OXR1-FL) and the short OXR1 isoform (OXR1-C). OXR1-C consists predominantly of the TLDc domain. Finally, both NCOA7 and OXR1-FL contain a Lysin Motif (LysM), which is involved in bacterial wall degradation and peptidoglycan interactions, and a Glucosyltransferases, Rab-like GTPase activators and Myotubularins (GRAM) domain, whose function has been linked to membrane-associated processes (Buist *et al.*, 2008; Jiang *et al.*, 2008). NCOA7 uniquely contains an Estrogen Receptor α binding domain (ERαbd), which interacts with different nuclear receptors, including estrogen receptor α (Shao *et al.*, 2002). Finally, TBC1D24 contains a Tre-2, Bub2 and Cdc16 (TBC) domain, which contain GTPase-activating properties (Gabernet-Castello *et al.*, 2013).

whether this role of EAK-7 requires the TLDc domain (Alam *et al.*, 2010; Williams *et al.*, 2010). Another TLDc domain family member NCOA7, while not induced by OS, also protects cells from oxidative damage, a function conferred by the TLDc domain (Durand *et al.*, 2007; Finelli, 2010). NCOA7 binds to nuclear receptors, such as estrogen receptor α (ERα) and peroxisome proliferator-activated receptor γ (PPARγ), through the ERα-binding domain, and enhances the transcriptional activities of these nuclear receptors after β-estradiol stimulation (Shao *et al.*, 2002; Arai *et al.*, 2008), but no studies have provided insight into the function of the TLDc domain of NCOA7. Recent studies have found that mutations in *TBC1D24* cause a range of different neurological conditions, particularly those associated with epilepsy (Table 1.1). Furthermore, TBC1D24 modulates neurite outgrowth, excitatory synapses formation, and spontaneous excitatory transmission, particularly the frequency of miniature excitatory postsynaptic currents *in vitro* (Corbett *et al.*, 2010; Falace *et al.*, 2010; Falace *et al.*, 2014). Knock-down of *Tbc1d24* decreases dendritic arborisation, and delays radial migration and multipolar-bipolar transition of cortical neurons in the intermediate zone *in vivo* (Falace *et al.*, 2014). One compound heterozygous mutation A509V in the TLDc domain is linked to Familial Infantile Myoclonic Epilepsy, and significantly impairs TBC1D24-mediated neurite outgrowth (Falace *et al.*, 2010). Interestingly, A509V has been predicted to alter the structure of the TLDc domain by creating steric hindrance with Cys530, which has previously been found to react directly with ROS (Falace *et al.*, 2010; Oliver *et al.*, 2011; Blaise *et al.*, 2012). Taken together, these data suggest that the TLDc domain participates in regulation of neurite outgrowth, though the mechanisms remain unclear.

To better understand the function of Oxr1 and the TLDc domain, previous work in the laboratory has identified a number of protein interacting partners of both full length Oxr1 (Oxr1-FL) and Oxr1-C isoforms by mass spectrometry, and confirmed by co-

Table 1.1 Disease-associated mutations in TBC1D24

Variant	Mutation	Protein Alteration	Phenotype	References
Stop-gain	c.468C > A	p.C156*	Familial malignant migrating partial seizures of infancy	(Milh *et al.*, 2013)
Missense	c.686T > C	p.F229S	Familial malignant migrating partial seizures of infancy	(Milh *et al.*, 2013)
Missense	c.439G>C	p.D147H	Familial Infantile Myoclonic Epilepsy	(Falace *et al.*, 2010)
Missense	c.1526C>T	p.A509V	Familial Infantile Myoclonic Epilepsy	(Falace *et al.*, 2010)
Missense	c.751T>C	p.F251L	Focal epilepsy, dysarthria, mild to moderate intellectual disability, cortical thickening	(Corbett *et al.*, 2010)
Frameshift	c.969_970delGT	p.S324Tfs*3	Early onset progressive encephalopathy, myoclonic epilepsy with dystonia, developmental and neurological retardation	(Guven and Tolun, 2013)
Missense	c.724C>T	p.R242C	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Missense	c.118C>T	p.R40C	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Frameshift	c. 1008delT	p.H336Qfs*12	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Intron	c.1206+5G>A	splicing	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Missense	c.58C>G	p.Q20E	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Missense	c.119G>T	p.R40L	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Missense	c.999G>T	p.L333F	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Missense	c.328G>A	p.G110S	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome	(Campeau *et al.*, 2014)
Missense	c.208G>T	p.D70Y	Nonsyndromic deafness DFNB86	(Rehman *et al.*, 2014)
Missense	c.878G>C	p.R293P	Nonsyndromic deafness DFNB86	(Rehman *et al.*, 2014)

immunoprecipitation studies using cerebellar and brain protein lysates (Finelli, 2010). In the presence of OS, a large number of protein interacting partners of Oxr1-FL and Oxr1-C are involved in OS response and RNA processing. Interestingly, under conditions of OS, FUS and TDP-43, two Oxr1-binding targets identified by mass spectrometry, form complexes with Oxr1-C, while FUS also interacts with Oxr1-FL (Figure 1.3) (Finelli, 2010). As discussed earlier, mutations in FUS and TDP-43 have been discovered in familial and sporadic cases of ALS and frontotemporal lobar degeneration (FTLD) (Arai *et al.*, 2006; Neumann *et al.*, 2006; Mackenzie *et al.*, 2007; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). While the interaction between Oxr1-C and FUS or TDP-43 is not dependent on presence of RNA, some pathogenic mutations in FUS and TDP-43 that were previously linked to ALS decrease binding affinity to Oxr1-C (Figure 1.3-1.4) (Finelli, 2010). In particular, the interaction between FUS^{P517L}, $TDP\text{-}43^{A321G}$, and $TDP\text{-}43^{D169G}$ and Oxr1-C are significantly decreased (Figure 1.4) (Finelli, 2010).

Importantly, recent studies indicate that changes in OXR1 expression are observed in patients suffering from neurodegenerative disorders. Expression of intermediate OXR1 isoforms, which contain the TLDc domain, in the spinal cord of ALS patients and Oxr1 in pre-symptomatic low-copy $SOD1^{G93A}$ ALS mice, a model of ALS, is significantly increased, suggesting that this up-regulation may serve as a protective response to OS during the progression of ALS and a novel early marker of neuropathological pathways (Oliver *et al.*, 2011). Furthermore, *OXR1* is significantly down-regulated in posterior cingulate cortex pyramidal neurons of PD patients, indicating that down-regulation of *OXR1* may contribute to ROS-mediated neurodegeneration in PD (Stamper *et al.*, 2008).

Although *Oxr1* plays a pivotal role in modulating critical cellular functions necessary to protect neurons from free radical damage, and expression of *OXR1* appears to be dysregulated in a variety of neurodegenerative diseases, little is known about the

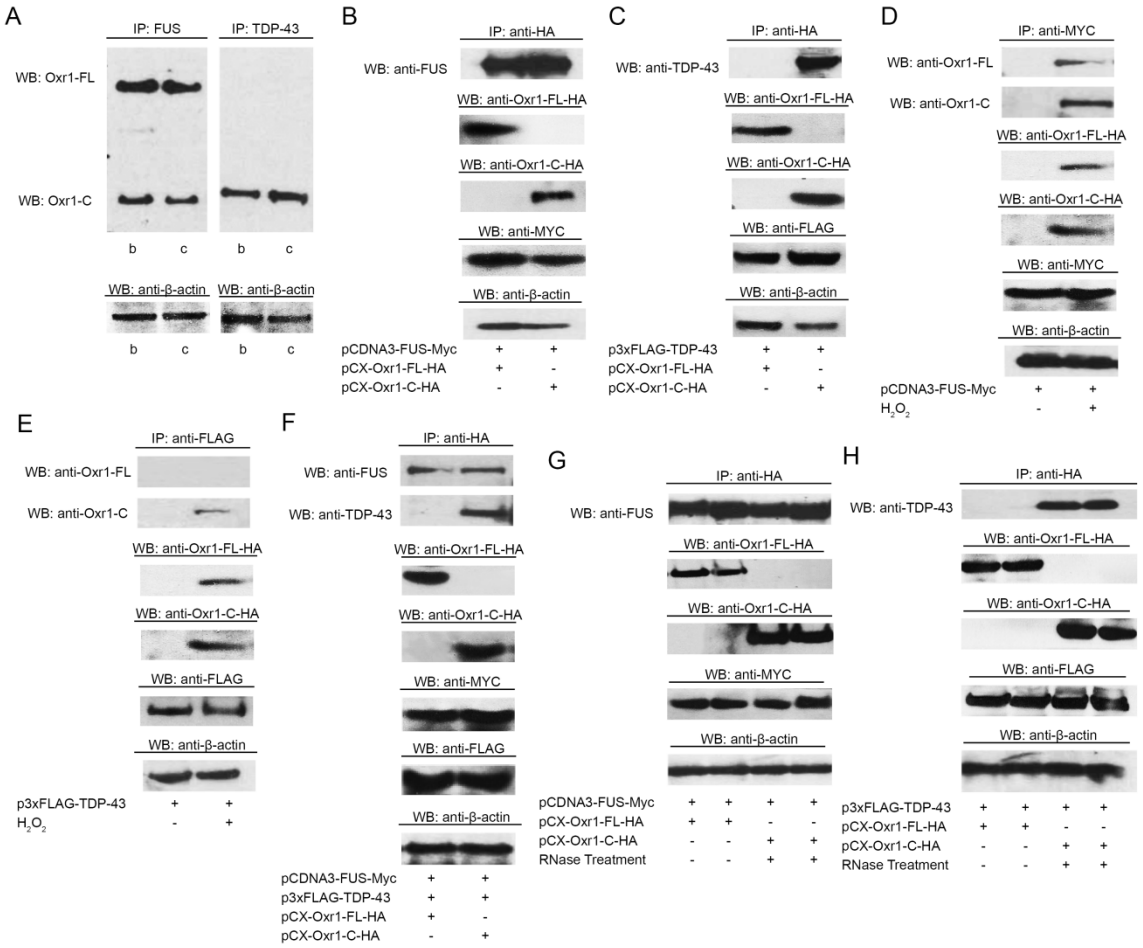

Figure 1.3. Oxr1 interacts with FUS and TDP-43

(**A**) Co-immunoprecipitation (IP) of Oxr1-FL and Oxr1-C with FUS and TDP-43 in brain (b) and cerebellum (c) protein extracts with TDP-43 binding specifically to Oxr1-C. (**B**) FUS binds to Oxr1-FL and-C while (**C**) TDP-43 only binds to Oxr1-C in co-transfected N2A cells. (**D-E**) FUS and TDP-43 interacts with endogenous Oxr1 only under oxidative stress conditions. (**F**) Co-IP of TDP-43 and FUS with Oxr1 in co-transfected N2A cells. (**G**) Co-IP of FUS or (**H**) TDP-43 with Oxr1 treated with 100 µg/ml RNAse for 1 hour shows the interaction between FUS or TDP-43 and Oxr1 is not dependent on RNA. (**A-H**) Adapted with permission from Finelli MJ (2010) Study of two mouse mutants to identify novel neurodegenerative pathways. In: Department of Physiology, Anatomy and Genetics - Medical Sciences Division. Oxford: University of Oxford, copyright (2010).

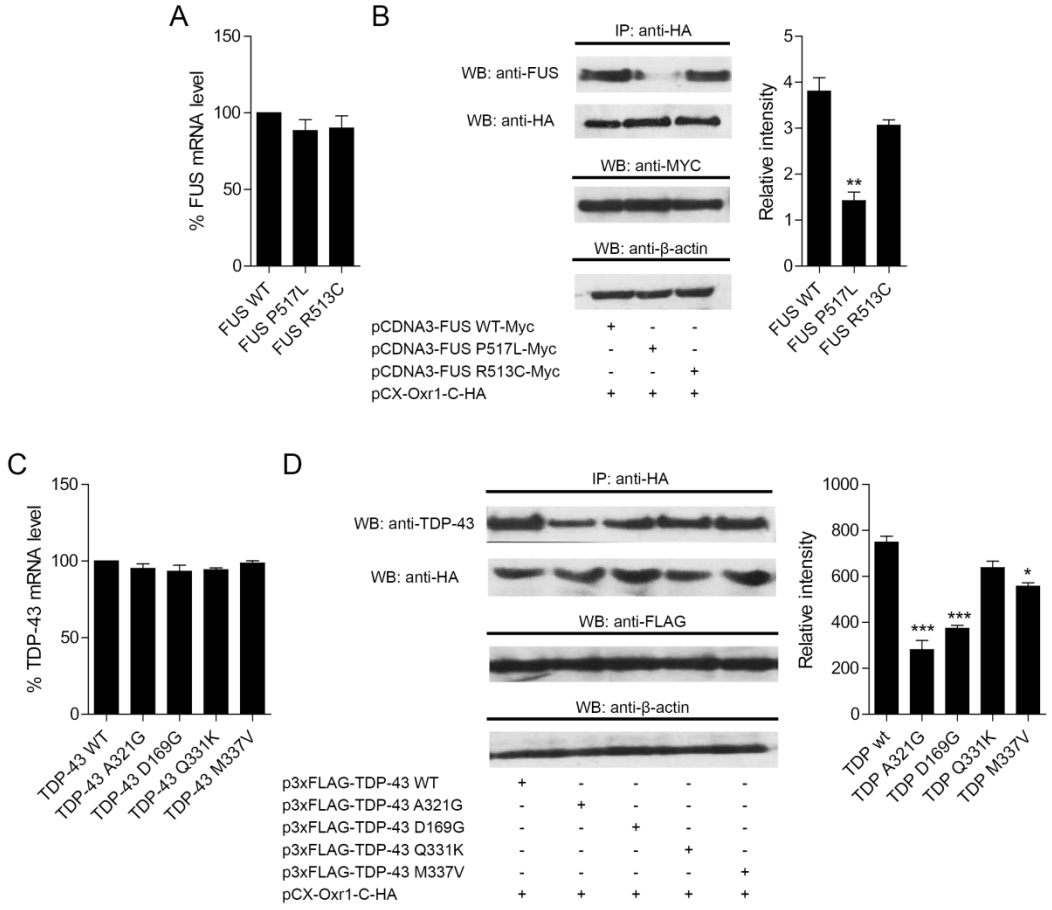

Figure 1.4. ALS-linked mutations in FUS and TDP-43 alter interaction with Oxr1-C
(**A**) No difference in transfection of wild-type or mutant FUS was observed by qRT-PCR. (**B**) Co-IP of Oxr1-C with FUS wild-type (WT) and mutants (P517L and R513C) in co-transfected N2A cells demonstrates the interaction between Oxr1-C and FUS P517L is altered. (**C**) No difference in transfection of wild-type or mutant TDP-43 was observed by qRT-PCR. (**D**) Interaction between Oxr1-C and TDP-43 A321G and D169G is altered. Co-IP of Oxr1-C with TDP-43 wild-type (WT) and mutants (A321G, D169G, Q331K, M337V) in co-transfected N2A cells demonstrates the interaction between Oxr1-C and TDP-43 A321G and D169G is altered. (**B,D**) Quantification of western blot and values are the mean ± SEM (*p<0.05; **p<0.01; ***p<0.001; 1-way ANOVA with Dunnett's Multiple Comparison Test). (**A-D**) Adapted with permission from Finelli MJ (2010) Study of two mouse mutants to identify novel neurodegenerative pathways. In: Department of Physiology, Anatomy and Genetics - Medical Sciences Division. Oxford: University of Oxford, copyright (2010).

mechanisms by which *Oxr1* confers neuronal sensitivity to OS, the neuroprotective potential of Oxr1 during pathogenesis of neurological conditions, and whether loss of Oxr1 in selective neuronal populations leads to *in vivo* neurodegeneration.

1.4 Aims of Thesis

In this thesis, I worked to elucidate mechanisms by which Oxr1 functions to protect neurons from OS-mediated damage and apoptosis, and explore the therapeutic potential of Oxr1 during pathogenesis of neurodegenerative disease through the use of cellular and animal models. My four principal aims were:

1) Examine the interaction between Oxr1, and ALS-associated proteins, FUS and TDP-43, and characterise functions of Oxr1 in concert with these proteins in response to OS
2) Investigate *in vivo* neuroprotective functions of Oxr1 during pathogenesis of neurodegenerative diseases.
3) Characterise downstream pathways of Oxr1 that confer neuronal sensitivity to OS
4) Determine whether specific loss of Oxr1 in motor neurons leads to a neurodegenerative phenotype *in vivo*

2 Materials and Methods

2.1 Ethics Statement

All animal experiments were conducted in adherence to the guidelines set forth by the UK Home Office regulations, and with the approval of the University of Oxford Ethical Review Panel.

2.2 Mouse Strains

Bella (*bel*) mice were originally derived from the progeny of an *N*-ethyl-*N*-nitrosourea (ENU)-injected BALB/c male and a C3H/HeH female, as described previously (Oliver *et al.*, 2011). Homozygous *bel/bel* mice were generated by intercrossing *bel/+* mice that have been backcrossed for over ten generations to C3H/HeH background. Mice were genotyped using D15Mit229, a polymorphic microsatellite marker that lies approximately 10kb from the *bel* deletion. Tg(*Prnp-Oxr1*)/+ mice, which were generated by Dr. Ben Davies (Wellcome Trust Centre for Human Genetics) as previously described (Oliver *et al.*, 2011), overexpress an HA-tagged *Oxr1* transgene driven by the mouse *Prnp* promoter (Borchelt *et al.*, 1996), and were maintained on a C57BL/6 background. Transgenic $SOD1^{G93A}$ mice were derived from the B6SJL-TgN(SOD1-G93A)1Gur (Jackson Laboratory) strain, and backcrossed onto, and maintained on a C57BL/6 background. N171-82Q transgenic Huntington's disease mice were obtained from Dr. Abraham Acevedo-Arozena (MRC Harwell) on a C57BL/6 background.

$Oxr1^{tm1a(EUCOMM)Wtsi}$ knockout-first conditional allele mice have LoxP sites flanking (flox) exon 15 and 16 of *Oxr1*, and were generated by the Wellcome Trust Sanger Institute and maintained on a C57BL/6 background. Transgenic mice with β-actin driving FLPe variant recombinase (B6.SJL-Tg(ACTFLPe)9205Dym/J) (Jackson Laboratory) was maintained on a C57BL/6 background. Rosa26 locus lacZ reporter mice (B6;129S4-

Gt(*ROSA*)26*Sor*^{tm1Sor}/J) (Jackson Laboratory) were maintained on a C57BL/6 background and used to detect expression pattern of specific promoter-drive *cre* transgene. ChAT-Cre transgenic mice (GENSAT, GM53), in which Cre recombinase is expressed solely in motor neurons under the control of the bacterial artificial chromosome (BAC) containing the endogenous Chat-promoter, was generated by Gong et al. (2007), obtained from UC Davis, and maintained on a C57BL/6 background.

The day of plug was designated as E0.5, and the day of birth was designated as P0. Mice received food and water *ad libitum*, and were housed on a 12-hour on/off light cycle.

2.3 Accelerating Rotarod Test

Mice were placed on a grooved plastic beam of a commercial rotarod device (Ugo Basile, Italy), which revolves at a default 5 rpm, facing in an orientation opposite to the rotation. Mice were trained once with a ramping session of 5-40 rpm over 5 minutes at P57 and tested for 1 trial each day on 3 consecutive days. The time latency to fall from the rod or complete two rotations on the rod without an attempt to run on the accelerating rod was recorded, and an average was obtained for the 3 trials for each time point.

2.4 Grip Strength

Mice were held by their tails, and gently placed on a commercial grip strength device (Chatillon), such that their forepaws gripped a metal mesh bar attached to the apparatus. The mice were then gently pulled across the metal mesh bar, which measured the maximum force exerted before the mice released their grip. The maximum force (g) was recorded for three trials with a 30-second rest period between each trial.

2.5 Tissue Collection and Preparation

For perfused tissue collection, mice were given a lethal intraperitoneal injection of pentobarbitone, followed by transcardial perfusion with 0.9% saline containing 10 U/ml heparin, and fixation with 4% paraformaldehyde (PFA). Brains and spinal cords were dissected, post-fixed overnight in 4% PFA at 4°C, cyroprotected in 30% sucrose, and mounted in OCT. Tissue sections were cut on a cryostat at 15µm. For fresh frozen tissue, the mice were sacrificed by cervical dislocation, and the brains and spinal cords were dissected, and subsequently frozen in OCT medium on dry ice, and stored at -80°C.

2.6 Cell Culture, Transfections, and Treatments

HeLa and Neuro2a (N2A) cells were maintained in Dulbecco's Modified Eagles Medium (DMEM-GlutaMAX) (Invitrogen) supplemented with 10% fetal bovine serum, 1mM non-essential amino acids (Invitrogen), and 1% Penicillin-Streptomycin solution (Invitrogen). Cells were transfected with DNA constructs using FuGene 6 (Roche Applied Science) diluted in Opti-MEM (Invitrogen), according to the manufacturer's instructions.

Primary granule cell cultures were carried out as previously described (Bilimoria and Bonni, 2008). Mouse pups at P7 were sacrificed by cervical dislocation and decapitated. The cerebella were dissected, and the meninges and large blood vessels were removed. The dissected cerebella were then washed with HHGN solution (1X HBSS (Invitrogen)/2.5 mM HEPES (pH 7.4)/35 mM glucose/4 mM sodium bicarbonate) 3 times, before being placed in trypsin-DNase solution (10mg/mL trypsin/2mg/mL DNase/HHGN solution) for 20 minutes at room temperature. After decanting the trypsin-DNase solution, the cerebella were washed three times with HHGN solution and dissociated in 0.1mg/mL DNase/BME medium (Invitrogen). The cells were centrifuged at 200 x g for 5 minutes at 4°C, and resuspended in culture medium (BME medium/25mM KCl/10% bovine growth

serum (Hyclone)/1X penicillin-streptomycin-glutamine). Approximately 750,000 granule cells were plated on previously prepared poly-L-ornithine-coated coverslips. After 24 hours, cytosine-1-β-D-arabinofuranoside was added for a final concentration of 10μM, and after day *in vitro* (DIV) 3, glucose was added to a final concentration of 25mM. To culture until DIV17, appropriate amounts of culture medium with 25mM glucose was added every 3 days until immunocytochemistry was performed.

For OS treatments, cells were treated with 1mM H_2O_2 (Sigma), or 0.5mM arsenite (Sigma) for the indicated durations, followed by recovery in fresh medium for 30 min. For proteasome inhibition experiments, cells were treated with 10μM MG-132 (Calbiochem) for 4 hours before subsequent OS treatments. For inhibition of autophagy-lysosomal degradation or arginine methylation, cells were treated with 1μM Concanamycin-A (Santa Cruz) or 100μM AMI-1 (Sigma), respectively for 12 hours before subsequent OS treatments.

2.7 Immunoprecipitation and Western Blotting

Tissue or cell extracts were prepared using appropriate amounts of RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40) or Lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton-X) for western blotting, and immunoprecipitation buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% CHAPS) for co-immunoprecipitation. Protein levels were quantified using BCA assays (Pierce Thermo Scientific) for equal loading. For co-immunoprecipitation, tissue protein extracts were first pre-cleared with G-Sepharose (Sigma) beads for 1 hr at 4°C. Next, the protein extracts were incubated either for 2 hours or overnight at 4°C with 2-8μg of antibody, after which appropriate volumes of G-Sepharose (Sigma) beads were added to the protein extracts. For co-immunoprecipitation of HA, MYC and FLAG-tagged proteins from cells, protein

extracts were incubated either for 2 hours or overnight at 4°C with appropriate volume of EZview™ Red anti-HA, MYC or FLAG Affinity Gel beads (Sigma), with no pre-clearing step. All immunoprecipitated proteins were subsequently washed 3-6 times with IP buffer, resuspended in 25µl of sample loading buffer, and boiled for 5 min at 100°C.

Protein samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Amersham). Membranes were subsequently blocked with 5% skim milk powder in PBST (PBS, 2% Tween-20) for 1 hr at room temperature (RT), before incubation with appropriate primary antibody diluted in PBST for either 1 hour at RT or overnight at 4°C. After washing 3 times with PBST, membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Amersham, 1:10,000), and blots were developed with the ECL kit (Amersham).

Primary antibodies used were as follow: mouse anti-FLAG (Sigma, 1:4500); rabbit anti-FUS (Bethyl, 1:10,000); rabbit anti-HA (Sigma, 1:10,000); rabbit anti-HO-1 (Enzo Life Sciences, 1:1,000); mouse anti-mono and dimethyl arginine (Abcam, 1:1000); mouse anti-Myc (Sigma, 1:2500); rabbit anti-Oxr1 (1:1000, (Oliver *et al.*, 2011)); rabbit anti-PRMT1 (Millipore, 1:1000); sheep anti-SOD1 (Calbiochem); rabbit anti-STAT3 (Cell Signalling, 1:2000); rabbit anti-p-STAT3 Tyr705 (Cell Signalling, 1:1000); rabbit anti-TDP-43 (Proteintech, 1:1000); and mouse anti-β-actin (Sigma, 1:6000).

2.8 Immunocytochemistry

Paraffin wax embedded sections were first treated with xylene/ethanol to remove the paraffin wax. For antigen-retrieval, paraffin wax, fresh frozen, and perfused sections were boiled in sodium citrate buffer for 20 minutes and washed in ddH_2O. HeLa and N2A cells were washed with PBS, followed by fixation with 4% PFA for 30 minutes, and permeabilisation with 0.1% Triton-X/PBS for 30 minutes at RT.

Fixed cells and brain sections were blocked in 5% Bovine Serum Albumin (BSA) (Sigma)/PBS/0.3% Triton-X at RT for 1 hour. Incubation with primary antibodies diluted in 5% BSA/1X PBS/0.3% Triton-X was conducted overnight at 4°C or for 1 hour at RT. After incubation with primary antibody, brain sections were washed in PBS, followed by incubation with Alexa fluor secondary antibodies (Invitrogen) at appropriate dilutions for 1-3 hours at RT. Brain sections were subsequently washed with PBS, and covered with VECTASHIELD® Mounting Medium with DAPI (Vector Lab) and a glass coverslip. After drying, clear nail polish was applied to the edges of the slides, and slides were stored at 4°C. Cells were washed in PBS, after which coverslips were mounted onto glass slides with VECTASHIELD® Mounting Medium with DAPI.

Primary antibodies and dilutions used include: rat anti-CD68 (1:400, Millipore Bioscience Research Reagents); rat anti-CTIP2 (1:500; Abcam); rabbit anti-DCP1A (1:200, Sigma); mouse anti-FLAG (1:100, Sigma); rabbit anti-FUS (1:250, Bethyl); mouse anti-GFAP (1:400, Sigma); rabbit anti-HA (1:100, Sigma); mouse anti-Myc (1:100, Sigma); mouse anti-NeuN (1:500; Millipore Bioscience Research Reagents); rabbit anti-TDP-43 (1:350, Proteintech); and goat anti-TIA-1 (1:200, Santa Cruz).

TUNEL staining for apoptosis was carried out on fresh-frozen brain sections with the *In Situ* Cell Death Detection Kit, TMR Red (Roche) according to the manufacturer's protocol. After final wash with PBS, sections were covered with VECTASHIELD® Mounting Medium with DAPI (Vector Lab) and a glass coverslip.

For all cell counting assays, at least 200 total cells were counted from a minimum of 4 different randomly chosen microscopic fields (20x magnification). Images were viewed on a Zeiss Axioskop 2 microscope, acquired using an AxioCam HR digital camera (Zeiss), and processed using AxioVision software (Zeiss) and Adobe Photoshop.

2.9 *In situ* Hybridisation

In situ hybridisation was performed by Dr. Peter Oliver. Digoxigenin (DIG)-labelled riboprobe synthesis and hybridisation on 12µM spinal cord sections was conducted as previously described (Wilkinson, 1998; Oliver *et al.*, 2011). Region of *Oxr1* mouse cDNA sequence for DIG-labelled synthesis was used as previously described (Oliver *et al.*, 2011).

2.10 Spinal Cord Histology

Experiments were performed with assistance from Dr. Peter Oliver and Benjamin Edwards. For nissl staining, cryostat tissue sections were air dried, fixed in 100% ethanol for 30 seconds, and hydrated in distilled water. Sections were subsequently stained for 3-5 minutes with 0.5% Cresyl violet solution and rinsed in distilled water. The sections were then dehydrated with a series of alcohol dilutions, with 90% ethanol dilution containing 0.1% acetic acid. Finally, the sections were placed in Histoclear II (National Diagnostics) before being mounted in DPX mountant (BDH). All motor neurons on nissl stained and matched sections of lumbar spinal cord segments (L1 and L6) were counted. 8 sections were used for motor neuron counts in Chapter 4 and 18 sections were used for motor neuron counts in Chapter 6. Images were viewed on a Zeiss Axioskop 2 microscope, acquired using an AxioCam HR digital camera (Zeiss), and processed using AxioVision software (Zeiss) and Adobe Photoshop.

2.11 Muscle Histology

All muscle histology experiments were conducted with assistance from Benjamin Edwards. For haematoxylin and eosin (H&E) stain, fresh muscle sections were placed in haematoxylin for an appropriate time no longer than 15 minutes, and washed in distilled

water. After staining, sections were subsequently bled in 50% ethanol/0.3% hydrocloric acid for 10 seconds, and washed in distilled water. The sections were then placed in eosin for 2 minutes, washed in distilled water, and dehydrated in 60%, 75%, and 100% ethanol. After dehydration, the sections were placed in Histoclear for 2 minutes before being covered with Histomount and a coverslip. For group atrophy analysis, total muscle area in atrophy was counted and divided by total muscle area.

For NMJ staining, fresh dissected muscles were teezed apart, and placed in PBS/0.2% Triton X-100 with an appropriate dilution of Alexa Fluor 488 conjugated α-bungarotoxin (Invitrogen) overnight at 4°C. The teezed muscle samples were subsequently washed in PBS three times, before being further teezed apart, placed on a slide, and covered with Histomount and a coverslip.

For fibre composition and group atrophy analyses, fresh dissected muscles were placed in OCT, cut in 15μm sections on the cryostat, and stored at -80°C. Muscle sections were blocked in 5% BSA/PBS/0.3% Triton-X at RT for 1 hour. Incubation with primary antibodies diluted in 5% BSA/1X PBS/0.3% Triton-X was conducted overnight at 4°C or for 2 hour at RT. Muscle sections were then washed in PBS, followed by incubation with appropriate Alexa fluor secondary antibodies (Invitrogen) (1:750) for 1-3 hours at RT. Brain sections were subsequently washed with PBS, and covered with VECTASHIELD® Mounting Medium with DAPI (Vector Lab) and a glass coverslip. After drying, clear nail polish was applied to the edges of the slides, and slides were stored at 4°C.

Primary antibodies and dilutions used include: mouse anti-MYHC1 (1:200, B8-F8, German Collection of Microorganisms and Cell Cultures); mouse anti-MYHC2A (1:200; SC-71, German Collection of Microorganisms and Cell Cultures); and mouse IgM anti-MYHC2B (1:100, BF-F3, German Collection of Microorganisms and Cell Cultures)

2.12 Liquid Chromatography-Mass Spectrometry

Samples were prepared for liquid-chromatography (LC)-mass spectrometry (MS) as previously described (Cruz *et al.*, 2013). After the co-immunoprecipitated samples were separated by 12% SDS-PAGE, the gel was stained by Coomassie stain (0.1% Coomassie R250 dye/10% acetic acid/40% methanol) and destained by 20% methanol/10% acetic acid. The protein bands were subsequently cut into small 1-2 mm^3 pieces, and washed twice with 50% methanol/5% acetic acid at room temperature. The gel pieces were then dehydrated with 100% acetonitrile, and dried in a vacuum centrifuge. The protein samples were then reduced with 10mM DTT at room temperature for 30 minutes, and alkylated with 100mM iodoacetamide at room temperature for 30 minutes. The gel pieces were then dehydrated with 100% acetonitrile, and dried in a vacuum centrifuge before being rehydrated with 100mM ammonium bicarbonate for 10 minutes at room temperature. After the rehydration, the gel pieces were again dehydrated with 100% acetonitrile, dried in a vacuum centrifuge, placed on ice. The gel pieces were then digested overnight at 37°C with appropriate volume of 20ng/μL sequencing grade trypsin (Promega) diluted in 50mM ammonium bicarbonate. The digested gel pieces were washed with 50mM ammonium bicarbonate for 10 minutes, before the supernatant was collected. The gel pieces were subsequently subjected to extraction with 50% acetonitrile/5% formic acid for 10 minutes, after which the supernatant was collected. After the first extraction, the gel pieces were further extracted with 85% acetonitrile/5% formic acid until the gel pieces were sufficiently dehydrated, with supernatant collection for each sample. The volume of the extracted supernatant was reduced to less than 5 μL by evaporation in a vacuum centrifuge, and adjusted to approximately 20 μL with 5% acetonitrile/0.1% formic acid. The samples were then sonicated in a water bath for 15 min at room temperature and centrifuged for 15 minutes at 4°C to pellet insoluble material.

Peptides in each sample were separated by LC and identified by MS, and LC-MS/MS was performed by Dr. Holger Kramer with an Ultimate 3000 nano-HPLC system (Dionex, Sunnyvale, CA, USA) and DCMS link 2.0 software, which was coupled directly with a 3D high capacity ion trap mass spectrometer (amaZon; Bruker Daltonics). All parameters were set and MS/MS analysis was conducted as previously noted with raw LC-MS/MS data being processed and Mascot compatible files being generated with DataAnalysis 4.0 software (Bruker Daltonics) (Cruz *et al.*, 2013). Finally, LC-MS/MS data was analysed with MASCOT (version 2.4) (Perkins *et al.*, 1999; Koenig *et al.*, 2008), and comparison with the UniProt_SwissProt database with a *Mus musculus* taxonomy restriction (v2012.09.07). MASCOT is an algorithm for the assignment of probability based scores to peptide-spectrum matches. The MASCOT score is $-10*\log_{10}(p)$, where p is the absolute probability of the peptide-spectrum match being a random event, and only significant protein hits were reported with a p-value<0.05. In this analysis only significant protein hits were reported with a p-value<0.05. The search parameters were designated as the following: 2+, 3+, and 4+ ions; peptide mass tolerance 0.3 Da; $^{13}C = 2$; fragment mass tolerance 0.6 Da; number of missed cleavages: two; instrument type: ESI-TRAP; fixed modifications: Carbamidomethylation (Cys); and variable modifications: Oxidation (Met).

2.13 Quantitative real-time polymerase chain reaction (qRT-PCR)

Preparations of total RNA, cDNAs and qRT-PCR were performed on four wild-type, Tg(*Prnp-Oxr1*)/+, $SOD1^{G93A}$ ALS, and $SOD1^{G93A}$/ Tg(*Prnp-Oxr1*) mice at P60, P90, and P135. Total RNA was extracted from lumbar spinal cord tissue with RNeasy Kits (Qiagen). Contaminating genomic DNA was removed using RNase-free DNase I (Qiagen). Complementary DNAs were synthesized with the RevertAid First Strand cDNA Synthesis Kit (Fermentas), using 1µg of total RNA for each reaction.

Primer sequences were designed using the Primer 3 program (http://frodo.wi.mit.edu/primer3/), or obtained from previous online publications and commercial companies, with the nucleotide sequences of the primers shown in Table 2.1. qRT-PCR reactions were subsequently carried out using SYBR Green PCR master mix (Applied Biosystems) with primers and cDNA added in optimized concentrations, using a StepOne real-time PCR machine (Applied Biosystems) with cycling conditions at 95°C for 10 seconds, followed by 40 cycles of 55°C for 15 seconds, 60°C for 10 seconds. Gene amplification specificity was verified by melting curve analyses. All values obtained were normalized with respect to mRNA levels of a control gene, *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*).

Table 2.1. Nucleotide sequences of quantitative RT-PCR primers

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
ApoE	TCTGGTGGAGCAAGGTCGCC	TTGCCCACTTCCTCCAGCCG
C1qa	CAACGTGGTTATCTTTGACAAGGT	GAAGTTGAAGTAATAGAAGCCGGG
C1qb	CACCAACGCGAACGAGAACT	GGCCAGGCACCTTGCA
C1qc	CTACTTCGTCTACTACACATCGCA	CACCATGCCATTGTAGTCATTGAC
Cd52	TCCTCCTCTTCCTCACTATCATTCT	GGCACATTAAGGTATTGGCAAAGA
Ctss	TACCAGGGTTCTTGTGGTGC	AGGGATATCAGCTTCCCCGT
Ctsz	AGACCGAATCAACATCAAGAGGAA	TAAACTTGTCACAGTCTTGGTCCT
FTH	GGCTGCCATCAACCGCCAGA	TCGGCATGCTCCCTCTCCTCA
FTL	GCCTGGTCAACTTGCACCTG	CCCCGCGATCGTTCTGAAAC
Gapdh	GCTACACTGAGGACCAGGTTGTC	AGCCCCGGCATCGAA
HexB	TTTCTGCTCCTTGGTACTTAGACC	TTGGAGTAAGGTTAGTTGCATCCA
hSOD1	GGCCAAAGGATHAAGAGAGGC	TGTGCGGCCAATGATGCAAT
Lyz2	TCCTGACTCTGGGACTCCTC	AGCCAGCCATTCCATTCCTT
Mpeg1	AGAAACCGGATCTACACAGTGAAA	GATTACGTGTGTGCCATAGTTGAG
MT-I	TGGACCCCAACTGCTCCTG	CTTGTCCGCGGCGCCTTTG
MT-III	AGACCTGCCCCTGTCCTACT	GCACACACAGTCCTTGGCAC
Oxr1-FL	CAGTCGTGACTGGACAGGTTT	ATGGGCTACATCTGGAGTCG
Serpina3n	CGAAACTGTACCCTCTGACTGTAT	TTGGCTATCTTGGCTATAAAGGGG
Vim	CGGAAAGTGGAATCCTTGCAGG	AGCAGTGAGGTCAGGCTTGGAA

2.14 Microarray Analysis

The microarray experiments were performed by Dr. Sheena Lee and all analyses were conducted together with Dr. Lee. First, the RNA integrity was analysed on a

BioAnalyzer (Agilent Laboratories, US) to confirm all samples had a RNA Integrity Number ≥ 8. Labelled and fragmented sense ssDNA for hybridisation were generated from 200ng of RNA with the Ambion WT Expression Kit and the Affymetrix GeneChip® WT Plus Reagent Kit for P60 and P90 samples, respectively, and with the Affymetrix WT Terminal Labelling and Controls Kit. The distribution of fragment lengths was then assessed with a BioAnalyser (Agilent Laboratories). Labelled and fragmented sense ssDNA was subsequently hybridized to the Affymetrix GeneChip Mouse Gene 2.0 ST Array and Affymetrix GeneChip Mouse Gene 1.0 ST Array for P60 and P90 samples, respectively. The chips were then washed and stained using the Affymetrix Hybridisation, Wash, and Stain Kit, according to the manufacturer's instructions, and processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000.

The microarray data was Robust Multi-array Average (RMA) normalized using GeneSpring GX12.6 (Agilent), and differentially expressed genes were identified using a moderated t-test without a multiple testing correction, or with a Benjamini and Hochberg multiple testing correction (limma), and false discovery rate of ≤0.05. The genes that were designated as "rescued" fulfilled the following criteria: 1) genes that had a >1.3 fold change and $p<0.05$ between $SOD1^{G93A}$ samples and both wild-type and Tg(*Prnp-Oxr1*) samples; and 2) genes that had a <1.2 fold change or $p>0.05$ between $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) samples and both wild-type and Tg(*Prnp-Oxr1*) samples. The Ingenuity Pathways Analysis tool (Ingenuity Systems, www.ingenuity.com) was used to gain additional *in silico* functional information. The functions tool in Ingenuity compares genes of interest to known biological functions and disease states, and was used to detect the most significant functional pathways. The canonical pathways tool in Ingenuity was used to identify whether genes of interest were found in common pathways, and the upstream analysis tool

in Ingenuity was used to predicted proteins of interest that may be activated or inhibited upstream of the differential gene expression.

2.15 β-Galactosidase Staining

β-Galactosidase staining was performed by Arran Babbs in the laboratory. Fresh frozen tissue sections were first fixed in 0.2% Glutaraldehyde in 1X PBS for 10 minutes on ice. After washing in 1X PBS and 0.02% NP-40/0.01% Sodium Deoxycholate/2mM $MgCl_2$ in 0.1M phosphate buffer pH 7.3, fixed tissue sections were immersed in 1mg/ml X-Gal staining solution (0.02% NP-40/0.01% Sodium Deoxycholate/5mM Potassium Ferricyanide/5mM Pottassium Ferrocyanide/2mM $MgCl_2$ in 0.1M phosphate buffer pH 7.3) overnight at 37°C in the dark. Stained tissue sections were post-fixed 4% PFA for 10 minutes, and washed with 1X PBS, and covered with Histomount and a coverslip.

2.16 Cloning and site-directed mutagenesis

Mouse *PRMT1* coding sequence (CCDS57547.1) and *Psmb10* coding sequence (CCDS28411.1) were cloned into pcDNA3.1 (Invitrogen) vector with either a C-terminal HA-tag (5'-TACCCATACGATGTTCCAGATTACGCT-3') or a C-terminal FLAG-tag (5'-GACTACAAAGACGATGACGACAAG-3'). The conserved upstream human *OXR1-C* sequence (NW_004078037.1; 21197233-21197631bp) was cloned into PGL4.0[luc2] (Promega) vector. All missense mutations were generated by site-directed mutagenesis using a QuikChange™ Site-Directed Mutagenesis Kit (Agilent Technologies), with primer sequences listed in Table 2.2. The mouse miR-137 over-expression construct was cloned using BLOCK-iT™ Pol II miR RNAi Expression Vector Kits (Invitrogen) according to manufacturer's instructions.

Table 2.2. Site-directed Mutagenesis Primers

Primer Name	Sequence: 5' – 3'
PBX mut F	GATTAGCTCTCTCTCTTGTCAAGGAAATCTTTGTCTCTAGCAAAC
PBX mut R	GTTTGCTAGAGACAAAGATTTCCTTGACAAGAGAGAGAGCTAATC
Foxa2 mut F	CTTTTTTGTTACTGGGTTACTTTTCCTATATTCATGTACTGATG
Foxa2 mut R	CATCAGTACATGAATATAGGAAAAGTAACCCAGTAACAAAAAAG
Foxo3 mut 1 F	GTATATTCATGTACTGATGTTTGGAGGTATAGGCTCTGTGTCTTTAAAC
Foxo3 mut 1R	GTTTAAAGACACAGAGCCTATACCTCCAAACATCAGTACATGAATATAC
FoxL1 mut F	CTCAGGACTATCATTGCTATGGCGACTACTTAGTGATTAGCTCTC
FoxL1 mut R	GAGAGCTAATCACTAAGTAGTCGCCATAGCAATGATAGTCCTGAG
LHX3 mut F	CAGTTCTTACGTGATTTTATGTAACGGAATTTGCCCTGGAAAGGAAC
LHX3 mut R	GTTCCTTTCCAGGGCAAATTCCGTTACATAAAATCACGTAAGAACTG
NF-κB mut F	GCAACCAAACTTAAGCATGTTAAATTCTCTCTTCCCTCCCCAGC
NF-κB mut R	GCTGGGGAGGGAAGAGAGAATTTAACATGCTTAAGTTTGGTTGC
Nkx2-5 mut 1F	GGGTACAGTGCAACCAAACTTGCGCATGGGAAATTCTCTCTTCCC
Nkx2-5 mut 1R	GGGAAGAGAGAATTTCCCATGCGCAAGTTTGGTTGCACTGTACCC
Nkx2-5 mut 2F	CAGTTCTTACGTGATTTTATGTAACTTGGTTTGCCCTGGAAAGGAAC
Nkx2-5 mut R	GTTCCTTTCCAGGGCAAACCAAGTTACATAAAATCACGTAAGAACTG
Sp1 mut F	CTTAAGCATGGGAAATTCTCTCTTAACTCCCCAGCTTTACAGCCTCCACTC
Sp1 mut R	GAGTGGAGGCTGTAAAGCTGGGGAGTTAAGAGAGAATTTCCCATGCTTAAG

2.17 Dual-Luciferase Assay

Transfected HeLa cells were lysed and assayed for firefly and renilla luciferase activity using the Dual-Luciferase Reporter Assay (Promega) according to manufacturer's instructions. Light emission from each sample was measured using a FLUOstar Optima plate reader (BMG Labtech) after a delay of 2 seconds, and a total integration of 10 minutes for the luciferase, with all experiments being performed in triplicate.

2.18 Statistical analysis

Results were analysed using Prism (GraphPad Software, Inc.) with the appropriate statistical tests. Data are presented as mean ± SEM (standard error of mean), with n indicating the number of independent samples used in each group for comparison.

3 Oxr1 regulates cytoplasmic localisation of FUS and TDP-43 under oxidative stress

3.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting both upper motor neurons in the cortex, and lower motor neurons in the brain stem and the spinal cord, leading to severe muscle wasting and death due to respiratory failure. Unfortunately, the pathogenic mechanisms underlying ALS are still not well-understood, and only one drug, riluzole, has been approved for ALS patients, yielding only a modest improvement in survival. In recent years, important progress has been made with the identification of genes that are mutated in familial ALS (fALS) and sporadic ALS (sALS) cases, including those encoding for antioxidant superoxide dismutase 1 (SOD1), RNA-binding proteins, fused in sarcoma (FUS) and transactive response DNA-binding protein 43kda (TDP-43), and an unknown protein, C9ORF72 (Andersen and Al-Chalabi, 2011; DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011).

Mutations in FUS and TDP-43 are responsible for approximately 10% of fALS cases and 1% of sALS cases (Gitcho *et al.*, 2008; Kabashi *et al.*, 2008; Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). Structurally similar to heterogeneous ribonucleoproteins (hnRNPs), FUS and TDP-43 shuttle between the nucleus and cytoplasm, and play an important role in regulating gene expression through transcription, pre-mRNA splicing, and microRNA processing (Zinszner *et al.*, 1997; Ayala *et al.*, 2008; Tan and Manley, 2009; Lagier-Tourenne *et al.*, 2010; Polymenidou *et al.*, 2011; Sephton *et al.*, 2011). FUS also plays an important role in neurons for the proper development of dendritic spines, dendritic transport of mRNA, and synaptic transmission at the neuromuscular junction, and ALS-associated mutations in FUS cause defects in growth cone and branching of axons (Fujii *et al.*, 2005; Fujii and

Takumi, 2005; Armstrong and Drapeau, 2013; Groen *et al.*, 2013). In addition, loss of TDP-43 leads to reduced axonal and dendritic branching, and aberrant formation of synaptic terminals at the neuromuscular junction (Feiguin *et al.*, 2009; Lu *et al.*, 2009).

Interestingly, while FUS and TDP-43 are normally localised and function in the nucleus, FUS-positive and TDP-43-positive cytosolic aggregates are pathological hallmarks of non-SOD1 fALS cases, of most sALS cases, as well as of a related neurological disorder, frontotemporal lobar degeneration (FTLD) with ubiquitin-positive inclusions (Arai *et al.*, 2006; Neumann *et al.*, 2006; Mackenzie *et al.*, 2007; Kwiatkowski *et al.*, 2009; Neumann *et al.*, 2009; Vance *et al.*, 2009; Deng *et al.*, 2010). While the exact consequence of sequestering FUS and TDP-43 to the cytoplasm remains unknown, increasing evidence suggests that ALS-linked mutations in FUS and TDP-43 not only cause relocalisation of FUS and TDP-43 from the nucleus to the cytoplasm, but also increase cellular toxicity (Johnson *et al.*, 2009; Barmada *et al.*, 2010; Kabashi *et al.*, 2010; Huang *et al.*, 2011a; Kino *et al.*, 2011; Wu *et al.*, 2013). These findings suggest that cytoplasmic aggregation of ALS-associated FUS and TDP-43 mutants may contribute to the disease pathology through either a loss of normal function in the nucleus or a toxic gain-of-function by accumulating in the cytoplasm.

Thus, recent work has sought to understand mechanisms governing nucleocytoplasmic transport of and aggregation of FUS and TDP-43. Arginine methylation by protein arginine N-methyltransferase 1 (PRMT1) and direct interaction with transportin regulate FUS shuttling between the nucleus and cytoplasm (Dormann *et al.*, 2010; Tradewell *et al.*, 2011; Yamaguchi and Kitajo, 2012). Recently, a study showed that Parkinson's disease-associated parkin increases TDP-43 accumulation and ubiquitination in the cytoplasm through a pathway dependent on Histone Deacetylase 6 (HDAC6) (Hebron *et al.*, 2013). Furthermore, stress, particularly oxidative stress (OS), recruits wild-

type and ALS-associated mutant FUS and TDP-43 to aggregate into cytoplasmic stress granules (Colombrita *et al.*, 2009; Johnson *et al.*, 2009; Bosco *et al.*, 2010a; Dormann *et al.*, 2010; Dewey *et al.*, 2011; Parker *et al.*, 2012; Sama *et al.*, 2013). In particular, OS causes cysteine oxidation of TDP-43 and subsequent formation of disulphide cross-links, which increase levels of insoluble TDP-43 (Cohen *et al.*, 2012). OS is a central feature of ALS, and stress granule markers have been found to colocalise with FUS-positive and TDP-43-positive aggregates in the spinal cord of ALS patients, suggesting that long-term stress can lead to pathological inclusions of FUS and TDP-43 in ALS (Volkening *et al.*, 2009; Barber and Shaw, 2010; Dormann *et al.*, 2010; Bentmann *et al.*, 2012).

Recent work has identified a previously uncharacterised protein oxidation resistance 1 (Oxr1) that protects neurons against OS-induced apoptosis through an unknown function of the highly conserved TLDc domain (Oliver *et al.*, 2011). In addition, OXR1 expression is up-regulated in the spinal cord of ALS patient and pre-symptomatic $SOD1^{G93A}$ ALS mice, suggesting that up-regulation of Oxr1 could be both an early neuroprotective response and an early marker of oxidative stress (Oliver *et al.*, 2011). Although Oxr1 directly interacts with reactive oxygen species (ROS), Oxr1 does not function as an enzyme, suggesting that in response to increased levels of ROS, Oxr1 may regulate downstream pathways to protect neurons against OS-mediated cell death. Interestingly, FUS and TDP-43 bind to the shortest Oxr1 isoform that still contains the TLDc domain (Oxr1-C), while FUS also interacts with the full-length Oxr1 isoform (Oxr1-FL) *in vivo* and under OS conditions *in vitro* (Figure 1.2) (Finelli, 2010). While the interaction between Oxr1-C and FUS or TDP-43 is not dependent on presence of RNA, pathogenic mutations in FUS and TDP-43 that are linked to ALS can alter binding affinity to Oxr1-C (Figure 1.2-1.3; 3.1) (Finelli, 2010). In particular, FUS^{P517L}, $TDP\text{-}43^{A321G}$, and $TDP\text{-}43^{D169G}$ showed significantly decreased interaction with Oxr1-C (Figure 1.3; 3.1)

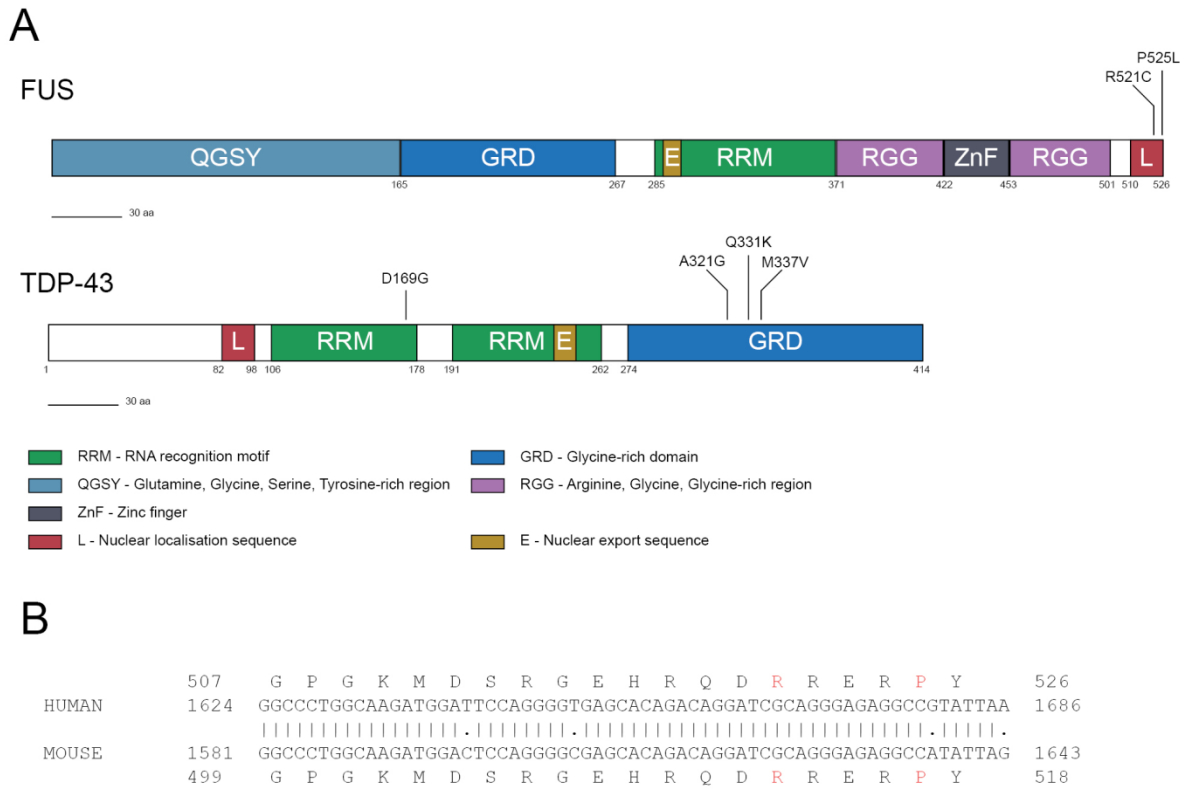

Figure 3.1. Schematic representation of FUS and TDP-43
(**A**) Schematic diagram of FUS and TDP-43 domains, and the localisation of ALS-linked mutations in FUS and TDP-43 that are used in the study. (**B**) Protein and nucleotide sequence conservation between human (top: GenBank accession number NM_004960.3) and mouse (bottom: accession number NM_139149.2), showing the correspondence between human and mouse amino acid sequence number for the ALS-associated mutations (in red).

(Finelli, 2010). Little is known about the mechanisms by which Oxr1 confers protection against OS damage, and whether Oxr1 participates in FUS- and TDP-43-associated pathways remains unknown.

3.1.1 Aims of chapter 3

Contributions of OS to pathobiology of ALS are well-documented, and OS has been shown to regulates the localisation of ALS-associated mutant FUS and TDP-43 (Barber and Shaw, 2010; Ferraiuolo *et al.*, 2011; Fiesel and Kahle, 2011). Furthermore, given that Oxr1 interacts with FUS and TDP-43, and that some ALS mutations in FUS and TDP-43 interfere with this interaction, Oxr1 may provide an important link in understanding the connection between OS and ALS pathogenesis. The goal of this chapter was to begin characterisation of Oxr1 function in concert with FUS and TDP-43 in response to OS, and in particular, to gain insight into the novel Oxr1 antioxidant network.

3.2 Results

3.2.1 Loss of *OXR1* increases cytoplasmic aggregation of FUS and TDP-43 under oxidative stress

Recent studies have shown that ALS-linked mutations in FUS and TDP-43 accentuate the intrinsic propensity of FUS and TDP-43 to form cytoplasmic aggregates, particularly under OS conditions (Colombrita *et al.*, 2009; Johnson *et al.*, 2009; Bosco *et al.*, 2010a; Fushimi *et al.*, 2011; Gal *et al.*, 2011; Kino *et al.*, 2011; McDonald *et al.*, 2011; Sun *et al.*, 2011; Parker *et al.*, 2012). Therefore, I first investigated whether Oxr1 is necessary for proper cellular localisation of FUS and TDP-43. Consistent with previous reports, I found that under H_2O_2-induced stress, wild-type FUS and TDP-43 form cytoplasmic aggregates and in particular, preferentially localise to stress granules, as

labelled by T-cell-restricted intracellular antigen-1 (TIA-1), a marker for these structures (Figure 3.2A-B) (Kedersha *et al.*, 1999). Interestingly, knock-down of *OXR1* isoforms by shRNA resulted in a significant increase in the percentage of HeLa cells with FUS- and TDP-43-positive cytoplasmic inclusion bodies after H_2O_2 treatment (Figure 3.2C). The shRNA construct has been previously shown to knock-down *OXR1* by 85% (Mattéa Finelli, unpublished data). No difference in localisation of FUS and TDP-43 was observed under non-stressed conditions (data not shown). I confirmed, however, that the percentage of cells with TIA-1-positive stress granules is not affected by knock-down of *OXR1* (Figure 3.2D), suggesting that increased mislocalisation of FUS and TDP-43 after loss of OXR1 is not due to alterations in stress granule formation.

Next, I examined whether loss of Oxr1 *in vivo* leads to cytoplasmic localisation of FUS and TDP-43 by conducting immunocytochemistry on cerebellum and cortex tissue of end-stage bella (*bel*) mice. In contrast with *in vitro* results, I was unable to identify neurons in either *bel* cerebellum or cortex expressing mislocalised FUS or TDP-43 (Figure 3.3A-B). Because previous findings suggest that only a small, but significant percentage of cerebellar granule cells experience DNA damage and undergo apoptosis *in vivo* (Oliver *et al.*, 2011), I further investigated whether exogenous OS treatment alters localisation of FUS or TDP-43 in cerebellar granule cells (GCs). Similar to *in vivo* findings, even after treatment with H_2O_2, I was unable to observe any striking changes in localisation of FUS or TDP-43 in *bel* cerebellar GCs after 17 days in culture (Figure 3.4A-B). Taken together, these results suggest that loss of *Oxr1* increases cytoplasmic mislocalisation of FUS and TDP-43 under OS *in vitro*; however, localisation of FUS and TDP-43 is not altered in end-stage *bel* cerebellar and cortical neurons.

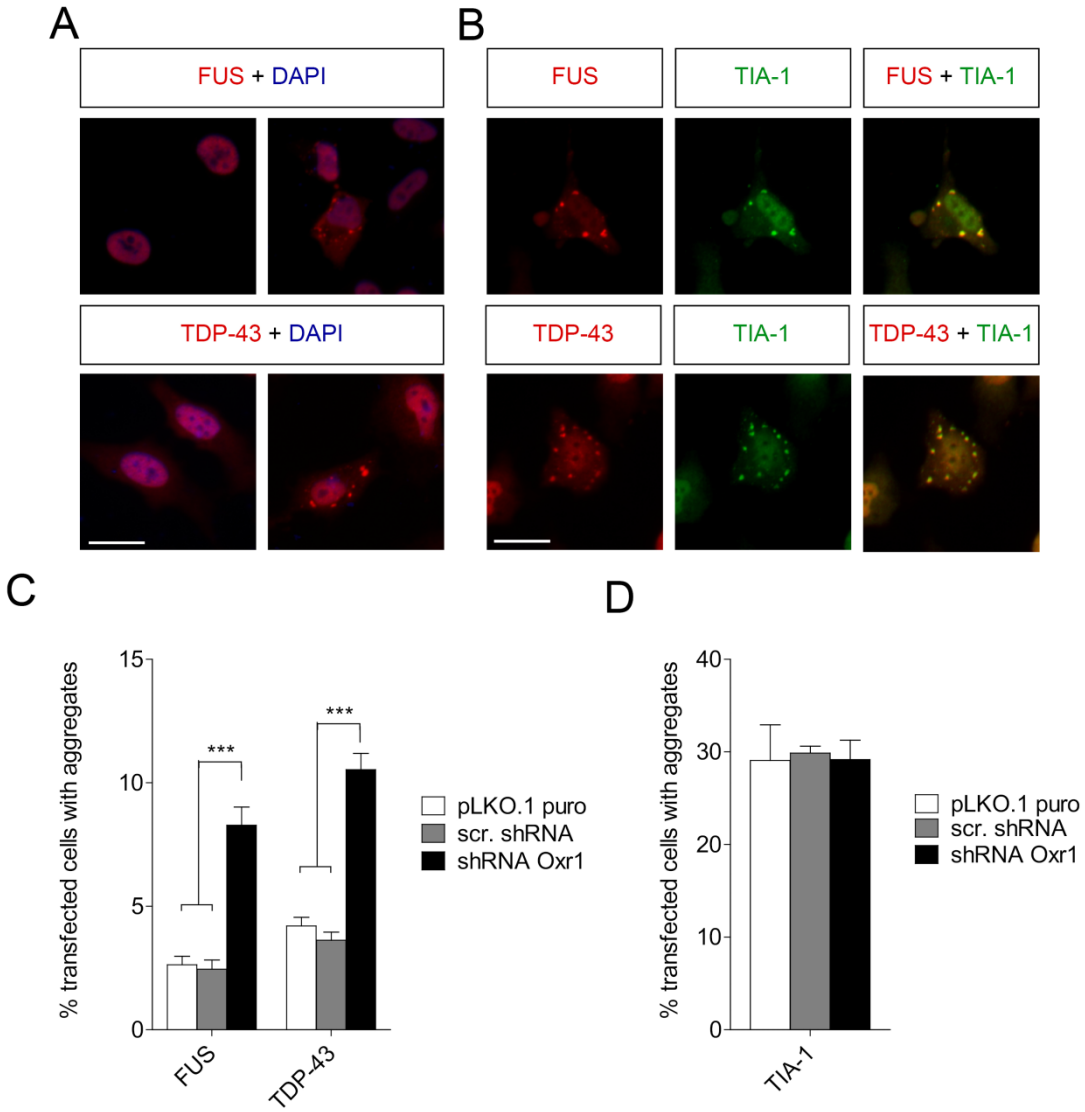

Figure 3.2. Knockdown of *OXR1* increases cytoplasmic aggregation of FUS and TDP-43 under H_2O_2-induced stress in HeLa cells

(**A**) Representative images of FUS and TDP-43 cytoplasmic aggregation under H_2O_2 treatment. (**B**) FUS and TDP-43 are recruited to TIA-1 positive stress granules under H_2O_2 treatment. (**C**) Quantification of cells forming FUS and TDP-43 aggregates under H_2O_2 treatment when transfected with *OXR1* shRNA or control constructs. (**D**) Quantification of cells forming TIA-1-positive aggregates under H_2O_2 treatment when transfected with *OXR1* shRNA or control constructs. (**A-B**) Scale bars: 25 µm. (**C-D**) Results are presented as mean ± SEM from 4 independent experiments. Statistical significance between co-transfection with pLKO.1 puro, scrambled (scr) shRNA, or shRNA for OXR1 was determined by one-way ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

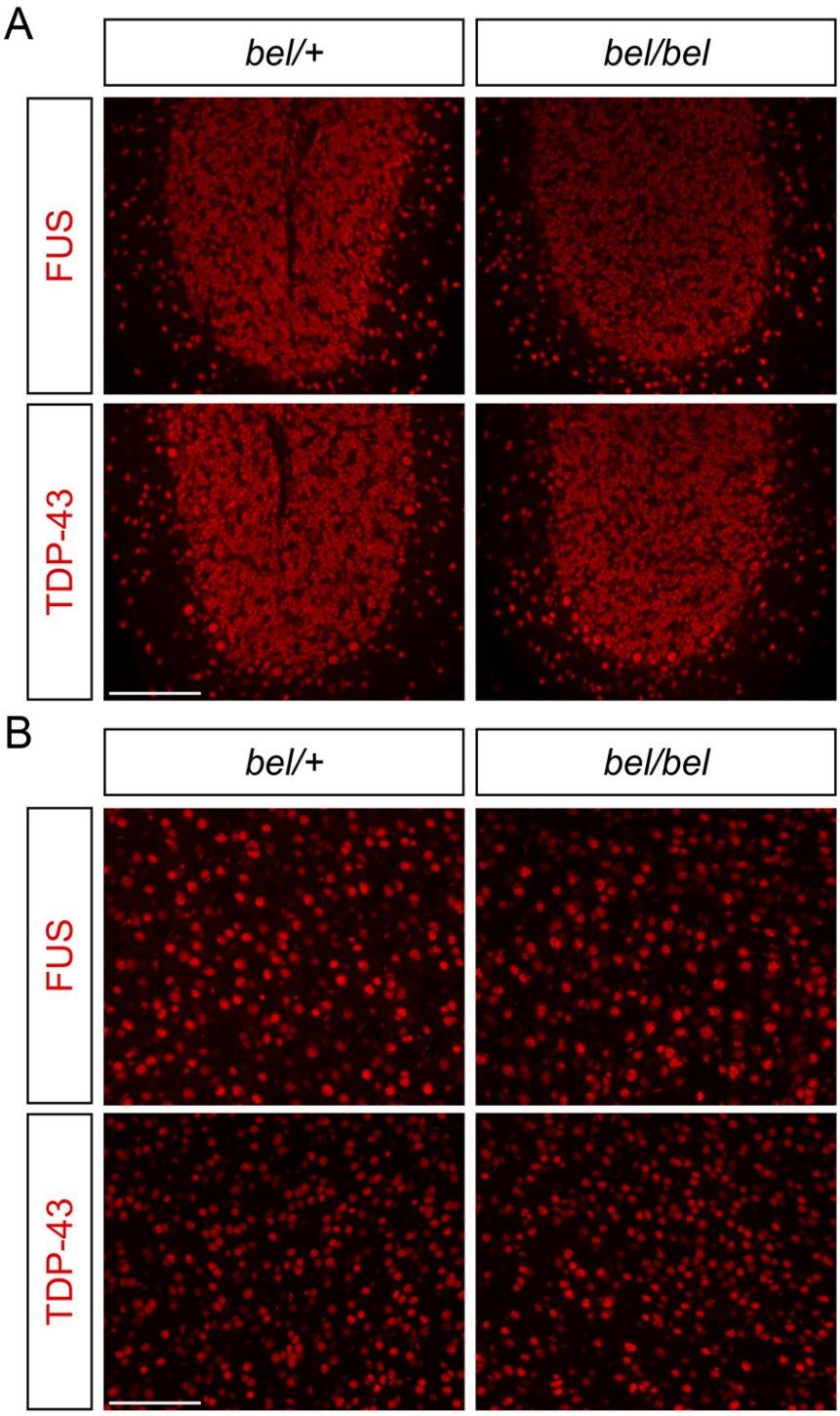

Figure 3.3. Expression of FUS and TDP-43 is not altered in *bel* mutant mice
FUS and TDP-43 expression in *bel*/+ and *bel/bel* mutant cerebellum is not altered and no cytoplasmic aggregation is observed. (**B**) FUS and TDP-43 expression in *bel*/+ and *bel/bel* mutant cortex remains unaltered and no cytoplasmic aggregation is observed. Scale bars: (**A-B**) 100 μm.

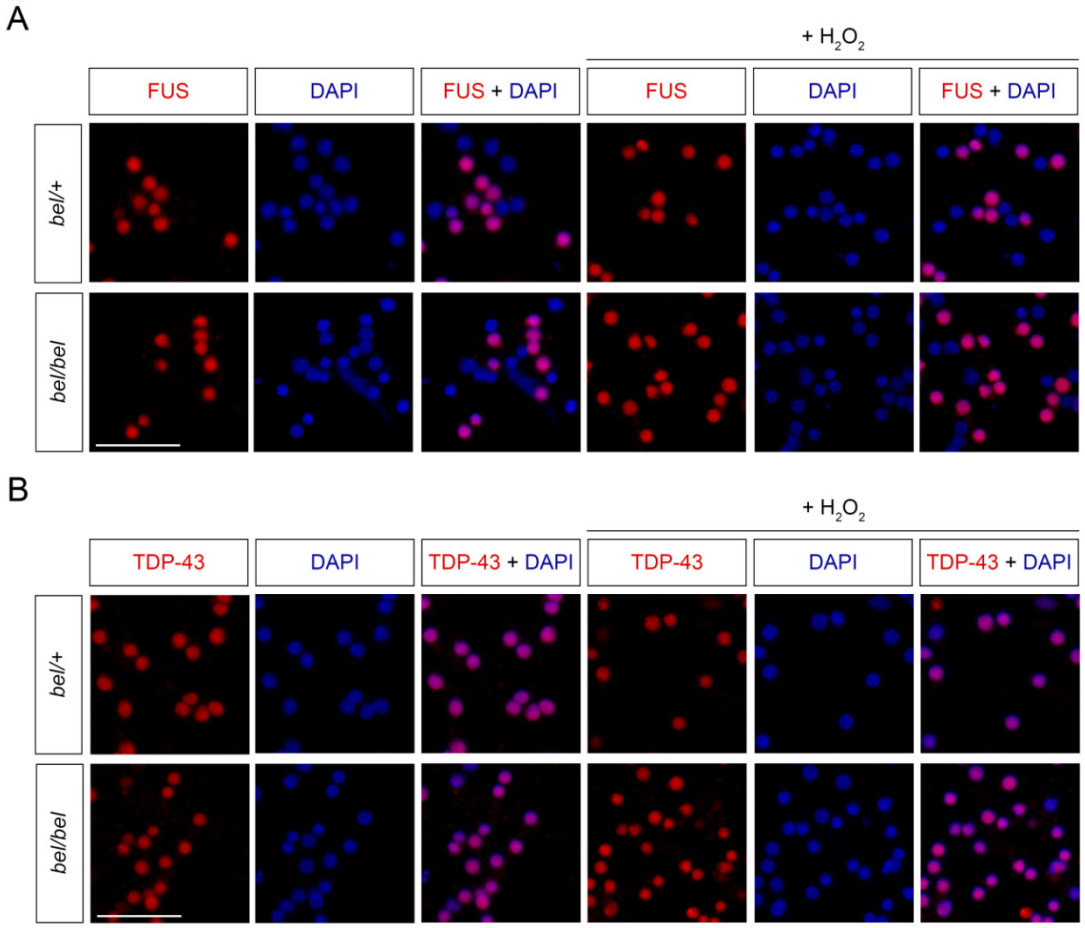

Figure 3.4. Loss of Oxr1 in *bel* mutants does not affect cellular localisation of wild-type FUS and TDP-43 in primary cerebellar granule cell cultures
(**A**) Localisation of wild-type FUS is not altered in primary culture of bel cerebellar granule cells with or without H_2O_2 treatment. (**B**) Localisation of wild-type TDP-43 is not altered in primary culture of *bel* cerebellar granule cells with or without H_2O_2 treatment. (**A-B**) Scale bar: 50 µm.

3.2.2 Over-expression of Oxr1-C reduces recruitment of wild-type and mutant FUS and TDP-43 to inclusion bodies in the cytoplasm under oxidative stress

I next examined whether over-expression of Oxr1-C alters cellular localisation of wild-type and ALS-linked mutant FUS and TDP-43 under OS. First, I confirmed findings from previous studies, that under OS conditions, transfected wild-type and mutant FUS and TDP-43 localise to cytoplasmic stress granules, labelled by TIA-1 (Figure 3.5A,C). Furthermore, consistent with a recent report, I found that FUS^{P517L} mutant is also occasionally recruited to processing bodies, labelled with mRNA-decapping enzyme 1A (DCP1A), a marker for processing bodies (Figure 3.5B) (Cougot *et al.*, 2004; Kino *et al.*, 2011). Interestingly, I found that over-expression of Oxr1-C with wild-type FUS and TDP-43 significantly reduces accumulation of FUS- and TDP-43-positive cytoplasmic inclusions after treatment with H_2O_2 and arsenite, both of which induce OS (Figure 3.6). However, over-expression of Oxr1-C does not affect the normal formation of stress granules under these conditions (Figure 3.6A). I found that over-expression of Oxr1-C significantly decreases cytoplasmic aggregation of FUS^{R513C}, TDP-43^{Q331K}, and TDP-43^{M337V} mutants (Figure 3.6). Interestingly, recruitment of FUS^{P517L}, TDP-43^{A321G}, and TDP-43^{D169G}, three mutants with reduced interaction with Oxr1-C, to cytoplasmic inclusions is not altered in the presence of Oxr1-C over-expression (Figure 3.6). Taken together, these findings suggest that interaction between Oxr1 and FUS or TDP-43 is responsible for proper cellular localisation of FUS and TDP-43, and over-expression of Oxr1 reduces mislocalisation of certain ALS-linked mutant FUS and TDP-43.

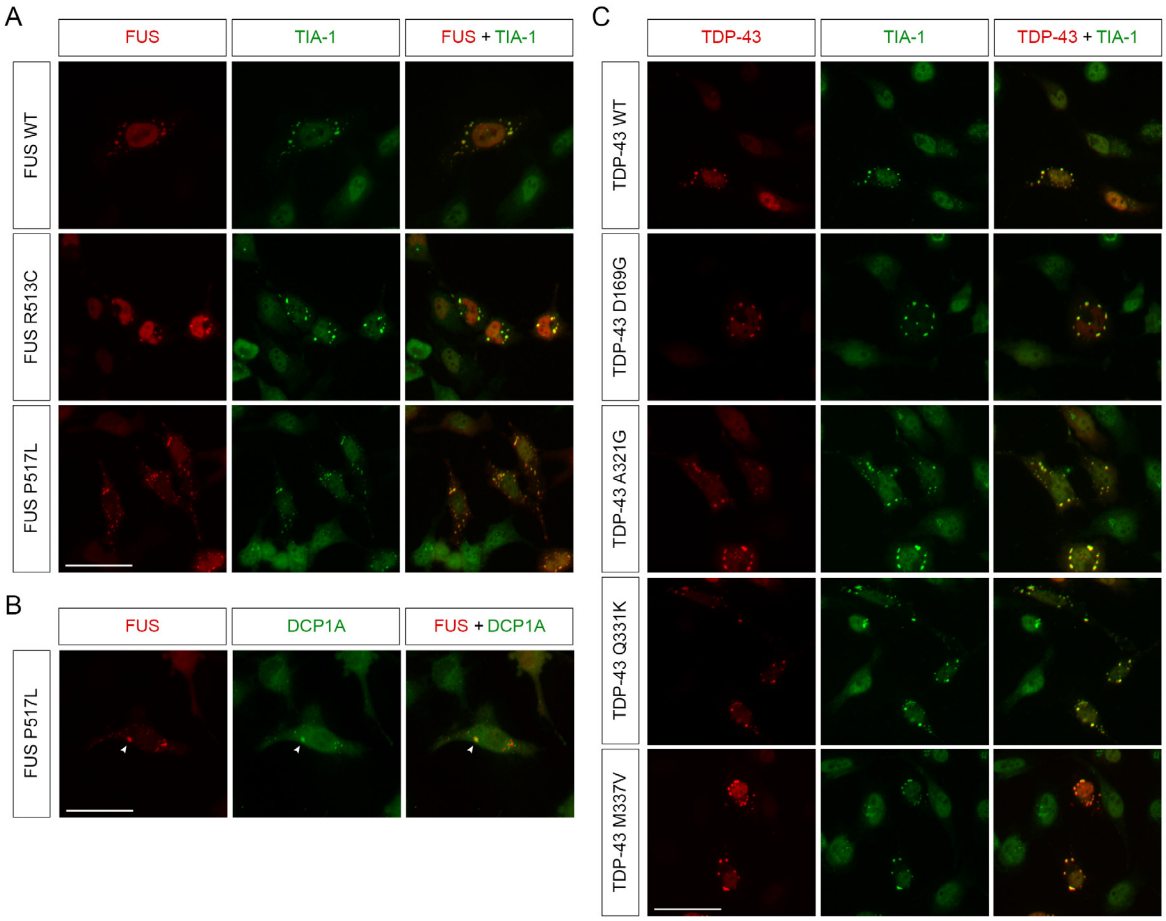

Figure 3.5. Wild-type and ALS-linked mutant FUS and TDP-43 localise to stress granules and processing bodies under oxidative stress conditions in HeLa cells
(**A**) Representative images of co-localisation of cytoplasmic aggregation of FUS wild-type and mutants with TIA-1-positive stress granules under H_2O_2 treatment. (**B**) Representative images of co-localisation of cytoplasmic aggregation of FUS^{P517L} mutant with DCP1A-positive processing bodies under H_2O_2 treatment. (**C**) Representative images of co-localisation of cytoplasmic aggregation of TDP-43 wild-type and mutants with TIA-1-positive stress granules under H_2O_2 treatment. (**A-C**) Scale bars: 50 µm.

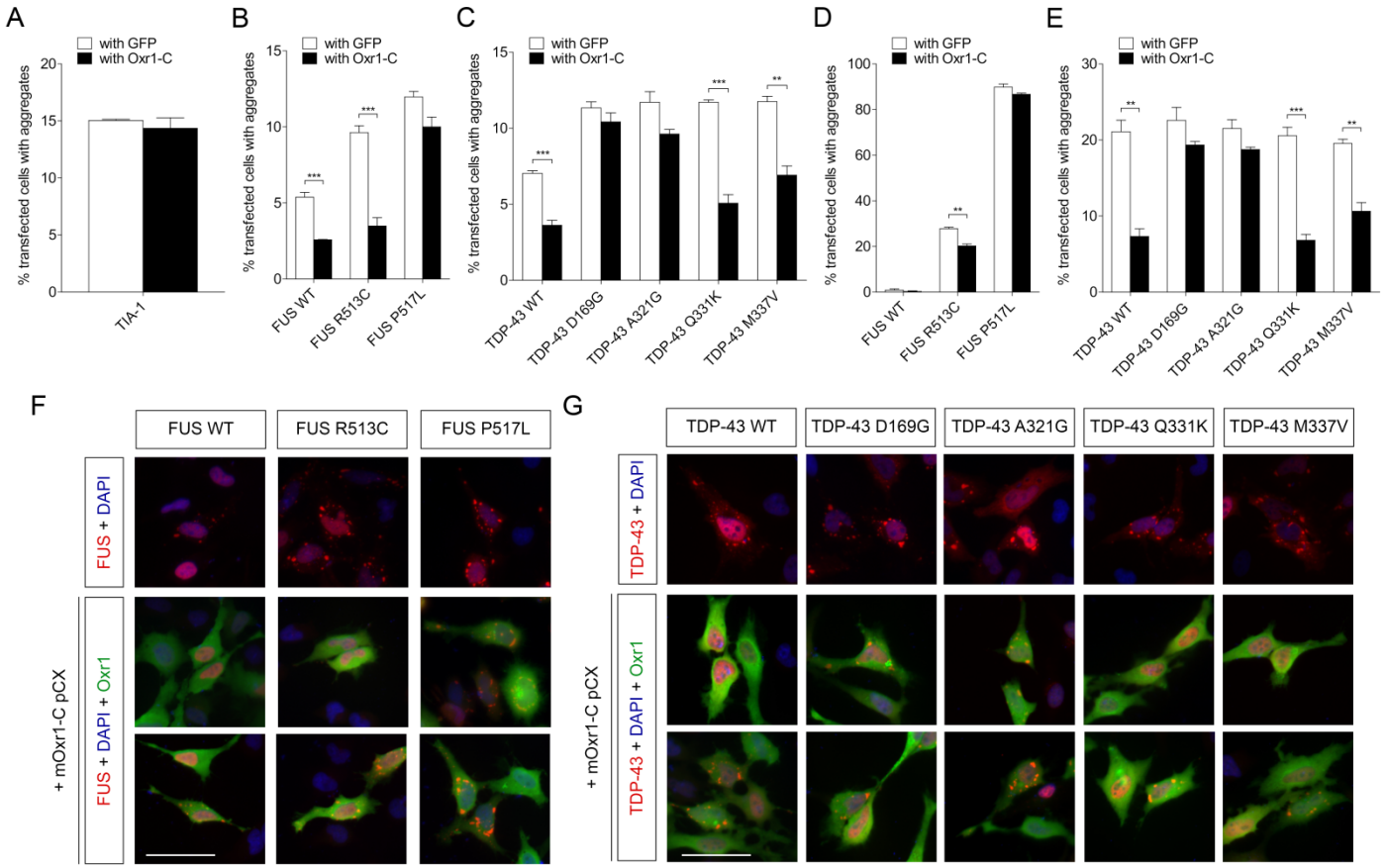

Figure 3.6. Over-expression of Oxr1 decreases recruitment of wild-type and ALS-linked mutant FUS and TDP-43 to cytosolic inclusion bodies

(**A**) Quantification of cells forming TIA-1-positive aggregates under H_2O_2 treatment when transfected with Oxr1-C. (**B**) Quantification of cells forming aggregates under H_2O_2 treatment when co-transfected with wild-type and mutant FUS, and with either GFP or Oxr1-C. (**C**) Quantification of cells forming aggregates under H_2O_2 treatment when co-transfected with wild-type and mutant TDP-43, and with either GFP or Oxr1-C. (**D**) Quantification of cells forming aggregates under arsenite treatment when co-transfected with wild-type and mutant FUS, and with either GFP or Oxr1-C. (**E**) Quantification of cells forming aggregates under arsenite treatment when co-transfected with wild-type and mutant TDP-43, and with either GFP or Oxr1-C. (**A-E**) Results are presented as mean ± SEM from 3 independent experiments. Statistical significance was determined by two-tailed unpaired Student's t-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (**F**) Representative images of cytoplasmic aggregation of wild-type and mutant FUS under H_2O_2 treatment. Over-expression of Oxr1-C significantly reduces aggregate formation for FUS^{WT} and FUS^{R513C}. (**G**) Representative images of cytoplasmic aggregation of TDP-43 wild-type and mutants under H_2O_2 treatment. Over-expression of Oxr1-C significantly reduces aggregate formation for $TDP\text{-}43^{WT}$, $TDP\text{-}43^{Q331K}$, and $TDP\text{-}43^{M337V}$. (**F-G**) Scale bars: 25 µm.

3.2.3 Oxr1-C decreases FUS and TDP-43 cytoplasmic inclusions independent of proteasome or autophagy degradation pathways

Proteasome and autophagy pathways have been implicated in the clearance of FUS and TDP-43 (Perrotti *et al.*, 2000; Kim *et al.*, 2009; Urushitani *et al.*, 2010; Wang *et al.*, 2010; Brady *et al.*, 2011; van Eersel *et al.*, 2011), therefore, I next examined whether over-expression of Oxr1-C targets FUS and TDP-43 for degradation. To investigate whether Oxr1-C function is dependent on proteasome pathways, I treated transfected HeLa cells with MG-132, a proteasome inhibitor, before subjecting the cells to exogenous peroxide-induced OS (van Eersel *et al.*, 2011). MG-132 reversibly binds to and inhibits the chymotrypsin-like site in the β5 subunit, and the caspase-like site in the β1 subunit at higher concentrations (Goldberg, 2012). I found that inhibition of proteasome function significantly increases cytoplasmic aggregation of mutant FUS and TDP-43, confirming mutant FUS and TDP-43 are degraded by proteasome pathways (Figure 3.7A-B). In addition, I found that MG-132 treatment only leads to a significant increase in the recruitment of wild-type TDP-43 to stress granules, suggesting wild-type FUS is degraded through an alternative mechanism (Figure 3.7A-B). Interestingly, even in the presence of proteasome inhibition, over-expression of Oxr1-C significantly reduces wild-type and mutant FUS and TDP-43 cytoplasmic aggregates (Figure 3.7A-B).

Next, to examine whether Oxr1-C function is dependent on autophagy, I treated transfected HeLa cells with concanamycin A, an autophagy-lysosomal protein degradation inhibitor, before stressing the cells with H_2O_2 (Kim *et al.*, 2009). Concanamycin A is a potent inhibitor of V-ATPases, which are proton pumps that regulate the acidification of the lysosome, a central component of autophagic processes (Huss *et al.*, 2002; Mijaljica *et al.*, 2011). In the absence of autophagy-dependent lysosomal protein degradation, I found a significant increase in the percentage of transfected cells with FUS- and TDP-43-positive

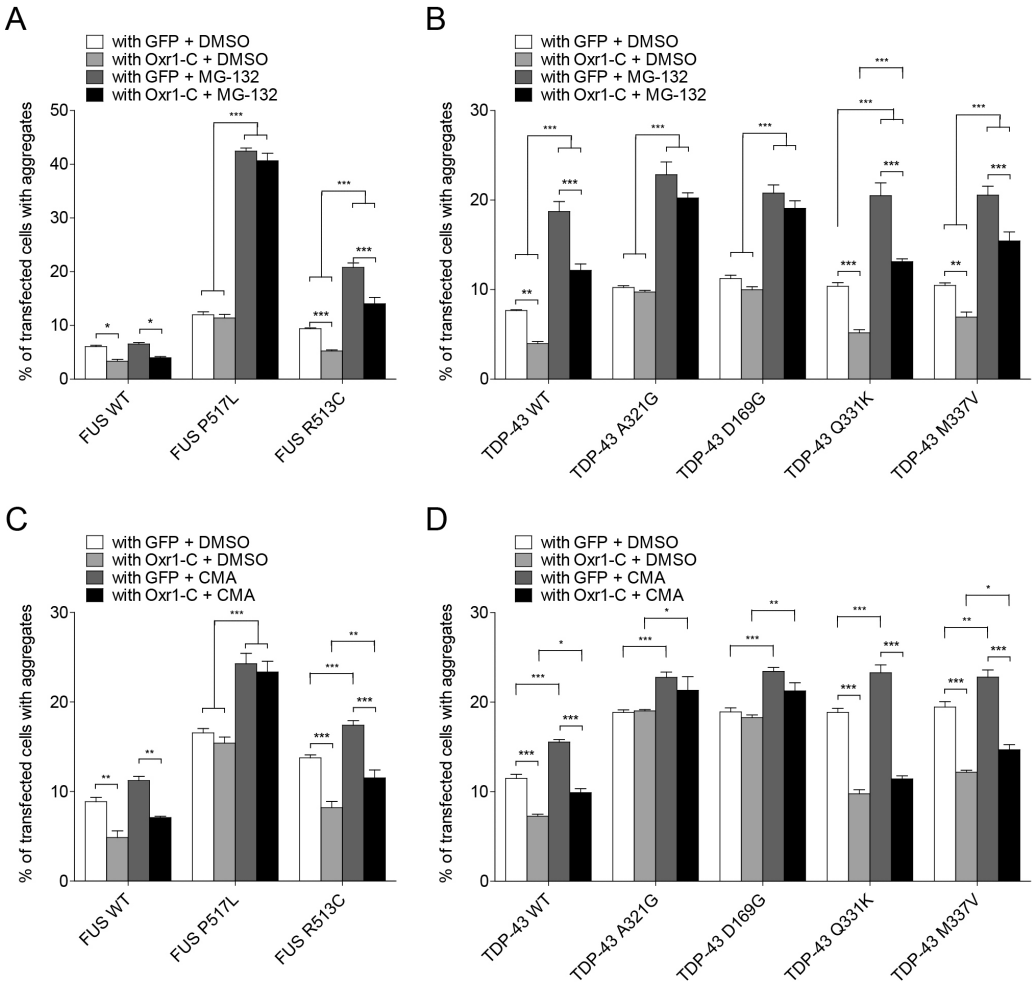

Figure 3.7. Reduction of cytoplasmic aggregation of wild-type and ALS-linked mutant FUS and TDP-43 by Oxr1-C does not depend on proteasome degradation or autophagy

(**A**) Quantification of HeLa cells forming aggregates under H_2O_2 treatment when co-transfected with FUS wild-type and mutants, or (**B**) TDP-43 wild-type and mutants, and with either GFP or Oxr1-C, after prior MG-132 treatment. (**A**) Quantification of HeLa cells forming aggregates under H_2O_2 treatment when co-transfected with FUS wild-type and mutants, or (**B**) TDP-43 wild-type and mutants, and with either GFP or Oxr1-C, after prior concanamycin A treatment. (**A-D**) Results are presented as mean ± SEM from 3 independent experiments. Statistical significance between co-transfection with GFP or Oxr1-C was determined by two-way ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

cytoplasmic inclusions for mutant FUS, and wild-type and mutant TDP-43, but no change for wild-type FUS-positive inclusions (Figure 3.7C-D). These findings suggest that while degradation of mutant FUS, and wild-type and mutant TDP-43 occurs through a mechanism involving autophagy, degradation of wild-type FUS is independent of autophagy (Figure 3.7C-D). In addition, I found that inhibition of autophagy does not ablate over-expression of Oxr1-C function in significantly decreasing the recruitment of wild-type and mutant FUS and TDP-43 to cytoplasmic aggregates (Figure 3.7C-D).

Noticeably, the percentage of transfected cells with cytoplasmic aggregation of FUS or TDP-43 mutants is not significantly reduced by over-expression of Oxr1-C after proteasome or autophagy inhibition for FUS^{P517L}, $TDP\text{-}43^{A321G}$, and $TDP\text{-}43^{D169G}$ mutants (Figure 3.7), which remains consistent with previous findings that these particular mutations impair binding with Oxr1-C. Taken together, these data demonstrate that Oxr1-C is able to decrease FUS and TDP-43 cytoplasmic aggregation in a manner that is independent of proteasome or autophagy protein degradation pathways.

3.2.4 FUS, TDP-43 and Oxr1-C interact with PRMT1, and Oxr1-C function depends on PRMT1 activity

Previous studies have found that arginine methylation of FUS by PRMT1 is important for FUS function and nuclear localisation (Du *et al.*, 2011; Tradewell *et al.*, 2011). Furthermore, because PRMT1 has also been identified as a binding partner of Oxr1-C (Finelli, 2010), I sought to examine whether PRMT1 function underlies mechanisms by which Oxr1 mediates cellular localisation of wild-type and mutant FUS and TDP-43. First, I investigated whether FUS, TDP-43, and Oxr1-C interact with PRMT1 by co-immunoprecipitation studies in N2A cells co-transfected with PRMT1 and either FUS, TDP-43, or Oxr1-C. Interestingly, I found that all three proteins, FUS, TDP-43, and Oxr1-

C bind to PRMT1 (Figure 3.8A-C). PRMT1 is a well-studied protein responsible for the majority of arginine methylation events in the cell (Gary *et al.*, 1996; Tang *et al.*, 2000); thus, I next tested the hypothesis that PRMT1 methylates arginine residues on FUS, TDP-43, and Oxr1-C. However, only FUS contains methylated arginine residues with or without OS (Figure 3.8D-E). These results suggest that interactions between PRMT1 and TDP-43 or Oxr1-C may be responsible for regulating downstream PRMT1 function.

To examine whether Oxr1-C function is dependent on PRMT1 activity, I treated transfected HeLa cells with AMI-1, a PRMT1-specific inhibitor, before treating the cells with H_2O_2 (Cheng *et al.*, 2004). In the absence of PRMT1 function, I reported similar findings of a small but significant increase in the number of transfected cells with FUS-positive cytoplasmic inclusions for wild-type and mutant FUS, but no difference in cytoplasmic aggregation of TDP-43 (Figure 3.8G-H) (Tradewell *et al.*, 2011). Interestingly, I found that inhibition of PRMT1 function prevents Oxr1-C from significantly decreasing cytoplasmic aggregation of wild-type and mutant FUS and TDP-43 (Figure 3.8G-H). I confirmed, however, that while treatment with AMI-1 is sufficient to inhibit PRMT1 function, evidenced by the decrease in endogenous FUS methylation, treatment with AMI-1 24 hours after transfection does not significantly alter methylation of over-expressed FUS (Figure 3.8F). In addition, inhibition of PRMT1 activity did not alter binding between FUS or TDP-43 with Oxr1-C (Figure 3.8I-J). Therefore, loss of Oxr1-C function under PRMT1 inhibition is not due to changes in methylation of FUS or altered binding between TDP-43 or FUS, and Oxr1-C. Taken together, these data indicate that proper function of PRMT1 is important for Oxr1-C function under OS.

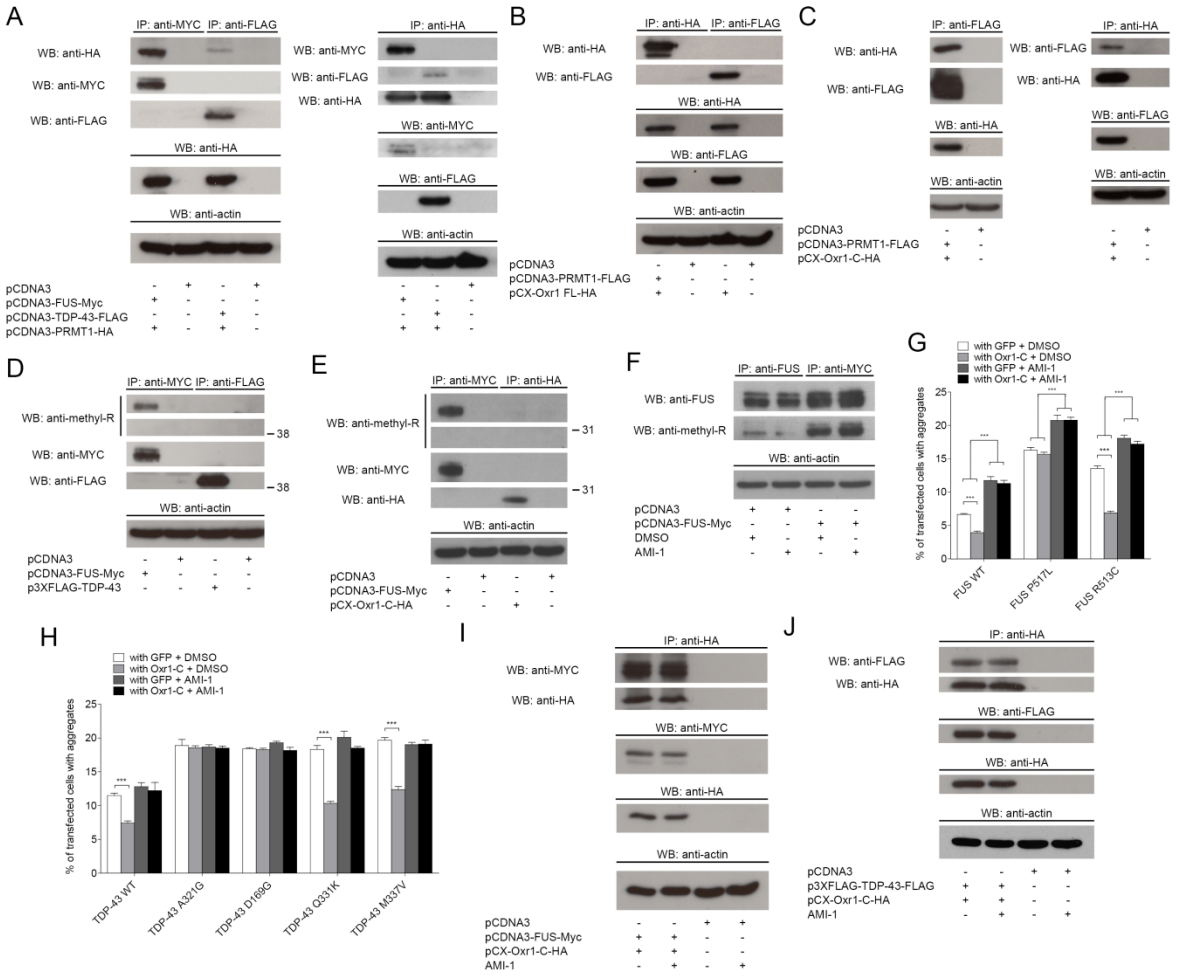

Figure 3.8. Reduction of cytoplasmic aggregation of wild-type and ALS-linked mutant FUS and TDP-43 by Oxr1-C depends on arginine methylation

(A) Co-IP of FUS and TDP-43 with PRMT1 in co-transfected N2A cells demonstrates FUS and TDP-43 interact with PRMT1. (**B**) Co-IP of Oxr1-FL and PRMT1 in co-transfected N2A cells shows that Oxr1-FL does not bind with PRMT1. (**C**) Co-IP of Oxr1-C and PRMT1 in co-transfected N2A cells shows that Oxr1-C interacts with PRMT1. Arginine residues of FUS, but not of TDP-43 (**D**) or Oxr1-C (**E**) are methylated. (**F**) Methylation of endogenous FUS is decreased, but methylation of transfected FUS is not altered after treatment with AMI-1, a potent inhibitor of PRMT1 function. (**G**) Quantification of cells forming aggregates under H_2O_2 treatment when co-transfected with FUS wild-type and mutants (R513C and P517L), and with either GFP or Oxr1-C with and without AMI-1 treatment. (**H**) Quantification of cells forming aggregates under H_2O_2 treatment when co-transfected with TDP-43 wild-type and mutants (D169G, A321G, Q331K, and M337V), and with either GFP or Oxr1-C with and without AMI-1 treatment. (**G-H**) Results are presented as mean ± SEM from 3 independent experiments. Statistical significance between co-transfection with GFP or Oxr1-C was determined by two-way ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Inhibition of PRMT1 activity by AMI-1 does not alter binding between Oxr1-C and FUS (**I**) or TDP-43 (**J**).

3.2.5 The TLDc domain is important for Oxr1-C-mediated proper localisation of FUS and TDP-43

The highly conserved TLDc domain is sufficient to protect neurons against OS and mutating a highly conserved glutamic acid residue in the TLDc domain to an alanine (EA mutant) renders Oxr1-C unable to confer protection in neurons against OS-induced apoptosis (Figure 3.9) (Finelli, 2010; Oliver *et al.*, 2011). The glutamic acid residue was originally chosen because this amino acid frequently plays an important role in the active site of enzymes (Takahashi *et al.*, 1967; Harris and Wilson, 1983; Hovde *et al.*, 1988; Liu *et al.*, 1996a; Finelli, 2010). To more closely investigate whether the TLDc domain is important for Oxr1-C function, I examined whether over-expression of Oxr1 EA-C still decreases cytoplasmic aggregation of wild-type and mutant FUS and TDP-43. I found that the percentage of transfected cells with FUS- and TDP-43-positive cytoplasmic inclusion bodies is not significantly reduced by over-expression of Oxr1 EA-C mutant, even for wild-type and ALS-linked mutations that do not alter binding with Oxr1-C (Figure 3.10A-B). I next examined whether loss of function in the Oxr1 EA-C mutant was due to impaired interactions with Oxr1 binding partners. However, I found that binding between Oxr1 EA-FL mutant and FUS, and between Oxr1 EA-C mutant and FUS, TDP-43, and PRMT1 is not altered (Figure 3.10C-D). Taken together, these data suggest that the highly conserved glutamate residue is crucial to proper Oxr1 function, but does not alter Oxr1 binding to its target proteins.

3.3 Discussion

Uncovering the pathogenic mechanisms underlying ALS will be particularly important for future therapeutic strategies. Here, I reported that knockdown of *OXR1* significantly increases cytoplasmic aggregation of wild-type FUS and TDP-43 under OS,

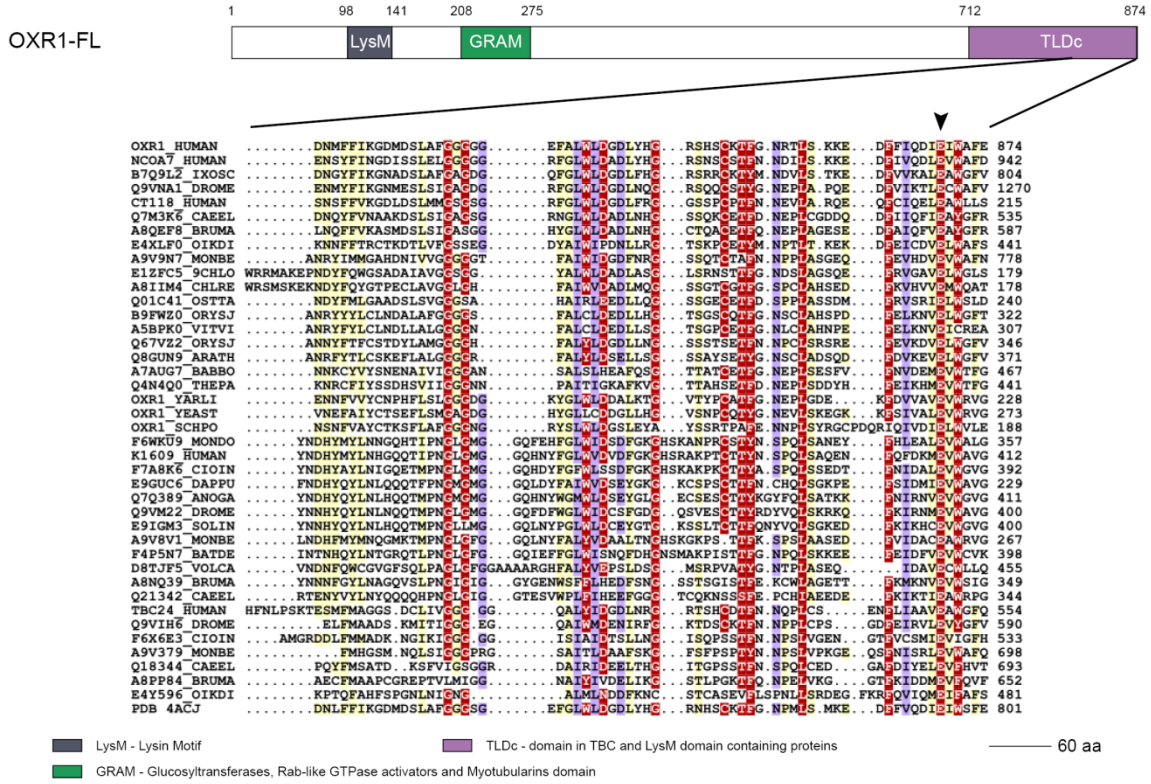

Figure 3.9. Schematic of the C-terminal end of the highly conserved TLDc domain
Sequence alignment of the C-terminal end of OXR1 and other TLDc domain family proteins. The glutamic acid residue mutated in Oxr1 EA-C is indicated with the arrow. Sequence alignment was performed and generously provided by Luis Sanchez-Pulido.

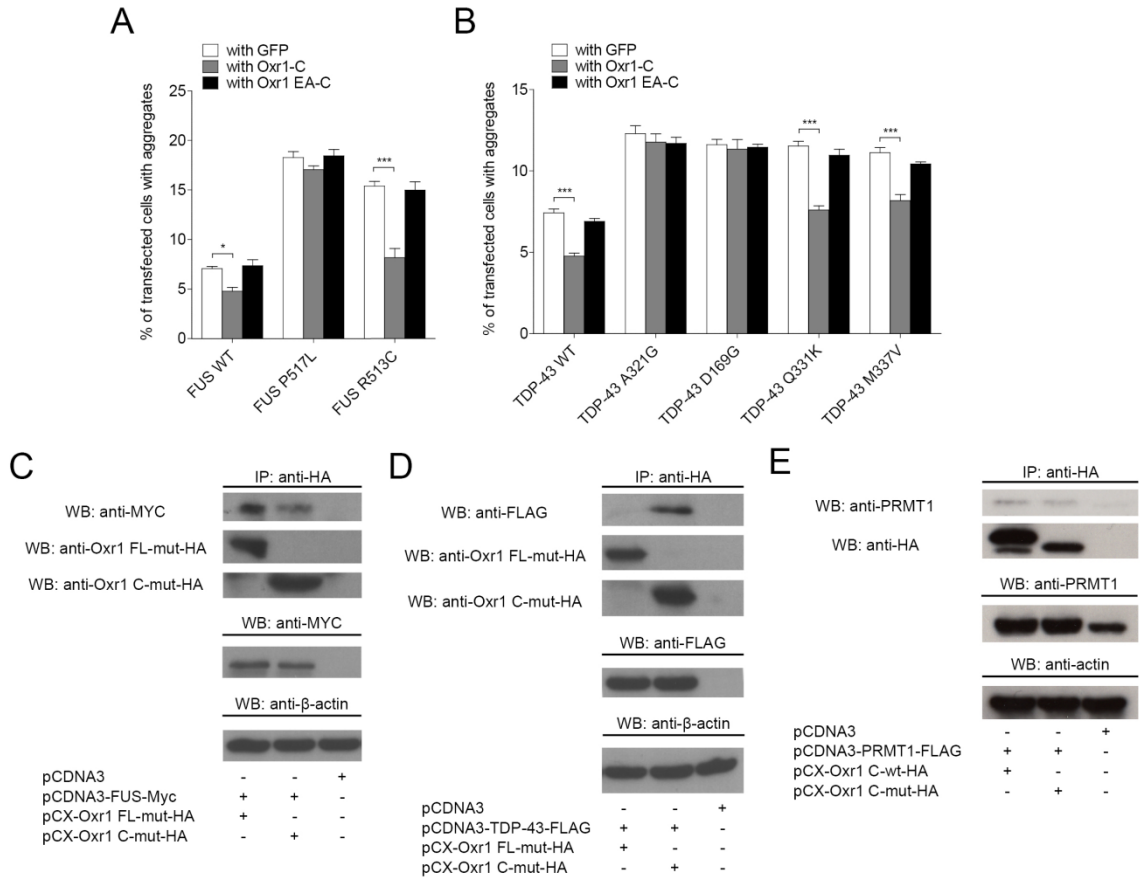

Figure 3.10. Mutation in highly conserved TLDc domain impairs Oxr1 function
(**A**) Quantification of cells forming aggregates under H_2O_2 treatment when co-transfected with FUS wild-type and mutants (R513C and P517L), and with either GFP, Oxr1-C, or Oxr1-EA-C. (**B**) Quantification of cells forming aggregates under H_2O_2 treatment when co-transfected with TDP-43 wild-type and mutants (D169G, A321G, Q331K, and M337V), and with either GFP, Oxr1-C, or Oxr1-EA-C (**A-B**) Results are presented as mean ± SEM from 3 independent experiments. Statistical significance between co-transfection with GFP, Oxr1, or Oxr1-C EA mutant was determined by two-way ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Co-IP of FUS (**C**), TDP-43 (**D**), or PRMT1 (**E**) with Oxr1-C and Oxr1 EA-C mutant in co-transfected N2A cells, demonstrating that interaction between FUS, TDP-43, or PRMT1 binding with Oxr1 is not altered by the EA mutation in the TLDc domain.

while over-expression of Oxr1 conversely ameliorates mislocalisation of wild-type and ALS-linked mutant FUS and TDP-43 in a manner dependent on PRMT1 arginine methylation activity. Furthermore, I demonstrated that the highly conserved TLDc domain plays an important role in regulating Oxr1-dependent cellular localisation of FUS and TDP-43 as over-expression of Oxr1 EA-C mutant no longer significantly reduces recruitment of wild-type and mutant FUS and TDP-43 to inclusion bodies in the cytoplasm. These experiments implicate Oxr1 as another protein responsible for proper nuclear localisation of both FUS and TDP-43, particularly under OS conditions, and a downstream pathway of PRMT1 to be involved in OS.

3.3.1 Oxr1 regulates nuclear export of FUS and TDP-43 under oxidative stress

Mutations in FUS and TDP-43 are responsible for approximately 10% of fALS cases and 1% of sALS cases, and cytosolic FUS and TDP-43 inclusions have been identified in patients harbouring these mutations (Arai *et al.*, 2006; Neumann *et al.*, 2006; Mackenzie *et al.*, 2007; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Mackenzie *et al.*, 2010). Mislocalised FUS and TDP-43 in cytoplasmic aggregations are also a pathological hallmark of sporadic ALS and a related neurological disorder, FTLD with ubiquitin-positive inclusions (Ito and Suzuki, 2011). Interestingly, stress granule markers, such as TIA-1, co-localise with the cytoplasmic inclusion bodies in patients with FUS and TDP-43 pathology (Colombrita *et al.*, 2009; Volkening *et al.*, 2009; Liu-Yesucevitz *et al.*, 2010; Ito and Suzuki, 2011). These findings and other *in vitro* studies find that cellular stress, such as OS, induces recruitment of FUS and TDP-43 to stress granules, and ALS-associated mutations lead to increased mislocalisation (Johnson *et al.*, 2009; Bosco *et al.*, 2010a; Liu-Yesucevitz *et al.*, 2010; Dewey *et al.*, 2011; Fushimi *et al.*, 2011; Ito and Suzuki, 2011; Kino *et al.*, 2011; McDonald *et al.*, 2011; Sun *et al.*, 2011; Parker *et al.*, 2012). The

mechanisms by which wild-type and mutant FUS and TDP-43 localise to cytoplasmic inclusion bodies are not well understood. Recent studies demonstrate that nuclear-cytoplasmic shuttling of FUS depends on a nuclear import receptor, Transportin, and arginine methylation by PRMT1 (Dormann *et al.*, 2010; Ito and Suzuki, 2011; Tradewell *et al.*, 2011), but little is known about the pathways regulating nuclear export of TDP-43.

I identified Oxr1 as a novel protein responsible for proper nuclear localisation of both FUS and TDP-43 by binding to them under OS conditions (Figure 1.2;3.2). In particular, knock-down of *OXR1* increases cytoplasmic aggregation of FUS and TDP-43, while over-expression of Oxr1 conversely decreases cytoplasmic aggregation of ALS mutant FUS and TDP-43 (Figure 3.2;3.6). While Oxr1 has previously been found in the mitochondria (Elliott and Volkert, 2004; Oliver *et al.*, 2011), Oxr1 appears to be a predominantly cytoplasmic protein when overexpressed, and preliminary data suggests that endogenous Oxr1 shuttles from the mitochondria into the cytoplasm during extended stress (Finelli, 2010). Therefore, findings that Oxr1 modulate FUS and TDP-43 localisation further corroborate evidence that suggests that Oxr1 plays an important role under OS in the cytoplasm.

Although knockdown of *OXR1* results in increased cytoplasmic aggregation of FUS and TDP-43 in HeLa cells under OS, no changes in cellular localisation of FUS and TDP-43 were observed in *bel* mice or in *bel* primary neuronal cultures treated with exogenous H_2O_2 stress (Figure 3.3-3.4). This discrepancy in findings may be due to a number of different limitations from the experimental design. Firstly, HeLa cells were originally selected for these experiments because previous studies have used this particular cell line to examine FUS and TDP-43 aggregation and localisation, the relatively large HeLa cell size made viewing aggregates easier, and HeLa cells are easily transfected with high efficiency (Dormann *et al.*, 2010; McDonald *et al.*, 2011; Parker *et al.*, 2012; Baron *et*

al., 2013). HeLa cells are an immortal cell line derived from cervical cancer cells (Scherer *et al.*, 1953). Thus, findings that OXR1 regulates FUS and TDP-43 localisation in HeLa cells may not translate faithfully to neurons *in vivo* due to differing cell types and because *in vitro* environments lack the complexity of cell-cell interactions and extracellular factors that exist *in vivo* (Toledo and Wahl, 2006). Conversely, there are also limitations from the *in vivo* and primary neuronal culture experiments. Given that onset of ALS symptoms generally begin in later decades of life, it is likely that endogenous FUS and TDP-43 pathology require extended amounts of OS and environmental stress (Dormann *et al.*, 2010). Therefore, because *bel* mice fail to survive beyond P26, *bel* cortical and cerebellar neurons may not have enough time and stress to induce FUS and TDP-43 mislocalisation. Similarly, primary cerebellar GC cultures were conducted at P7, and cultured until day *in vitro* 17 to correspond with end-stage *bel* mice before GC cultures were treated with exogenous peroxide stress. Thus, GC cultures may still have limited accumulation of stress necessary to observe localisation of FUS and TDP-43 to cytoplasmic aggregates. Finally, GC cultures may not be the best model to observe ALS-associated FUS and TDP-43 as degeneration of motor neurons is the central clinical feature of ALS patients. In the future, we can address this discrepancy to determine whether Oxr1 regulates FUS and TDP-43 localisation by confirming these findings *in vitro* neuronal cell lines, such as NSC-34 motor neuron-like cells (Cashman *et al.*, 1992), and by examining FUS and TDP-43 expression in a conditional knockout mouse model that specifically deletes *Oxr1* in spinal motor neurons and survives into adulthood; the conditional knockout mouse model will be discussed in detail in Chapter 6.

Furthermore, Oxr1 protects against ROS-induced apoptosis in neurons and OS levels can induce cytoplasmic aggregation of FUS and TDP-43 (Johnson *et al.*, 2009; Liu-Yesucevitz *et al.*, 2010; Ito and Suzuki, 2011; Kino *et al.*, 2011; Oliver *et al.*, 2011);

therefore, it could be alternatively possible that levels of Oxr1 expression regulate cellular levels of OS, which subsequently result in a secondary effect of mislocalisation of FUS and TDP-43. Because certain ALS-linked mutations reduce FUS and TDP-43 binding with Oxr1 (Finelli, 2010), and over-expression of Oxr1 is unable to reduce cytoplasmic aggregation of these mutants under OS (Figure 3.6), the data supports the former hypothesis that direct interaction between Oxr1 and FUS or TDP-43 regulates cellular localisation of FUS and TDP-43. Taken together, these findings suggest that Oxr1 may serve as a crucial link between OS and nuclear localisation of FUS and TDP-43.

3.3.2 Interplay between PRMT1, Oxr1, proteasome, and autophagy in regulating FUS and TDP-43 cytoplasmic aggregation under oxidative stress

In addition to understanding nuclear localisation of FUS and TDP-43, recent work has also begun to investigate the pathways regulating cytoplasmic aggregation of FUS and TDP-43. The proteasome and autophagy have been identified as two important protein degradation pathways for FUS and TDP-43, and inhibition of either pathway leads to increased aggregation of wild-type and ALS-associated mutant TDP-43 (Perrotti *et al.*, 2000; Kim *et al.*, 2009; Urushitani *et al.*, 2010; Wang *et al.*, 2010; Brady *et al.*, 2011; van Eersel *et al.*, 2011). Furthermore, parkin and HDAC6 regulate TDP-43 ubiquitination and accumulation in the cytoplasm (Hebron *et al.*, 2013). Consistent with these previous studies, I confirmed that wild-type and ALS-linked mutant TDP-43 are degraded in proteasome- and autophagy-dependent pathways (Figure 3.7). Although I found that cytoplasmic aggregation of over-expressed FUS does not significantly differ even when proteasome and autophagy are inhibited, I demonstrated for the first time that ALS-linked FUS mutants are degraded in a pathway dependent on both proteasome and autophagy (Figure 3.7A,C). This difference of degradation for wild-type and ALS-linked mutant FUS,

suggests that ALS-associated mutations in FUS may alter the normal pathways by which FUS is degraded, thereby facilitating recruitment to cytosolic inclusions, particularly under increased levels of ROS. Interestingly, over-expression of Oxr1 reduces wild-type and ALS-linked mutant FUS and TDP-43 even under inhibition of proteasome and autophagy, thus establishing a downstream pathway of Oxr1 that is independent of protein degradation.

In addition, recent studies have found that arginine methylation of FUS by PRMT1 regulates FUS localisation, and in particular, inhibition of arginine methylation prior to over-expressing wild-type and ALS-linked mutant FUS reduced FUS aggregation in the cytoplasm (Du *et al.*, 2011; Tradewell *et al.*, 2011; Yamaguchi and Kitajo, 2012). Interestingly, as I found here, and as previously reported, inhibition of PRMT1 and arginine methylation after the transfection of FUS constructs results in increased recruitment of wild-type and ALS-mutant FUS to cytoplasmic aggregates (Figure 3.8F) (Tradewell *et al.*, 2011; Yamaguchi and Kitajo, 2012). There are conflicting reports about whether PRMT1 and arginine methylation increase or reduce cytoplasmic FUS and FUS-positive cytosolic inclusions (Tradewell *et al.*, 2011; Yamaguchi and Kitajo, 2012), but the results shown herein demonstrate clearly that loss of PRMT1 function leads to increased aggregation of FUS in the cytoplasm, especially under OS (Figure 3.8). Furthermore, given that methylation is a permanent cellular event, these results suggest that wild-type and ALS-linked mutant FUS localisation is also dependent on a pathway requiring PRMT1 function independent of methylation of FUS arginine residues.

Surprisingly, Oxr1 not only interacts with PRMT1, but Oxr1-mediated reduction of wild-type and ALS-associated mutant FUS cytoplasmic aggregation is completely ablated after PRMT1 function is inhibited (Figure 3.8). Furthermore, although inhibition of PRMT1 activity did not affect recruitment of wild-type and ALS-linked TDP-43 to cytoplasmic aggregates, over-expression of Oxr1 no longer reduces TDP-43-positive

cytosolic inclusions when PRMT1 function is inhibited (Figure 3.8). Taken together, these results implicate a PRMT1-dependent pathway downstream of Oxr1 function under OS and suggest that this pathway may also regulate FUS nucleocytoplasmic localisation without altering arginine methylation of FUS.

In the future, it will be particularly important to establish whether or not the interaction between PRMT1 and Oxr1, and which downstream targets of PRMT1 are necessary for proper FUS and TDP-43 localisation under OS. Recent studies have identified cyclin-dependent kinases, glycogen synthase kinase 3, and c-Jun N-terminal kinase (JNK) as modulators of TDP-43 accumulation in stress granules under OS (Meyerowitz *et al.*, 2011; Moujalled *et al.*, 2013). Interestingly, JNK has also been identified as an upstream regulator of Oxr1 expression in *Anopheles gambiae* (Jaramillo-Gutierrez *et al.*, 2010). Thus, further experiments are required to identify whether any of these kinases may be targets of PRMT1 and work in concert with Oxr1 under OS to regulate recruitment of FUS and TDP-43 to cytoplasmic aggregates.

3.3.3 Importance of TLDc domain in Oxr1 function

Together with OXR1, four other proteins, NCOA7, TBC1D24, KIAA1609, and C20ORF118, form the TLDc domain family proteins. The highly conserved TLDc domain is sufficient to confer protection against OS-induced apoptosis (Durand *et al.*, 2007; Oliver *et al.*, 2011). Furthermore, Oxr1 reacts directly with ROS through a highly conserved cysteine residue in the TLDc domain, but does not function as an enzyme, suggesting that the TLDc domain may modulate Oxr1 function by sensing levels of ROS (Oliver *et al.*, 2011). Interestingly, another mutation in the TLDc domain of TBC1D24, A509V, is associated with familial infantile myoclonic epilepsy, and inhibits neurite outgrowth *in vitro* (Falace *et al.*, 2010). A recent crystallization of zebrafish Ncoa7 TLDc domain

suggests that the alanine to valine residue mutation linked to epilepsy creates steric hindrance that could alter the protein's conformation and the highly conserved cysteine residue's reactivity (Falace *et al.*, 2010; Oliver *et al.*, 2011; Blaise *et al.*, 2012). Unfortunately, the TLDc domain structure does not share significant structural similarity with any other known proteins, and its function remains unclear (Blaise *et al.*, 2012).

Here, I showed that the highly conserved TLDc domain of Oxr1 also plays a critical role in regulating cellular localisation of FUS and TDP-43 under OS (Figure 3.10). Using the recently crystallized structure of zebrafish Ncoa7 TLDc domain (Blaise *et al.*, 2012), we modelled the TLDc domain of human OXR1 and found that a highly conserved glutamate residue, Glu869, interacts with Ser712 (Figure 3.11) (Blaise *et al.*, 2012). Interestingly, over-expression of Oxr1 EA-C mutant no longer significantly decreases cytoplasmic aggregation of wild-type and mutant FUS and TDP-43 under OS, although the interaction between Oxr1 EA-C and FUS, TDP-43, or PRMT1 is not altered (Figure 3.10). In addition, mutating this highly conserved glutamate residue to an alanine residue not only is predicted to ablate bonding to Ser712, but also renders Oxr1 unable to protect cerebellar granule cells from OS-induced apoptosis (Figure 3.10B) (Finelli, 2010). Taken together, these data confirm the importance of TLDc domain of Oxr1 in protecting against ROS-induced damage, and in mediating FUS and TDP-43 localisation under stress, and implicate this particular glutamate residue as important for Oxr1 function.

3.3.4 Function of the FUS, TDP-43, and Oxr1-C complex

Although Oxr1 has been previously shown to regulate neuronal sensitivity to OS, its function in the central nervous system is still not well understood (Oliver *et al.*, 2011). The function of FUS and TDP-43 are only beginning to be elucidated with a number of recent studies identifying FUS- and TDP-43-regulated mRNA targets (D'Ambrogio *et al.*,

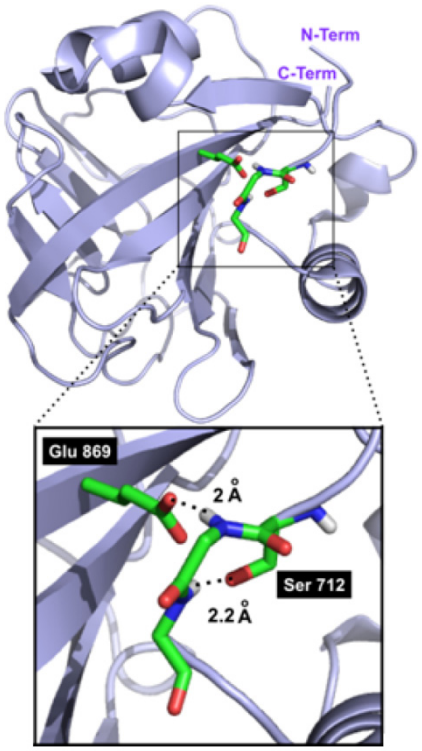

Figure 3.11. Mutation in highly conserved TLDc domain alters Oxr1 protein folding
Model of OXR1 showing bonding between Glu869 and Ser712, an interaction that should be ablated when the glutamine residue is mutated to an alanine residue in mOxr1 EA-C. Model was generated by Luis Sanchez-Pulido.

2009; Hoell *et al.*, 2011; Polymenidou *et al.*, 2011; Sephton *et al.*, 2011; Tollervey *et al.*, 2011; Xiao *et al.*, 2011; Colombrita *et al.*, 2012; Fiesel *et al.*, 2012; Hazelett *et al.*, 2012; Ishigaki *et al.*, 2012; van Blitterswijk *et al.*, 2013). Interestingly, certain ALS-linked mutations in FUS and TDP-43 lead to aberrant splicing, some of which are loss-of-function and others gain-of-function (D'Ambrogio *et al.*, 2009; Kino *et al.*, 2011; Fiesel *et al.*, 2012; van Blitterswijk *et al.*, 2013). Of the ALS-linked FUS and TDP-43 mutants, FUS^{R513C}, TDP-43^{Q331K}, and TDP-43^{M337V} mutants were the only mutants for which binding to Oxr1 is not altered, and recruitment to cytoplasmic aggregates is decreased by Oxr1 over-expression (Figure 1.4;3.6). Therefore, if Oxr1 regulates FUS and TDP-43 mediated splicing, over-expression of Oxr1 is most likely to ameliorate the splicing defects of FUS^{R513C}, TDP-43^{Q331K}, and TDP-43^{M337V} mutants. No splicing dysregulation has been confirmed *in vitro* for FUS^{R513C}, TDP-43^{Q331K}, and TDP-43^{M337V} mutants (D'Ambrogio *et al.*, 2009; Kino *et al.*, 2011; Fiesel *et al.*, 2012; van Blitterswijk *et al.*, 2013); thus, I have not examined whether Oxr1 modulates RNA splicing through its interaction with FUS and TDP-43. As the mechanisms by which ALS-linked mutations in FUS and TDP-43 affect RNA splicing become better understood, it will be important to determine whether over-expression of Oxr1 also ameliorates FUS- and TDP-43-dependent RNA splicing defects.

Furthermore, FUS and TDP-43 have both been implicated in both dendritic and axonal development (Fujii *et al.*, 2005; Fujii and Takumi, 2005; Feiguin *et al.*, 2009; Lu *et al.*, 2009; Groen *et al.*, 2013). In addition, ALS-linked mutant FUS increases interaction with the protein survival motor neuron (SMN), and sequesters SMN to the cell body, leading to axonal defects, a phenotype that is rescued by over-expression of SMN (Groen *et al.*, 2013). Another TLDc domain family member, TBC1D24, has also been implicated in modulating neurite outgrowth, and a mutation in the TLDc domain ablates the neurite outgrowth phenotype (Corbett *et al.*, 2010; Falace *et al.*, 2010; Falace *et al.*, 2014).

Therefore, it is possible that Oxr1 regulates proper neurite development through a FUS- and TDP-43-dependent pathway.

Finally, FUS and TDP-43 also regulate mitochondria function, with ALS-associated mutations in FUS and TDP-43 causing decreased mitochondrial length, increased mitochondrial fission and decreased mitochondrial fusion, and decreased axonal mitochondrial transport (Shan *et al.*, 2010; Xu *et al.*, 2010; Tradewell *et al.*, 2011; Xu *et al.*, 2011a; Cozzolino *et al.*, 2013; Wang *et al.*, 2013). Under OS, Oxr1 is up-regulated in the mitochondria, and during extended periods of OS, Oxr1 shuttles from the mitochondria into the cytoplasm (Elliott and Volkert, 2004; Finelli, 2010). Given the links between mitochondrial dysfunction, OS, and neurodegeneration (Lin and Beal, 2006; Cozzolino *et al.*, 2013), Oxr1 very likely participates in an important pathway in regulating mitochondrial function, particularly under OS. Therefore, future experiments will be important to determine whether over-expression of Oxr1 alleviates mitochondrial dysfunction caused by ALS-linked mutant FUS and TDP-43.

In summary, I uncovered a novel function of Oxr1 in regulating FUS and TDP-43 accumulation in cytoplasmic aggregates under OS, and demonstrated that this particular function is both PRMT1- and TLDc domain-dependent. Interestingly, I found that over-expression of Oxr1 reduces aggregation of ALS-associated mutant FUS and TDP-43, suggesting novel neuroprotective functions of Oxr1 in FUS and TDP-43 neuropathology. Therefore, understanding the process by which ALS mutations affect wild-type FUS and TDP-43 function and facilitate recruitment of FUS and TDP-43 to cytosolic inclusions, and mechanisms through which Oxr1 alleviates these phenotypes will be crucial to uncovering neurodegenerative pathways of ALS and developing future treatments for patients suffering from ALS and other FUS- and TDP-43-related neurological disorders.

4 Neuronal overexpression of Oxr1 is neuroprotective *in vivo*

4.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that leads to the progressive loss of upper motor neurons in the cortex, and lower motor neurons in the brain stem and the spinal cord, leading to severe muscle wasting and death due to respiratory failure (Hardiman *et al.*, 2011). Although majority of ALS patients die 3 years after the onset of symptoms, riluzole remains the only approved drug for ALS patients, yielding a modest extension of 3-6 months in life expectancy (Hardiman *et al.*, 2011). While most ALS cases are sporadic, approximately 10% of the cases are familial, and mutations in Cu/Zn super oxide dismutase (*SOD1*) account for 20% of the familial ALS cases (Ferraiuolo *et al.*, 2011). Transgenic mice over-expressing human $SOD1^{G93A}$ recapitulate many clinical features of ALS patients, including defects in motor control, muscle denervation, and progressive degeneration of spinal motor neurons (Gurney *et al.*, 1994). Tremendous progress has been made using the $SOD1^{G93A}$ ALS mice and other ALS mouse models, uncovering pathogenic pathways, including oxidative stress (OS), mitochondrial dysfunction, excitotoxicity, protein aggregation, and neuroinflammation, but the mechanisms by which ALS manifests are still unclear (Ferraiuolo *et al.*, 2011; Swarup and Julien, 2011).

One important pathway, OS is a central component of ALS pathophysiology, particularly SOD1-mediated ALS (Barber and Shaw, 2010; Ferraiuolo *et al.*, 2011; D'Amico *et al.*, 2013). ROS-mediated damage to DNA, lipids, and proteins has been found in the cortex and spinal cord of ALS patients (Beal *et al.*, 1997; Pedersen *et al.*, 1998). As described in Chapter 1, increasing lines of evidence suggest that mutations in SOD1 cause a toxic gain-of-function that generates ROS through an unknown mechanism (Barber and Shaw, 2010; Ferraiuolo *et al.*, 2011). ALS mutant SOD1 also localise to the mitochondrial

intermembrane space, increasing ROS production, decreasing cytochrome oxidase activity and leading to motor neuron degeneration (Goldsteins *et al.*, 2008; Igoudjil *et al.*, 2011). Furthermore, astrocytes and glia that express mutant SOD1 release toxic factors, including ROS, which also cause motor neuron death (Harraz *et al.*, 2008; Marchetto *et al.*, 2008).

Complementing evidence that OS contributes to neuron dysfunction and death in ALS, a meta-analysis of therapeutic strategies found that drugs targeting antioxidant pathways at time of symptom onset are most effective in prolonging survival in mutant SOD1 mice (Benatar, 2007). Recent studies have identified a number of pharmacological compounds and genetic modifiers targeting the generation of ROS that increase lifespan and delay disease pathogenesis in ALS mouse models, including several targeting the generation of ROS (Turner and Talbot, 2008; Riboldi *et al.*, 2011; McGoldrick *et al.*, 2013). Treatment with SS-31, a cell-permeable peptide antioxidant that is targeted to the inner mitochondrial membrane and has ROS scavenging properties, Diacetylbis(N(4)-methylthiosemicarbazonato)copper(II), a compound that passes through the blood brain barrier (BBB) and possess antioxidant properties, and VK-28 and M30, two BBB-permeable iron chelators, protect against OS-damage in the lumbar spinal cord, and significantly improve motor function and survival by approximately 10% in $SOD1^{G93A}$ ALS mice (Wada *et al.*, 1994; Zhao *et al.*, 2004a; Petri *et al.*, 2006; Soon *et al.*, 2011; Wang *et al.*, 2011a). Loss of NOX1 or NOX2, two proteins involved in ROS production, both extend lifespan and delay disease onset in in $SOD1^{G93A}$ ALS mice, with a dramatic 74% increased survival for *NOX2* knock-down (Wu *et al.*, 2006; Marden *et al.*, 2007). Furthermore, treatment with apocynin, a NOX inhibitor, significantly increases lifespan of $SOD1^{G93A}$ ALS mice by approximately 90% at higher dosage regimens (Harraz *et al.*, 2008). Interestingly, over-expression of Nrf2, an important transcription factor regulating expression of antioxidant enzymes, in astrocytes increases glutathione levels, delays

disease onset, and extends survival by 16% (Kobayashi and Yamamoto, 2005; Vargas *et al.*, 2008). Therefore, understanding antioxidant defence networks is particularly important for the development of future therapeutic strategies.

Originally identified in a screen for human genes that protects against OS-induced damage, *oxidation resistance 1* (*OXR1*) is highly conserved in all eukaryotes, and confers cellular sensitivity to OS-mediated apoptosis (Volkert *et al.*, 2000; Elliott and Volkert, 2004; Durand *et al.*, 2007; Oliver *et al.*, 2011; Murphy and Volkert, 2012). Loss of Oxr1 in bella mice leads to severe cerebellar ataxia, oxidative damage and neuronal apoptosis in the granule cell layer of the cerebellum, and death before postnatal day 26 (P26) (Oliver *et al.*, 2011). Furthermore, over-expression of Oxr1 protects neurons against OS-induced cell death *in vivo* and *in vitro*, and the function of a highly conserved, but uncharacterised C-terminal TLDc domain is sufficient for this neuroprotective function of Oxr1 (Oliver *et al.*, 2011). Interestingly, intermediate isoforms of OXR1 expression are up-regulated in the spinal cord of ALS patients, and full-length isoform of Oxr1 (Oxr1-FL) is up-regulated in pre-symptomatic $SOD1^{G93A}$ ALS mice. Therefore, these data suggest that up-regulation of Oxr1 may not only serve as an early marker of OS, but also be neuroprotective during neurodegenerative disease progression (Oliver *et al.*, 2011).

4.1.1 Aims of chapter 4

OS is a central component of ALS disease pathogenesis, and Oxr1 regulates neuronal sensitivity to OS-induced damage and apoptosis (Barber *et al.*, 2006; Lin and Beal, 2006; Ferraiuolo *et al.*, 2011; Oliver *et al.*, 2011). In addition, over-expression of Oxr1 protects neurons against exogenous H_2O_2 stress, and up-regulation of OXR1 has been observed in ALS patients and an ALS mouse model (Oliver *et al.*, 2011). These results suggest that up-regulation of Oxr1 may serve to be neuroprotective *in vivo*,

particularly during ALS pathogenesis. The goal of this chapter was to investigate whether over-expression of Oxr1 in neurons from an early pre-symptomatic stage delays OS-related neuronal dysfunction and apoptosis in the $SOD1^{G93A}$ ALS mouse model.

4.2 Results

4.2.1 Neuron-specific Oxr1 over-expression in Tg(*Prnp-Oxr1*) mice

Oxr1 is highly expressed in the central nervous system, and we confirmed this expression is specific to neurons (Figure 4.1A) (Oliver *et al.*, 2011). Thus, to study whether or not over-expression of Oxr1 is neuroprotective *in vivo*, we first generated a transgenic mouse (Tg(*Prnp-Oxr1*)/+), in which the mouse prion protein *Prnp* promoter drives over-expression of HA-tagged *Oxr1-FL* (Figure 4.1B). The *Prnp-Oxr1* transgenic construct includes the *Prnp* promoter, including non-coding *Prnp* exon 1 and a small amount of noncoding *Prnp* exon 2, followed by the cDNA sequence of *Oxr1-FL* with a C-terminal HA-tag, and 3' untranslated regions of *Prnp* exon 3 and 3' genomic sequence downstream of *Prnp* locus, both of which include important 3' signalling sequences (Figure 4.1B). We received three independent founder mice positive for the transgene, and we next investigated which of the founder lines had the highest over-expression of Oxr1-FL by western blotting for Oxr1 as expression levels may be determined by genomic position or copy number. Founder line 1 had the highest over-expression of Oxr1-FL in both the brain and spinal cord (Figure 4.1C-D), with over-expression as early as E13.5 in the brain and spinal cord (Figure 4.1E-F). Furthermore, adult progeny from the first founder line have an approximately 5-fold increase in Oxr1-FL expression when compared with wild-type littermates (Figure 4.1G-H). Next, to confirm neuron-specific Oxr1 over-expression, we conducted immunocytochemistry on spinal cord and brain sections, labelling with HA and NeuN, a marker for neurons (Mullen *et al.*, 1992). We found that all

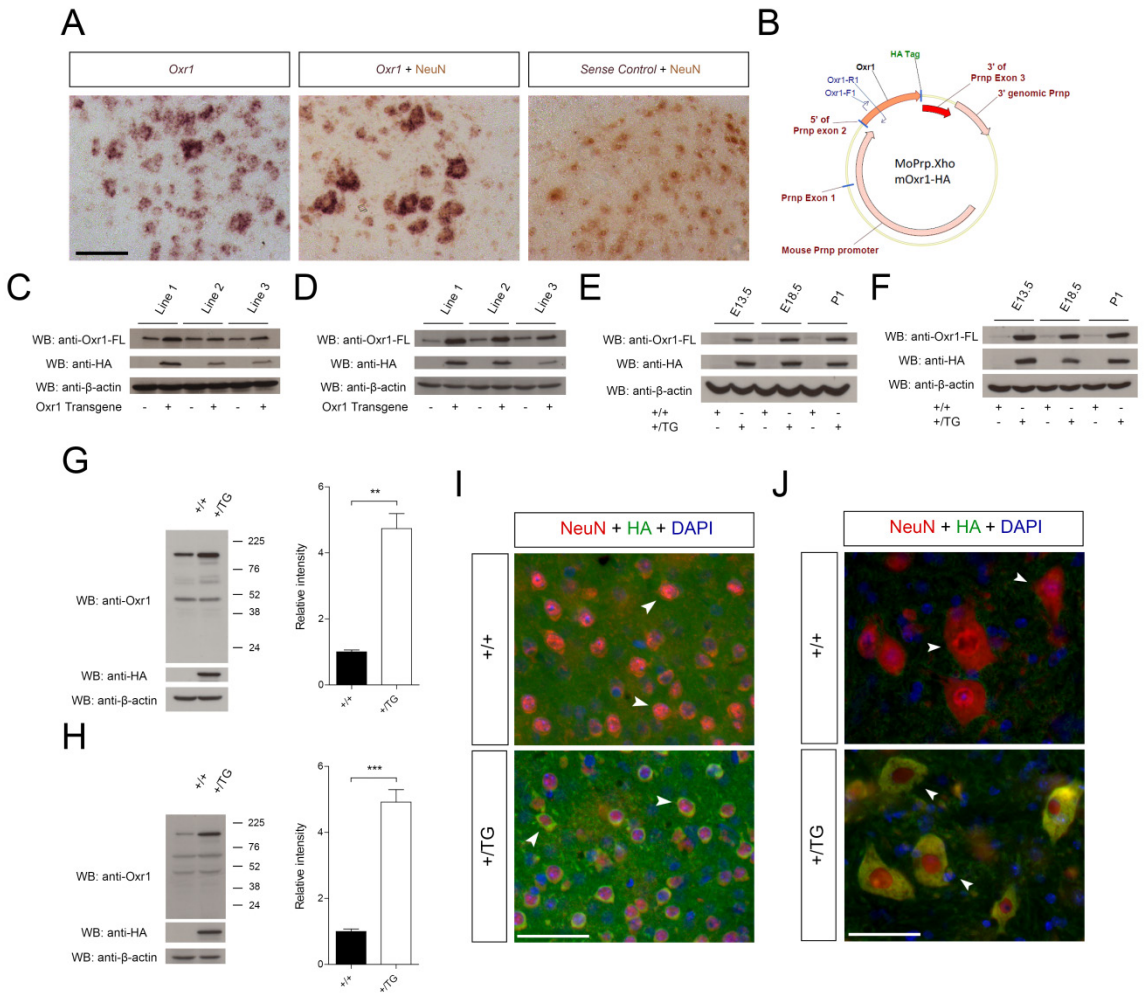

Figure 4.1. *Prnp*-promoter driven Oxr1 over-expression in transgenic mouse model
(**A**) Double *in situ* hybridisation and immunocytochemistry demonstrating endogenous Oxr1 is specifically expressed in neurons in the spinal cord. *In situ* hybridization was conducted by Dr. Peter Oliver. (**B**) Schematic of the transgenic construct, depicting position of the *Prnp* promoter, the Oxr1 transgene, and the 3'UTR and 3' genomic sequence downstream of the *Prnp* locus. Dr. Peter Oliver and Ben Edwards cloned the construct. *Prnp*-promoter driven over-expression of HA-tagged Oxr1 is highest in the first of three different founder lines in (**C**) the brain and (**D**) the spinal cord. HA-tagged Oxr1 is up-regulated by E13.5 in the transgenic founder line 1 (+/TG) in the brain (**E**) and the spinal cord (**F**), and by approximately 5-fold in the brain (**G**) and the spinal cord (**H**) of +/TG mice when compared with that of +/+ mice. (**G-H**) Quantification of Oxr1-FL, and values are the mean ± SEM (n=3 per genotype; **p<0.01, ***p<0.001, two-tailed student's t-test). Immunocytochemical staining for NeuN, and HA-tagged Oxr1-FL, in the (**I**) brain and (**J**) spinal cord, demonstrating that *Prnp*-promoter driven Oxr1-FL over-expression in +/TG mice is specific to neurons (arrows). (**I-J**) Scale bars: 50µm.

neurons also expressed cytoplasmic HA-tagged Oxr1-FL, and no HA-stained cells were found in any other populations in the brain or spinal cord (Figure 4.1I-J). Finally, we determined whether over-expression of Oxr1 has any detrimental effects. We observed no overt muscle or neuromuscular junction pathology in Tg(*Prnp-Oxr1*) mice when compared with wild-type mice (Figure 4.2A-B). Consequently, Tg(*Prnp-Oxr1*) mice do not perform differently from wild-type mice on rotarod or grip strength (Figure 4.2C-F). Together, we confirmed that Oxr1 over-expression does not cause any overt pathology, and established Tg(*Prnp-Oxr1*) mice as a model to investigate neuroprotective effects of Oxr1.

4.2.2 Oxr1 over-expression in neurons extends survival and delays motor dysfunction in SOD1^{G93A} ALS mice

Because OXR1 isoforms are up-regulated in spinal cord of ALS patients and SOD1^{G93A} ALS mice (Oliver *et al.*, 2011), we chose to investigate whether over-expression of Oxr1 delays or prevents neurodegeneration by crossing cross Tg(*Prnp-Oxr1*) mice with SOD1^{G93A} ALS mice. We first confirmed that the progeny of the cross, SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice, over-expressed both mutant SOD1^{G93A} and HA-tagged Oxr1-FL, with mutant SOD1^{G93A} expression levels comparable to that of SOD1^{G93A} ALS mice, and Oxr1-FL expression levels comparable to that of Tg(*Prnp-Oxr1*) mice in both the brain and the spinal cord at P60, P90, and P135 (Figure 4.3).

We next assessed whether the time to reach end-stage as determined by hind limb paralysis is extended by Oxr1 over-expression in neurons. Surprisingly, we found that while median survival for female SOD1^{G93A} ALS mice (n=11) is 149 days, median survival for female SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (n=10) is 177.5 days, a significant increase in lifespan of approximately 19% ($p<0.001$) (Figure 4.4A). Similarly, median survival for male SOD1^{G93A} ALS mice (n=12) is 149 days, while median survival for male

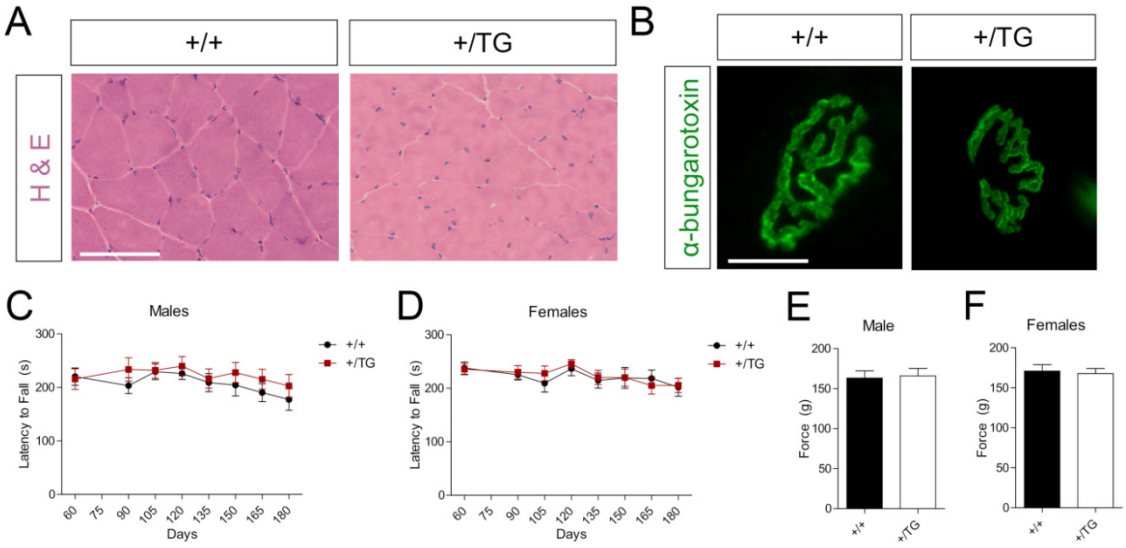

Figure 4.2. No overt pathology observed in Tg(*Prnp-Oxr1*) mice
(**A**) Representative images of H&E stained gastrocnemius muscle fibres revealing no muscle atrophy in +/TG mice compared with +/+ mice. Scale bar: 100µm. (**B**) Representative images of NMJ morphology revealing no changes in NMJ morphology in +/TG mice when compared with +/+ mice. Scale bar: 25µm. (**A-B**) Muscle experiments were conducted together with Ben Edwards and NMJ scoring was conducted solely by Ben Edwards. (**C-D**) No differences were observed in motor function of +/TG mice by rotarod. Values are the mean ± SEM (n=7-15 per genotype; Mann-Whitney U tests). (**E-F**) No differences were observed in muscle strength of +/TG mice by grip strength test. Values are the mean ± SEM (n=9-15 per genotype; Mann-Whitney U tests). Muscle strength experiments were conducted by Dr. Peter Oliver.

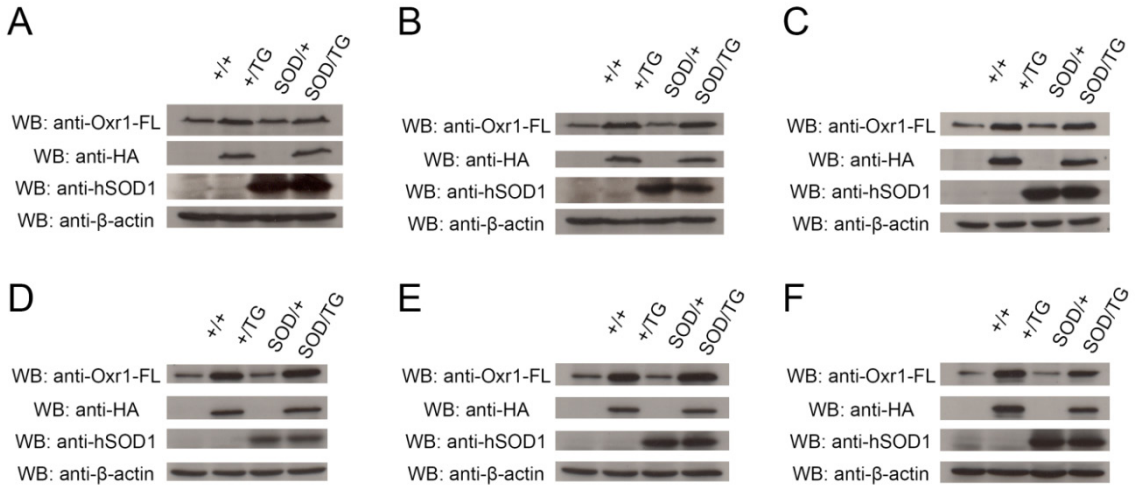

Figure 4.3. Characterization of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice
Western blot confirmation of stable transgene over-expression of HA-tagged Oxr1-FL and mutant hSOD1^{G93A} in the brain at (**A**) P60, (**B**) P90, and (**C**) P135 in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (SOD/TG), produced by crossing SOD1^{G93A} ALS (SOD/+) mice and +/TG mice. Western blot confirmation of stable transgene over-expression of HA-tagged Oxr1-FL and mutant hSOD1^{G93A} in the spinal cord at (**D**) P60, (**E**) P90, and (**F**) P135 in SOD/TG mice.

SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (n=12) is 174 for males (n=12), a significant increase in lifespan of approximately 17% ($p<0.001$) (Figure 4.4B). In addition, to determine whether neuronal over-expression of Oxr1 ameliorates motor function in SOD1^{G93A} ALS mice, we measured rotarod performance at multiple time-points. We found that average latency to fall for SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice is significantly longer than that of SOD1^{G93A} ALS mice at all time-points beginning at P60 ($p<0.05$) for females, and P90 for males ($p<0.001$) (Figure 4.4C-D). Taken together, these data demonstrates that over-expression of Oxr1 in neurons delays motor dysfunction and extends life-span of SOD1^{G93A} ALS mice.

In parallel, we also examined whether over-expression of Oxr1 in neurons ameliorates weight loss commonly observed in SOD1^{G93A} ALS mice. We found no significant differences in weight for Tg(*Prnp-Oxr1*) mice when compared to their wild-type counterparts, but SOD1^{G93A} ALS mice weigh significantly less only at P135 when compared to either wild-type or Tg(*Prnp-Oxr1*) mice. In addition, SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice have difficulty gaining weight, with significantly lower body weight beginning at P120 for females and P90 for males, when compared to wild-type or Tg(*Prnp-Oxr1*) mice, and no differences in weight were observed between SOD1^{G93A} ALS mice and SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (Figure 4.4E-F). Although Oxr1 over-expression in neurons increases lifespan of SOD1^{G93A} ALS mice, neuronal Oxr1 over-expression does not improve body weight during ALS disease progression.

4.2.3 Neuron-specific over-expression of Oxr1 increases motor neuron survival, and decreases oxidative stress in the spinal cord of SOD1^{G93A} ALS mice

Given that SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice survive longer than SOD1^{G93A} ALS mice, we next examined whether over-expression of Oxr1-FL in neurons also affects motor neuron survival in the spinal cord. Nissl staining of motor neurons confirmed a

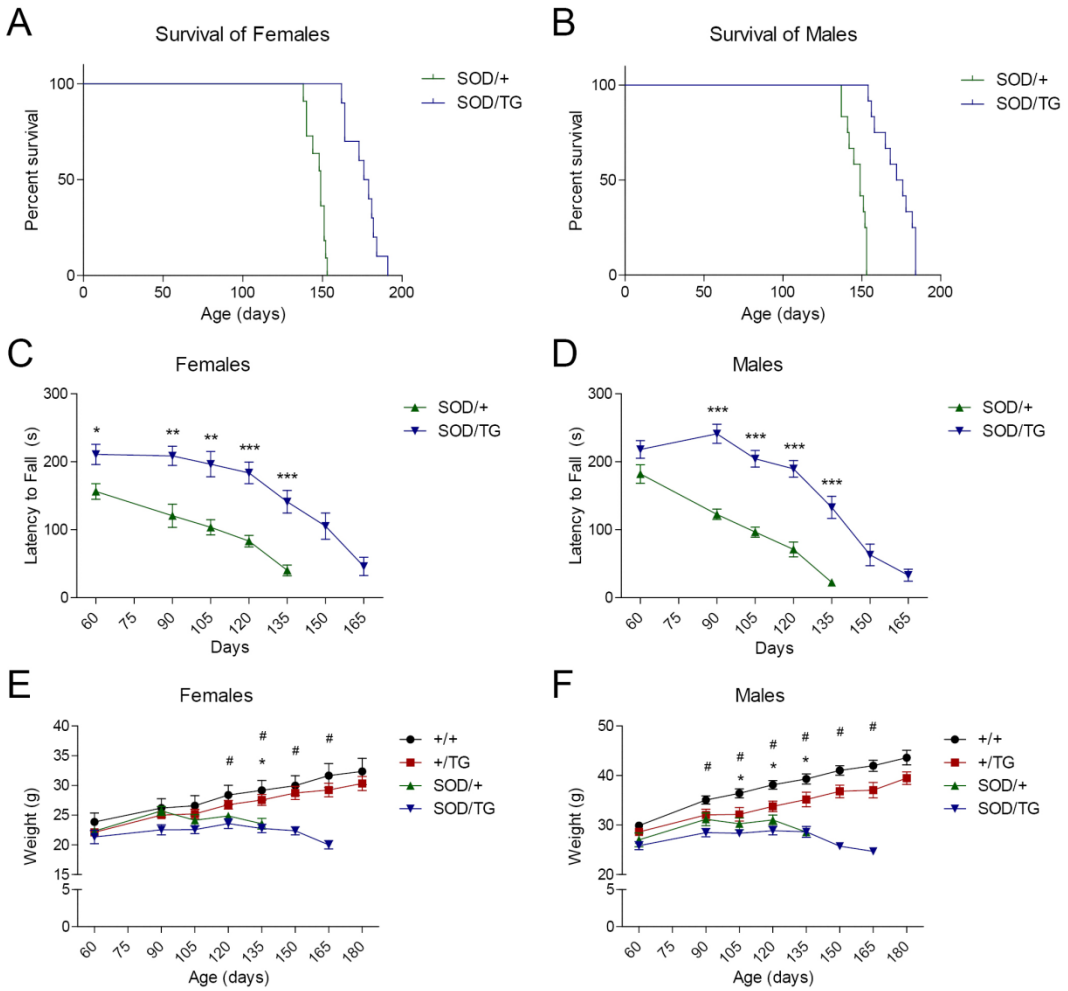

Figure 4.4. Neuronal over-expression of Oxr1 extends lifespan and improves motor function of SOD1^{G93A} ALS mice

(**A**) Kaplan-Meier curve showing probability of survival in female SOD1^{G93A} transgenic mice (SOD/+) and female SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (SOD/TG). Oxr1 over-expression increases median survival from 149 days for SOD/+ females (n=11) to 177.5 days for SOD/TG females (n=10). (**B**) Kaplan-Meier log rank test for survival, showing Oxr1 increases median survival from 149 days for SOD/+ males (n=12) to 174 days for SOD/TG males (n=12). (**A-B**) Survival curves were significantly different by Mantel-Cox test, $p<0.0001$ ($\chi^2 = 22.05$) for females, and $p<0.0001$ ($\chi^2 = 24.99$) for males. SOD/TG (**C**) females and (**D**) males have significantly improved motor performance (s) on accelerating rotarod when compared with SOD/+ females and males, respectively. (**C-D**) Values are mean ± SEM of motor performance (s) for mice still alive at each respective time point. (n=6-11 per genotype; *$p<0.05$, **$p<0.01$, ***$p<0.001$, Mann-Whitney U tests). (**E**) SOD/TG females and (**F**) males weigh significantly less beginning at P120 and P90, respectively, when compared with wild-type (+/+) and Tg(*Prnp-Oxr1*) (+/TG) mice. (**E**) SOD/+ females and (**F**) males weigh significantly less beginning at P135 and P105, respectively, when compared with +/+ and +/TG mice. No differences were observed between +/+ and +/TG mice. (**E-F**) Values are mean ± SEM of weight for mice still alive at each respective time point. (n=6-15 per genotype; *$p<0.05$, +/+ and/or +/TG vs. SOD/+; $^{\#}p<0.05$, +/+ and/or +/TG vs. SOD/TG, 1-way ANOVA at each time point, followed by Bonferroni post-hoc tests for individual comparisons between genotypes).

significant reduction in the lumbar spinal cord of SOD1^{G93A} ALS mice at P135, when compared to that of wild-type and Tg(*Prnp-Oxr1*) mice (n=3-4; $p<0.01$) (Figure 4.5A-B). Interestingly, we found significantly higher counts of motor neurons in the lumbar spinal cord of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice when compared to that of SOD1^{G93A} ALS mice at P135 (n=3; $p<0.05$), while no differences in spinal motor neuron survival were observed between SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice and either wild-type or Tg(*Prnp-Oxr1*) mice (n=3-4) (Figure 4.5A-B). Furthermore, Oxr1 confers neuronal sensitivity to OS, thus, we next investigated whether over-expressing Oxr1 in neurons affects OS in SOD1^{G93A} ALS mice by immunoblotting for heme-oxygenase 1 (HO-1), an OS marker (Ferrante *et al.*, 1997b; Ferrante *et al.*, 1997a). HO-1 is significantly induced in SOD1^{G93A} ALS mice at P135 by approximately 2-fold, when compared to that of wild-type and Tg(*Prnp-Oxr1*) mice (n=3; $p<0.01$) (Figure 4.5C-D). However, HO-1 induction is significantly reduced in SOD1^{G93A}/ Tg(*Prnp-Oxr1*) mice at P135 (n=3; $p<0.05$) when compared to SOD1^{G93A} ALS mice, and not statistically significant when compared to that of wild-type and Tg(*Prnp-Oxr1*) mice (Figure 4.5C-D). Together, these results establish Oxr1 as a modulator of motor function and disease pathogenesis in the spinal cord of SOD1^{G93A} ALS mice.

4.2.4 Survival of corticospinal motor neurons is unaffected in SOD1^{G93A} ALS mice

Because degeneration of corticospinal motor neurons (CSMN), have been described in ALS patients and in SOD1^{G93A} ALS mice (Tandan and Bradley, 1985; Ozdinler *et al.*, 2011), we next sought to determine whether up-regulation of Oxr1 in neurons delays neurodegeneration in the cortex of SOD1^{G93A} ALS mice by immunocytochemical staining for CTIP2, a marker for CSMN (Arlotta *et al.*, 2005). Surprisingly, we found that even at end-stage P135 SOD1^{G93A} ALS mice, no CSMN degeneration was observed when compared to wild-type and Tg(*Prnp-Oxr1*) mice (n=3)

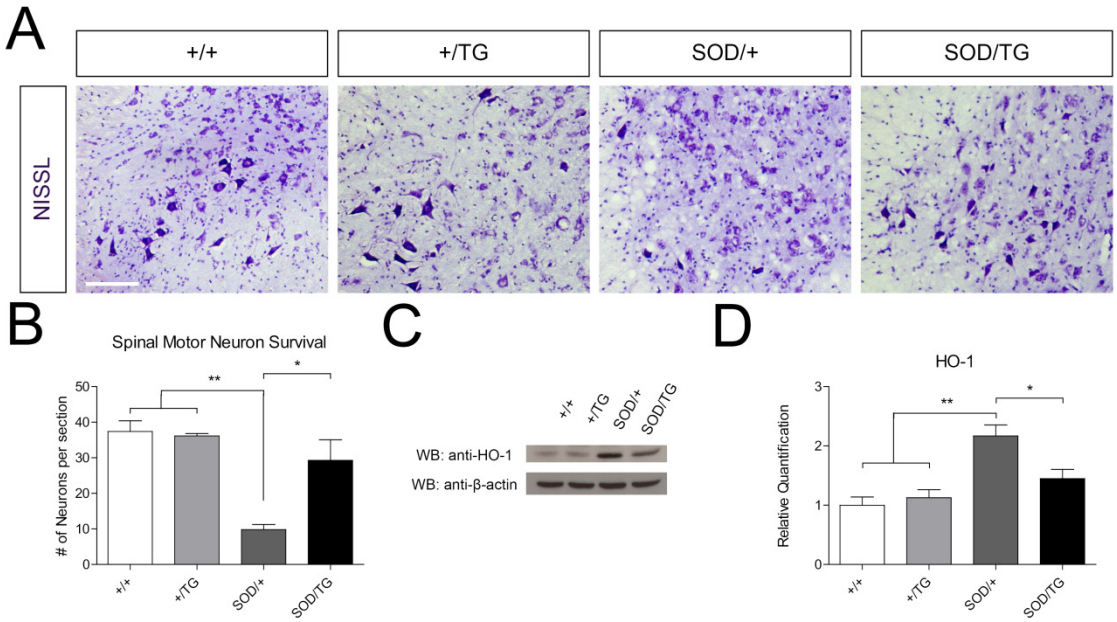

Figure 4.5. Neuronal over-expression of Oxr1 increases spinal motor neuron survival in SOD1^{G93A} ALS mice

(**A**) Representative images of Nissl stained motor neurons in lumbar spinal cord of female mice cross-sections at P135. Scale bar: 100µm. Nissl staining was conducted by Dr. Peter Oliver and all motor neuron counts were completed by me. (**B**) Motor neuron survival counts in lumbar spinal cord cross-sections showing increased survival in SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice when compared with SOD1^{G93A} ALS (SOD/+) mice at P135 (n=3-4). Values for motor neuron survival: wild-type (+/+) - 37.4 ± 3.0; Tg(*Prnp-Oxr1*) (+/TG) - 36.2 ± 0.6; SOD/+ - 9.8 ± 1.4; SOD/TG - 29.3 ± 5.8. (**C**) Representative images of protein levels of oxidative stress marker Heme oxygenase-1 (HO-1) at P135 in male mice. (**D**) Quantification of HO-1 expression using Image J (n=3) showing decreased induction of HO-1 in SOD/TG mice when compared with SOD/+ mice at P135. (**B,D**) Values are the mean ± SEM. (*p<0.05, **p<0.01; One-way ANOVA, with Tukey's post-hoc tests).

(Figure 4.6). Similarly, CSMN survive in similar numbers in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice when compared to wild-type and Tg(*Prnp-Oxr1*) mice (n=3) (Figure 4.6). Taken together, these results suggest that in SOD1^{G93A} ALS and SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice, survival of CSMN is largely unaffected at P135.

4.2.5 Neuronal over-expression of Oxr1 improves muscle strength and delays muscle pathology in SOD1^{G93A} ALS mice

Severe muscle atrophy is a downstream consequence of motor neuron loss in ALS patients and in SOD1^{G93A} ALS mice (Jokelainen, 1977; Telerman-Toppet and Coers, 1978; Gurney *et al.*, 1994), thus we investigated whether increased motor neuron survival from over-expression of Oxr1 delays muscle dysfunction in SOD1^{G93A} ALS mice. To examine whether neuron-specific over-expression of Oxr1 improves muscle strength in SOD1^{G93A} ALS mice, we analysed the performance of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice on a grip strength monitor. We observed significantly improved performance in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice when compared with SOD1^{G93A} ALS mice (Figure 4.7).

Next, we analysed muscle pathology of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice at P90 to determine whether Oxr1 over-expression delays onset of muscular dysfunction at this early symptomatic stage of SOD1^{G93A} ALS mice. We screened changes to neuromuscular junction (NMJ) morphology in extensor digitorum longus (EDL), gastrocnemius, soleus, and tibialis anterior (TA) muscles, and found that neuronal over-expression of Oxr1 significantly delays changes in gastrocnemius NMJ morphology in SOD1^{G93A} ALS mice ($p<0.05$) (Figure 4.8). Next, we found that at P90, SOD1^{G93A} ALS mice have significantly increased muscle atrophy in the EDL muscle ($p<0.01$) when compared to wild-type and Tg(*Prnp-Oxr1*) mice, an increase that is reduced in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice ($p<0.01$) (Figure 4.9A-B). Similarly, we found that at P90, SOD1^{G93A} ALS mice have

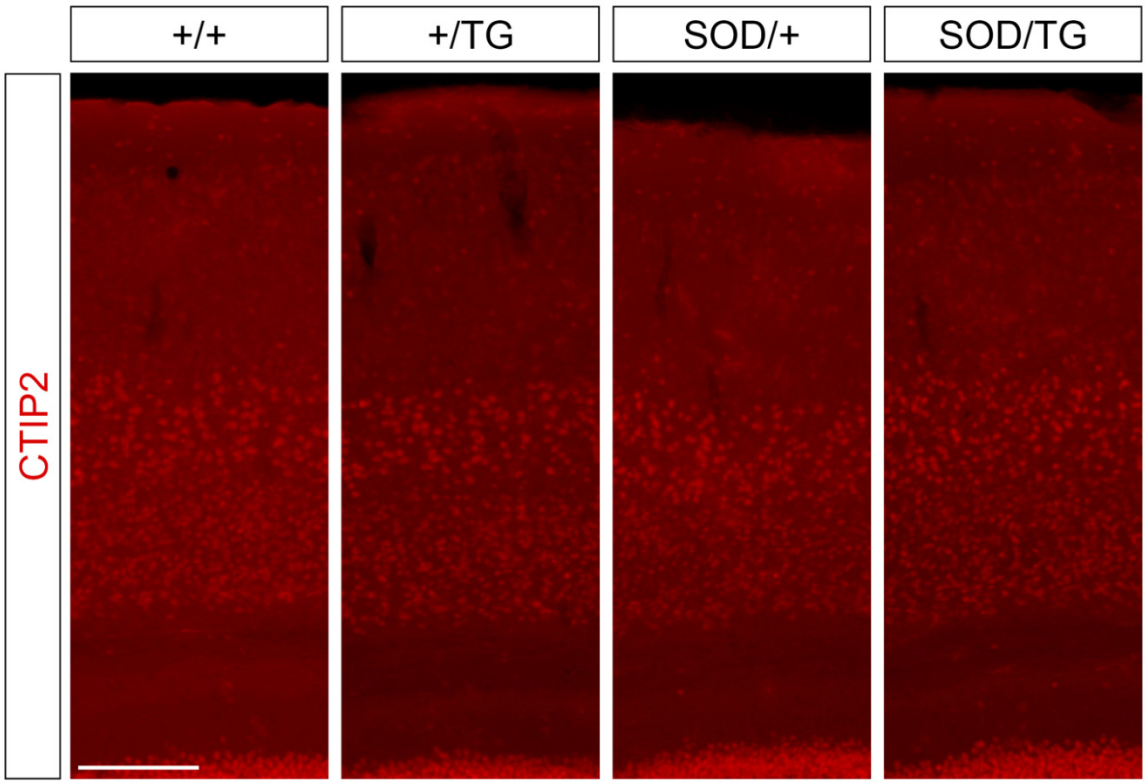

Figure 4.6. Lack of corticospinal motor neuron degeneration in SOD1^{G93A} ALS mice and SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice
Immunocytochemical staining for CTIP2, a marker of corticospinal motor neurons (CSMN), on cortical sections reveals no overt differences in survival of CSMN in male wild-type (+/+), Tg(*Prnp-Oxr1*) (+/TG), SOD1^{G93A} ALS (SOD/+), and SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice (n=3). Scale bar: 200µm.

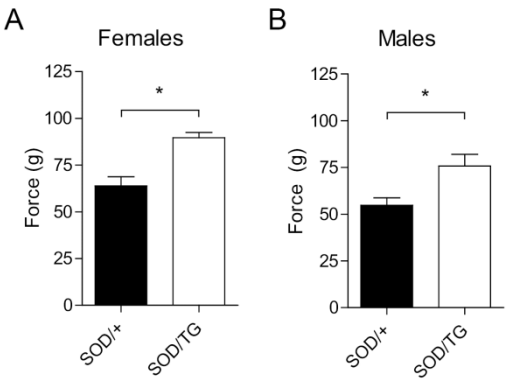

Figure 4.7. Neuronal over-expression of Oxr1 improves muscle strength in SOD1^{G93A} ALS mice

(**A**) SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) females and (**B**) males show significant improvements in muscle strength on a grip strength monitor when compared with SOD1^{G93A} ALS (SOD/+) females and males, respectively at P120. (**A-B**) Values are mean ± SEM. (n = 5-20 for each genotype; *p<0.05, Mann-Whitney U tests). Grip Strength experiments were conducted by Dr. Peter Oliver.

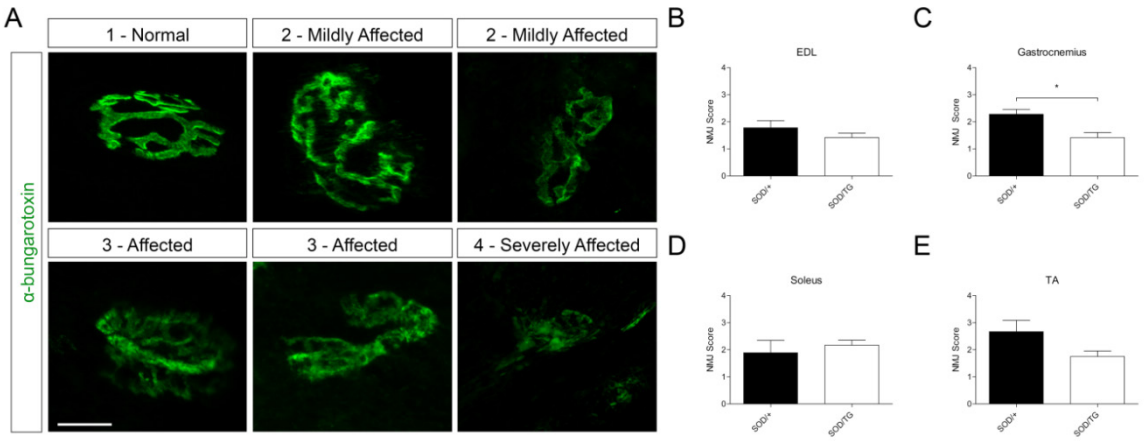

Figure 4.8. Neuronal over-expression of Oxr1 improves neuromuscular junction degeneration in gastrocnemius muscle of SOD1^{G93A} ALS mice

(**A**) Representative images of NMJs for each score: 1-Normal, 2-Mildly Affected, 3-Affected, and 4-Severely Affected. Scale bar: 25µm. NMJ scoring for (**B**) EDL, (**C**) Gastrocnemius, (**D**) Soleus, (**E**) and TA muscles for male wild-type (+/+), Tg(*Prnp-Oxr1*) (+/TG), SOD1^{G93A} ALS (SOD/+), and SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice showed NMJ morphology is significantly improved in the SOD/TG gastrocnemius when compared with SOD/+ gastrocnemius. (**B-E**) Values are mean ± SEM (n=6-8 for each genotype; *p<0.05, Mann-Whitney U tests). NMJ tissue processing was conducted together with Ben Edwards and NMJ scoring was conducted solely by Ben Edwards.

significantly increased muscle atrophy in the gastrocnemius muscle ($p<0.01$) when compared to wild-type and Tg(*Prnp-Oxr1*) mice, an increase that is reduced in $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice ($p<0.05$) (Figure 4.9C-D). No muscle atrophy was observed in soleus muscles in wild-type, Tg(*Prnp-Oxr1*), $SOD1^{G93A}$ ALS, and $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice (Figure 4.9E-F). Finally, increased TA muscle atrophy was observed in $SOD1^{G93A}$ ALS mice when compared to wild-type and Tg(*Prnp-Oxr1*) mice, but this increase is not reduced by neuronal Oxr1 over-expression (Figure 4.9G-H).

Due to denervation of muscles from motor neuron death, changes in muscle fibre type composition is a common pathological symptom (Pette and Staron, 2001), thus we determined whether Oxr1 over-expression in neurons ameliorates any muscle fibre type changes by immunocytochemical staining for Type I, IIA, and IIB fibres. After analysing the muscle composition at P90 for EDL, gastrocnemius, soleus, and TA muscles, we found that only EDL muscles have significant changes in muscle fibre type composition in $SOD1^{G93A}$ ALS mice, when compared with wild-type and Tg(*Prnp-Oxr1*) mice (Figure 4.10A-E). EDL muscles of $SOD1^{G93A}$ ALS mice have significantly decreased Type IIB fibres and increased Type IIA fibres, consistent with the findings that EDL muscles of $SOD1^{G93A}$ ALS mice become more fatigue resistant during disease progression (Figure 4.10B) (Scott *et al.*, 2001; Kieran *et al.*, 2004; Kieran *et al.*, 2005). Interestingly, no significant differences were observed in the EDL muscle composition of $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice, when compared to wild-type and Tg(*Prnp-Oxr1*) mice (Figure 4.10B). Staining for oxidative enzyme succinate dehydrogenase further corroborates the finding that over-expression of Oxr1 in neurons delays abnormal fatigue-resistant characteristics of and increased oxidative capacity in EDL muscles of $SOD1^{G93A}$ ALS mice. We observed a marked increase in dark-stained muscle fibres in $SOD1^{G93A}$ ALS mice, indicative of increased oxidative capacity, which was not observed in

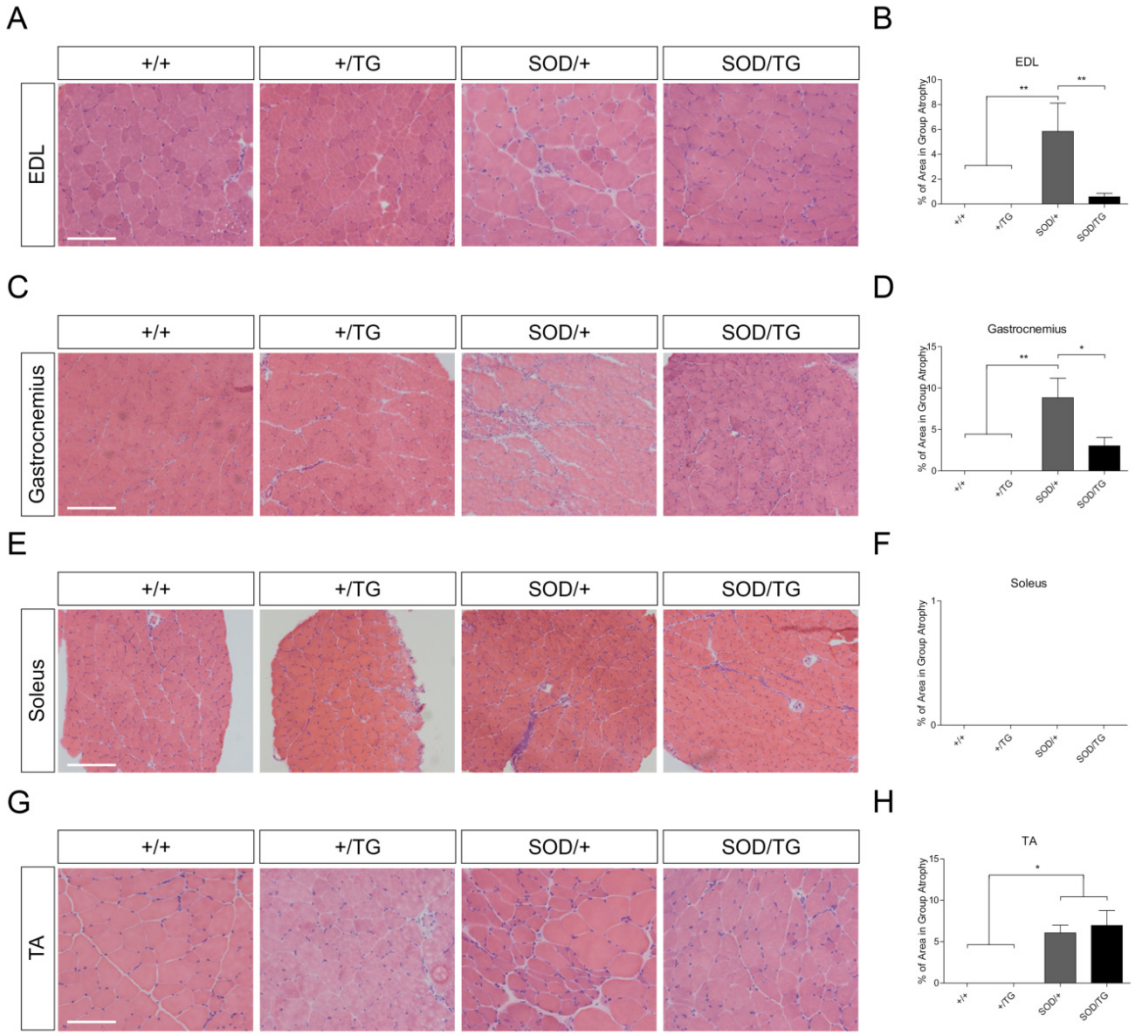

Figure 4.9. Over-expression of Oxr1 in neurons delays muscle group atrophy in EDL and gastrocnemius of P90 SOD1^{G93A} ALS mice

(**A**) Representative images of H&E stained EDL muscle fibres for male +/+, +/TG, SOD/+, and SOD/TG mice. (**B**) Quantification of EDL group atrophy shows significantly increased group atrophy in SOD/+ mice when compared with +/+ and +/TG mice, but significantly reduced group atrophy in SOD/TG mice when compared with SOD/+ mice. (**C**) Representative images of H&E stained gastrocnemius muscle fibres for male +/+, +/TG, SOD/+, and SOD/TG mice. (**D**) Quantification of gastrocnemius group atrophy shows significantly increased group atrophy in SOD/+ mice when compared with +/+ and +/TG mice, but significantly reduced group atrophy in SOD/TG mice when compared with SOD/+ mice. (**E**) Representative images of H&E stained soleus muscle fibres for male +/+, +/TG, SOD/+, and SOD/TG mice. (**F**) Quantification of soleus group atrophy shows no significant increase in group atrophy in SOD/+ or SOD/TG mice when compared with +/+ and +/TG mice. (**G**) Representative images of H&E stained TA muscle fibres for male +/+, +/TG, SOD/+, and SOD/TG mice. (**H**) Quantification of TA group atrophy shows significantly increased group atrophy in SOD/+ mice when compared with +/+ and +/TG mice, with no significantly reduced group atrophy in SOD/TG mice when compared with SOD/+ mice. (**A, C, E, G**) Scale bars: 100µm. (**B, D, F, H**) Values are mean ± SEM. (n=3-6 for each genotype; *p<0.05, **p<0.01, 1-way ANOVA, followed by Tukey's post-hoc tests). Muscle processing was conducted together with Ben Edwards, muscle images were taken and atrophy counts were conducted by Ben Edwards.

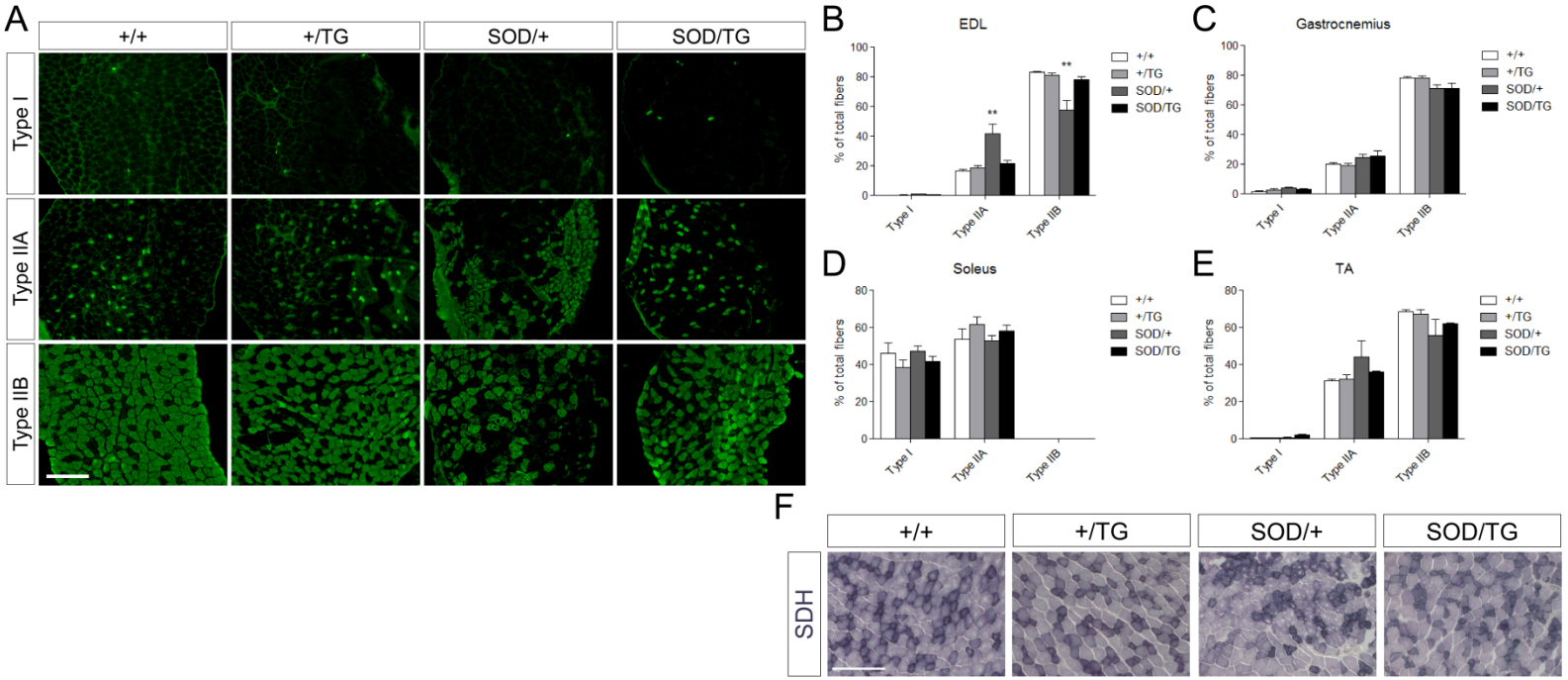

Figure 4.10. Over-expression of Oxr1 in neurons delays fatigue-resistant changes of EDL muscles in SOD1^{G93A} ALS mice
(**A**) Representative images of EDL muscle of P90 male wild-type (+/+), Tg(*Prnp-Oxr1*) (+/TG), SOD1^{G93A} ALS (SOD/+), and SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice, stained for Type I, Type IIA, and Type IIB fiber types. Scale Bar: 200µm. (**B**) Percentage of muscle fiber composition for Type I, Type IIA, and Type IIB fiber types for P90 EDL for each genotype, showing a significant increase in Type IIA fibres and a significant decrease in Type IIB fibres in SOD/+ mice when compared with +/+, +/TG, and SOD/TG mice. (**C**) Percentage of muscle fiber composition for Type I, Type IIA, and Type IIB fiber types for P90 gastrocnemius for each genotype, showing no changes in SOD/+ mice compared with +/+, +/TG, and SOD/TG mice. (**D**) Percentage of muscle fiber composition for Type I, Type IIA, and Type IIB fiber types for P90 soleus for each genotype, showing no changes in SOD/+ mice compared with +/+, +/TG, and SOD/TG mice. (**E**) Percentage of muscle fiber composition for Type I, Type IIA, and Type IIB fiber types for P90 TA for each genotype, showing no changes in SOD/+ mice compared with +/+, +/TG, and SOD/TG mice. (**F**) Representative images of male +/+, +/TG, SOD/+, and SOD/TG EDL muscle fibres stained for oxidative enzyme succinate dehydrogenase at P90, showing increased oxidative capacity only in SOD/+ mice. Scale Bar: 100µm. (**B-E**) Values are mean ± SEM. (n=3-7 for each genotype; **p<0.01, 2-way ANOVA, followed by Bonferroni post-hoc tests).

SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice, when compared to that of wild-type and Tg(*Prnp-Oxr1*) mice (Figure 4.10F). These data suggest that neuron-specific over-expression of Oxr1 is sufficient to improve muscle pathology in SOD1^{G93A} ALS mice.

4.3 Discussion

Understanding mechanisms by which OS contributes to the pathogenesis of different neurodegenerative diseases will be crucial for the development of future therapies. Here, we demonstrated that neuronal over-expression of Oxr1 *in vivo* protects against neurodegeneration in SOD1^{G93A} ALS mice, extending lifespan, improving motor function, and increasing spinal motor neuron survival. Moreover, we found that neuron-specific up-regulation of Oxr1 also improves muscle pathology in EDL and gastrocnemius muscles of SOD1^{G93A} ALS mice. Taken together, these results establish Oxr1 as a novel genetic modifier of ALS, particularly for SOD1-related ALS cases, with implications for Oxr1 as a neuroprotective protein in other neurodegenerative diseases.

4.3.1 Oxr1 modifies disease progression in SOD1^{G93A} ALS mice

Here, we established Oxr1 as a novel genetic modifier of ALS and found that neuron-specific up-regulation of Oxr1 extends lifespan, delays motor deficits, and improves spinal cord and muscle pathology. Recent studies have identified a number of genetic modifiers that increase lifespan and delay pathogenesis in ALS transgenic mouse models (Turner and Talbot, 2008; Riboldi *et al.*, 2011). Alongside Activating transcription factor 3 (ATF3), which is induced under stress conditions; Vascular endothelial growth factor (VEGF), a trophic factor; VEGF receptor 2 (VEGFR-2); X-box binding protein 1 (XBP-1), a transcription factor regulating unfolded protein response; and X-linked inhibitor of apoptosis protein (XIAP) (Inoue *et al.*, 2003; Storkebaum *et al.*, 2005; Wootz

et al., 2006; Wang *et al.*, 2007; Hetz *et al.*, 2009; Seijffers *et al.*, 2014); Oxr1 is one of very few genetic modifiers that when over-expressed or deleted specifically in neurons increase survival of $SOD1^{G93A}$ ALS mice. Furthermore, Oxr1 is the first antioxidant that significantly improves lifespan of $SOD1^{G93A}$ ALS mice when specifically over-expressed in neurons. Neuron-specific over-expression of Oxr1 increases survival of $SOD1^{G93A}$ ALS mice by approximately 19% for females, and 16.8% for males (Figure 4.4). Oxr1 over-expression in neurons not only increases survival of motor neurons in the lumbar spinal cord, but also improves motor function on the rotarod at all time-points (Figure 4.4-4.5). In addition, Oxr1 over-expression decreases expression of HO-1, an isoform of heme oxygenase that is up-regulated in response to OS in motor neurons of both $SOD1^{G93A}$ ALS mice and ALS patients (Figure 4.5) (Ferrante *et al.*, 1997b; Ferrante *et al.*, 1997a).

Interestingly, however, although degeneration of CSMN in the cortex has been observed in ALS patients and in the $SOD1^{G93A}$ ALS mice (Tandan and Bradley, 1985; Ozdinler *et al.*, 2011), we found no overt loss of CSMN in end-stage $SOD1^{G93A}$ ALS mice (Figure 4.6). These discrepancies may be due to the fact that our double-transgenic mice are on a mixed CBAB6F1/C57BL/6 background as opposed to the previous study on the $SOD1^{G93A}$ ALS mice, which were conducted on a congenic C57BL/6 background (Ozdinler *et al.*, 2011). Nonetheless, these findings support the hypothesis that over-expression of Oxr1 during ALS disease progression is neuroprotective.

Furthermore, the death of motor neurons in conjunction with SOD1-mediated toxicity in muscles lead to degeneration of neuromuscular junctions and severe muscle wasting in ALS patients and ALS mouse models (Jokelainen, 1977; Telerman-Toppet and Coers, 1978; Gurney *et al.*, 1994; Azzouz *et al.*, 1997; Dobrowolny *et al.*, 2008). Muscle atrophy is particularly prominent in gastrocnemius muscles, and intramuscular delivery of cardiotrophin-1 delays this particular skeletal muscle degeneration and improves motor

function (Mohajeri *et al.*, 1998; Bordet *et al.*, 2001). Here, we found that Oxr1 over-expression in neurons significantly decreases gastrocnemius muscle atrophy during ALS disease progression (Figure 4.9). In addition, EDL muscles are normally fast-contracting muscles that easily fatigue after repeated stimulation, but become slow-contracting and fatigue resistant during ALS disease progression (Kieran *et al.*, 2004; Kieran *et al.*, 2005). We also found that neuronal over-expression of Oxr1 also delays muscle fibre type composition changes in EDL muscles of $SOD1^{G93A}$ ALS mice from predominantly Type IIB fibres to 50% Type IIA fibres, suggesting that EDL muscles of $SOD1^{G93A}$ ALS mice are becoming slow-contracting and more fatigue resistant (Figure 4.10). Future research should also investigate whether neuronal Oxr1 over-expression improves muscle physiology, specifically decreased muscle force and motor unit survival, in $SOD1^{G93A}$ ALS mice (Kieran *et al.*, 2004; Kieran *et al.*, 2005; Dobrowolny *et al.*, 2008). Together with ATF3 and Flk1 (Storkebaum *et al.*, 2005), Oxr1 is one of three proteins, when over-expressed in neurons, delays motor dysfunction and improves muscle pathology.

VEGF and *Ephrin A4* (*EPHA4*) have been previously identified as a disease modifier of ALS in humans (Lambrechts *et al.*, 2003; Van Hoecke *et al.*, 2012). In particular, certain haplotypes in the *VEGF* promoter sequence were linked to lower gene transcription and VEGF levels *in vivo*, and subsequently, an increased risk of ALS (Lambrechts *et al.*, 2003). EPHA4 expression not only correlates inversely with disease pathogenesis, but two loss-of-function mutations in *EPHA4* were found to be associated with uncharacteristically increased survival (Van Hoecke *et al.*, 2012). Given that OXR1 expression is significantly increased in the spinal cord of ALS patients (Oliver *et al.*, 2011), and over-expression of Oxr1 improves motor function and extends lifespan in $SOD1^{G93A}$ ALS mice, it will be important to investigate whether any *de novo* mutations in *OXR1* affect disease progression and survival of ALS patients.

4.3.2 Role of Oxr1 in therapeutic strategies for neurodegenerative diseases

Compared to other organs, the brain requires high levels of oxygen metabolism, but has relatively limited antioxidant capacity, which makes neurons particularly sensitive to OS (Halliwell and Gutteridge, 2007). Furthermore, OS is a common pathobiology of all major neurodegenerative diseases, including AD, ALS, HD, and PD, and contributes substantially to neuronal dysfunction and death. (Dexter *et al.*, 1989; Beal *et al.*, 1997; Browne *et al.*, 1997; Hensley *et al.*, 1998; Pedersen *et al.*, 1998; Browne *et al.*, 1999; Butterfield *et al.*, 2002; Jenner, 2003; Barber *et al.*, 2006; Lin and Beal, 2006). Therefore, OS metabolites and antioxidant proteins could serve as a biomarker for early diagnosis and disease progression (Migliore *et al.*, 2005; Weir *et al.*, 2011; Gerlach *et al.*, 2012; Haas *et al.*, 2012; D'Amico *et al.*, 2013). For example, studies have found increased levels of DJ-1, an antioxidant protein that is mutated in some forms of early-onset recessive PD, in the blood plasma and cerebrospinal fluid of PD patients when compared with healthy controls (Waragai *et al.*, 2006; Waragai *et al.*, 2007). Conversely, another study reported decreased DJ-1 levels in PD patients in cerebrospinal fluid, and no changes in DJ-1 levels in blood plasma (Hong *et al.*, 2010). Due to limitations of current technologies to detect these biomarkers, including variation in sample processing, and low number of patients in each case, it is likely there are other confounding effects that underlie these inconsistencies (Shi *et al.*, 2010). Oxr1-FL is up-regulated in the spinal cord of pre-symptomatic low-copy $SOD1^{G93A}$ ALS mice (Oliver *et al.*, 2011), suggesting Oxr1 could serve as an early marker of OS and neurodegeneration. Thus, further research is needed to determine whether Oxr1 isoforms are altered in other pre-symptomatic neurodegenerative disease mice models, and during disease progression for each model.

Recent evidence also demonstrated proteins associated with different neurodegenerative disease regulate common OS pathways, and therapeutic strategies

targeting these common pathways both upstream and downstream of OS delays the onset and progression of neurodegenerative diseases (Halliwell, 2001; Moosmann and Behl, 2002; Tan *et al.*, 2003; Uttara *et al.*, 2009). In particular, DJ-1, and SOD1 are both modulators of the Nrf2 pathway, which regulates a large network of antioxidant genes through an upstream antioxidant response element (ARE) in response to OS (Milani *et al.*, 2013). DJ-1 regulates Nrf2 stability by preventing its interaction with Kelch-like ECH-associated protein 1 (Keap1), which is responsible for Nrf2 ubiquitination and subsequent degradation (Clements *et al.*, 2006; Gan *et al.*, 2010). ALS-linked SOD1 down-regulates *Nrf2* mRNA and Nrf2 target genes *in vitro*, and embryonic motor neuron cultures from $SOD1^{G93A}$ ALS mice also show decreased levels of Nrf2 and increased susceptibility to p75 neurotrophin receptor-induced apoptosis (Kirby *et al.*, 2005; Pehar *et al.*, 2007). Over-expression of Nrf2, inhibition of Keap1, or over-expression of Maf-S, a protein that forms a heterodimer with Nrf2 to activate transcription of antioxidant target genes, improves motor function and increases survival of α-synuclein-induced neurodegeneration in a *Drosophila* PD model (Barone *et al.*, 2011). In addition, astrocyte-specific over-expression of Nrf2 delays neuronal dysfunction and prolongs survival of PD mouse models (Chen *et al.*, 2009; Gan *et al.*, 2012). Similarly, specific over-expression of Nrf2 in astrocytes not only extends the lifespan of, but also improves muscle denervation and astrogliosis in $SOD1^{G93A}$ ALS mice (Vargas *et al.*, 2008; Vargas *et al.*, 2013).

While no ARE motifs have been identified upstream of Oxr1 (Malhotra *et al.*, 2010; Chorley *et al.*, 2012), changes in OXR1 expression has found in patients suffering from and in pre-symptomatic mouse models of diverse neurodegenerative diseases, suggesting that Oxr1 could play a central role in all major neurodegenerative OS pathways. Intermediate isoforms of OXR1 are up-regulated in the spinal cord biopsy samples of ALS patients, while *OXR1* is significantly down-regulated in the cortex of PD patients (Stamper

et al., 2008; Oliver *et al.*, 2011). Given that Oxr1 is neuroprotective, these findings suggest that in ALS patients, OXR1 may be up-regulated in response to increased OS, while in PD patients, *OXR1* may contribute to the ROS-mediated neurodegeneration. Importantly, we demonstrated that Oxr1 over-expression is neuroprotective *in vivo*, and improves lifespan of $SOD1^{G93A}$ ALS mice (Figure 4.4). Thus, it will be necessary to explore whether neuroprotective properties of Oxr1 can be extended to diverse neurological disorders by crossing Tg(*Prnp-Oxr1*) mice with other neurodegenerative disease mouse models, such as the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) PD mouse model and the R6/2 transgenic HD mouse model, both of which exhibit OS-related pathology (Arai *et al.*, 1990; Mangiarini *et al.*, 1996; Sriram *et al.*, 1997; Tabrizi *et al.*, 2000).

4.3.3 Oxr1 as a therapeutic target for ALS

While we presented exciting evidence that Oxr1 over-expression has therapeutic benefits in improving pathology and lifespan in $SOD1^{G93A}$ ALS mice, translating advances made in understanding molecular mechanisms governing ALS into clinically-effective therapies continue to be difficult due to limitations in mouse models used for preclinical testing and in both clinical diagnosis and design of clinical trials. Many compounds and genetic modifiers have been shown to increase survival of SOD1 mutant mouse models, but none have translated into successful therapeutic strategies for ALS patients (Benatar, 2007). Firstly, mutations in SOD1 only account for a very small proportion of all ALS patients (approximately 2%), and the relevance of SOD1 mutant mouse models are still unknown in recapitulating the pathways that are affected in sporadic ALS (Benatar, 2007; Sorenson, 2007). Furthermore, a majority of preclinical testing has been performed using $SOD1^{G93A}$ ALS mice, which we also used for our experiments (Benatar, 2007). Given the lack of our understanding of molecular mechanisms underlying ALS, it remains unclear

whether $SOD1^{G93A}$ only models one particular case of SOD1-linked familial ALS. These limitations suggest that demonstrating that Oxr1 over-expression increases survival of $SOD1^{G93A}$ ALS mice may have limited implications for other forms of ALS. Therefore, in the future, crossing Tg(*Prnp-Oxr1*) mice with additional ALS mouse models can determine whether Oxr1 is a potential genetic modifier of different forms of ALS *in vivo* (Turner and Talbot, 2008; Riboldi *et al.*, 2011; McGoldrick *et al.*, 2013).

Another important limitation of these preclinical studies is that many of these studies have been conducted with early initiation of drug therapy, or deletion or over-expression of genetic modifiers at a pre-symptomatic stage (if not earlier) in the SOD1 mouse models, such as the study discussed in this Chapter (Benatar, 2007). Findings from studies using this experimental design have given tremendous insight into molecular mechanisms underlying ALS, but have proven difficult to predict the efficacy of the therapy after the onset of disease, which is particularly important because currently, there is limited technology to identify ALS patients before onset of symptoms. Therefore, in the future, to further determine the therapeutic potential of OXR1 for ALS patients, we will need to examine whether up-regulation of Oxr1 after symptom onset of $SOD1^{G93A}$ ALS mice and other ALS mouse models still delays disease progression.

Finally, there are limitations of clinical trial design and technological barriers to successful translation of therapies from bench to bedside (Mitchell *et al.*, 2010; Bowser *et al.*, 2011). Delays between clinical manifestation and successful diagnosis, compounded by factors including clinical heterogeneity, may have drastic consequences, as some patients may bypass a critical window for therapeutics without treatment (Mitchell *et al.*, 2010; Bowser *et al.*, 2011). This delay in diagnosis contributes to enrolment challenges in clinical trial design, and along with other issues, for example, pharmacodynamics interactions with riluzole and a lack of well-defined prognostic outcome measures, have

contributed to unsuccessful attempts to identify additional therapies for ALS (Mitsumoto *et al.*, 2004; Aggarwal and Cudkowicz, 2008). Furthermore, as functional neuronal abnormalities have been identified during development in rodent models of ALS (Bendotti *et al.*, 2001; Raoul *et al.*, 2002; Raoul *et al.*, 2006; Bories *et al.*, 2007; Ferraiuolo *et al.*, 2007; Lobsiger *et al.*, 2007; van Zundert *et al.*, 2008), development of novel early diagnosis techniques could allow intervention in humans at the pre-clinical stage, decades prior to the onset of symptoms. These potential diagnostic techniques range from the discovery and use of protein biomarkers in cerebrospinal fluid or blood to the development of more sensitive electrophysiological tests and brain imaging markers (Henley *et al.*, 2005; Bowser *et al.*, 2011; Gordon and Meininger, 2011; Turner *et al.*, 2013). Early identification of ALS patients will allow for greater potential to achieve maximum therapeutic efficacy and permit better disease-monitoring, increasing the likelihood of success in clinical trials. Due to the multiplicity of disease-mediating factors and complexity of the disease, an effective treatment for ALS will require a multi-faceted approach from pre-symptomatic diagnosis to implementation of varied therapeutic strategies (Benatar, 2007; Glass *et al.*, 2012; Riley *et al.*, 2012).

In summary, in this chapter, we demonstrated for the first time that up-regulation of Oxr1 is neuroprotective *in vivo*. Interestingly, I found that over-expression of Oxr1 in neurons is sufficient to delay neurodegeneration and muscle pathology, and improve motor function in ALS. Taken together, these results suggest that therapeutic strategies targeting up-regulation of OXR1 in neurons could prove to be important for ALS patients, with wide-reaching implications for other neurodegenerative diseases.

5 Downstream pathways of Oxr1 function during ALS pathogenesis

5.1 Introduction

In Chapter 4, we demonstrated that neuronal over-expression of Oxr1 extends survival and ameliorates motor dysfunction of $SOD1^{G93A}$ ALS mice. Furthermore, Oxr1 over-expression in neurons delays oxidative stress, and improves motor neuron survival during ALS pathogenesis. Surprisingly, Oxr1 over-expression in neurons is also sufficient to delay neuromuscular junction degeneration, gastrocnemius muscle atrophy, and fatigue-resistant changes to extensor digitorum longus (EDL) muscles. Therefore, we hypothesize that Oxr1 functions upstream of many important molecular pathways to confer protection in neurons against SOD1-mediated apoptosis.

5.1.1 Aims of chapter 5

Little is currently known about Oxr1 function, with evidence suggesting that Oxr1 protects neurons against oxidative stress (OS), but does not act as an antioxidant enzyme (Oliver *et al.*, 2011). Moreover, over-expression of Oxr1 is neuroprotective *in vivo*, as shown in Chapter 4. Taken together, the data suggest that Oxr1 may be sensitive to increased levels of ROS, and subsequently regulate cellular survival pathways. The goal of this chapter was to gain insight into the mechanisms by which Oxr1 functions through 1) characterisation of pathways that are downstream of Oxr1 function and responsible for delaying ALS disease progression in $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice and assessment of Oxr1 over-expression on the transcriptome through microarray analysis; and 2) identification of novel protein interacting partners of over-expressed Oxr1 in the brain and spinal cord through co-immunoprecipitation and mass spectrometry analysis.

5.2 Results

5.2.1 Transcriptomic analysis reveals that over-expression of Oxr1 in neurons delays early gene expression changes in spinal cord of ALS mice at P60

Because little is known about the mechanisms by which Oxr1 functions and Oxr1 over-expression in neurons from an early embryonic time-point extends survival of $SOD1^{G93A}$ ALS mice (Figure 4.1;4.4), we first chose to investigate downstream pathways of Oxr1 function that modulate ALS disease pathogenesis by performing microarray analysis on RNA extracted from lumbar spinal cord tissue of wild-type, Tg(*Prnp-Oxr1*), $SOD1^{G93A}$ ALS, and $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice at an early pre-symptomatic stage (P60). We only identified subtle changes in the transcriptome of wild-type, Tg(*Prnp-Oxr1*), $SOD1^{G93A}$ ALS, and $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice; however, the correlation plot of normalized gene expression shows that only $SOD1^{G93A}$ ALS mice are grouped separately at P60 (Figure 5.1A). This result suggests that even at the pre-symptomatic stage, the spinal cord transcriptome is already becoming altered by $SOD1^{G93A}$ aberrant functions in $SOD1^{G93A}$ ALS mice.

Using the most stringent analysis, a moderated t-test with a Benjamini and Hochberg multiple testing correction (limma), we found that only two genes were changed under Oxr1 over-expression in neurons, *Oxr1*, and *Prnp* at this time-point. Up-regulation of *Prnp* is most likely due to the particular probeset recognizing noncoding sequences within the first and second exon of *Prnp* that precede the HA-tagged *Oxr1* transgene in the *Prnp-Oxr1* construct used to generate Tg(*Prnp-Oxr1*) mice (Figure 4.1;Table 5.1). Furthermore, only one gene is altered in the $SOD1^{G93A}$ spinal cord, when compared to wild-type and Tg(*Prnp-Oxr1*) spinal cords: *eukaryotic translation initiation factor 4A2* (*Eif4a2*) (Table 5.2). However, using limma, we identified no genes as being "rescued," significantly changed by >1.3 fold change in the $SOD1^{G93A}$ spinal cord, but not

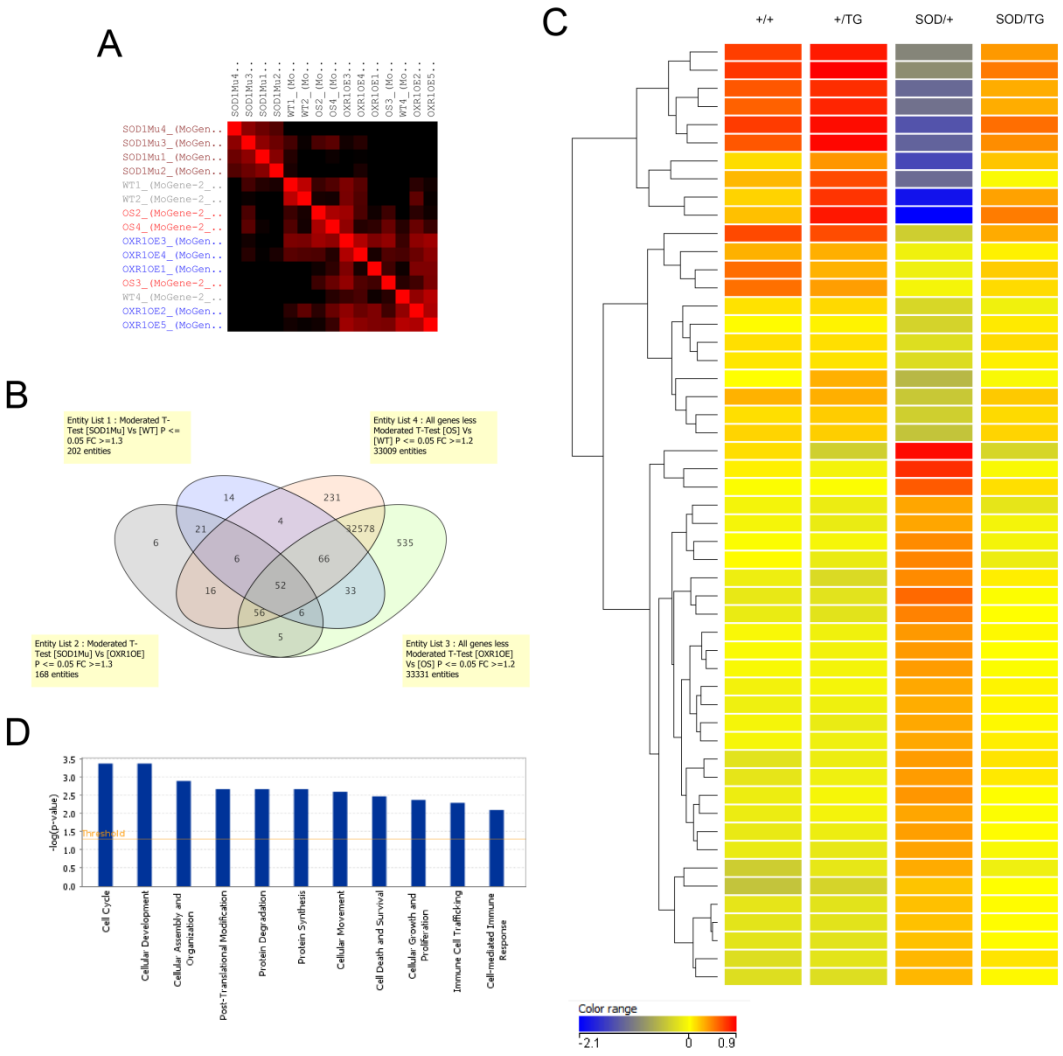

Figure 5.1. Over-expression of Oxr1 in neurons delays early subtle transcriptome changes in the spinal cord of SOD1^{G93A} ALS mice at P60

(**A**) Correlation plot of RMA normalized gene expression reveals that subtle transcriptome changes are specific to SOD1^{G93A} ALS mice (SOD1Mu), when compared with wild-type (WT), Tg(Prnp-Oxr1) (OXR1OE), SOD1^{G93A}/Tg(*Prnp-Oxr1*) (OS) mice. (**B**) Venn diagram illustrates the common expression overlap from microarray analysis to identify 52 "rescued" genes, those that are significantly changed ($p \leq 0.05$) by >1.3 fold change in the SOD1^{G93A} (SOD1Mu) spinal cord, but not significantly changed ($p > 0.05$) or by <1.2 fold change in the SOD1^{G93A}/Tg(*Prnp-Oxr1*) (OS) spinal cord. (**C**) Gene expression heat map of normalized signal intensity values for all genes identified as "rescued" by neuronal Oxr1 up-regulation from wild-type (+/+), Tg(Prnp-Oxr1) (+/TG), SOD1^{G93A} ALS (SOD/+), SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice. (**D**) Pathway analysis of 52 "rescued" genes demonstrates that Oxr1 over-expression in neurons delays SOD1^{G93A}-induced changes in a number of different molecular pathways. Significance of identified pathways is presented as a –log(p-value), where $p \leq 0.05$ is –log(p-value)≥1.3. RNA samples were prepared by Dr. Mattea Finelli, microarray experiment was performed by Dr. Sheena Lee, and analysis was conducted by Dr. Sheena Lee and me.

Table 5.1. Gene expression altered by Oxr1 over-expression in neurons at P60 using limma

Transcripts Cluster Id	p (Corr)	p value	Fold change	Up / Down	Gene description	Gene symbol
17311286	1.45E-04	4.30E-09	2.1	up	oxidationresistance1	Oxr1
17376541	1.47E-04	8.67E-09	1.7	up	Prion protein | prion protein dublet	Prnp | Prnd

Table 5.2. Mutant SOD1-induced gene expression changes in lumbar spinal cord at P60 using limma

	SOD Vs. WT				SOD Vs. TG					
Transcripts Cluster Id	p (Corr)	p	Fold change	Up / Down	p (Corr)	p	Fold change	Up / Down	Gene description	Gene symbol
17324398	2.71E-02	8.03E-07	3.0	up	5.11E-04	4.54E-08	3.2	up	Small nucleolar RNA, H/ACA box 81 | eukaryotic translation initiation factor 4A2	Snora81 | Eif4a2

Table 5.3. Mutant SOD1-induced gene expression changes in lumbar spinal cord at P60 using a moderated t-test without testing correction

	SOD Vs. WT			SOD Vs. TG				
Transcripts Cluster Id	p value	Fold change	Up / Down	p value	Fold change	Up / Down	Gene description	Gene symbol
17514340	4.59E-02	5.1	down	1.74E-04	7.8	down		
17514333	2.72E-02	4.4	down	3.27E-05	6.5	down		
17514349	2.18E-02	4.4	down	2.52E-04	4.9	down		
17514352	3.72E-02	3.7	down	2.34E-04	4.6	down		
17514328	1.39E-02	3.6	down	1.09E-04	4.0	down		
17514337	1.44E-02	3.4	down	1.31E-04	3.9	down		
17514330	2.43E-02	3.2	down	4.22E-04	3.6	down		
17514346	2.71E-02	3.2	down	6.33E-04	3.6	down		
17388663	1.43E-02	3.2	down	6.26E-05	3.8	down		
17324398	1.25E-06	3.0	up	2.87E-08	3.0	up	Small nucleolar RNA, H/ACA box 81 | eukaryotic translation initiation factor 4A2	Snora81 | Eif4a2
17514343	4.72E-02	2.8	down	1.06E-04	3.7	down		
17514326	1.36E-02	2.1	down	3.46E-03	2.1	down		
17407412	6.24E-03	1.9	up	2.06E-03	1.7	up	Small proline-rich protein 1A	Sprr1a
17275060	2.18E-04	1.8	up	5.45E-06	1.5	up	microRNA5099	Mir5099
17312079	7.95E-04	1.8	up	2.70E-02	1.3	up		
17491632	1.51E-04	1.8	down	3.97E-05	2.1	down		
17532627	1.34E-03	1.8	down	3.11E-04	1.8	down		
17542034	4.43E-02	1.7	up	1.16E-03	2.4	up	Cerebellar degeneration related antigen 1	Cdr1

17514355	1.09E-02	1.7	down	5.11E-04	1.7	down		
17243953	4.45E-04	1.6	up	1.28E-04	1.7	up		
17240834	1.32E-02	1.6	up	1.27E-03	1.4	up		
17248348	4.12E-02	1.6	up	1.03E-03	1.5	up		
17245223	2.29E-02	1.6	up	9.27E-04	1.8	up	Lysozyme2	Lyz2
17373634	1.48E-02	1.6	down	3.11E-02	1.3	down		
17380392	3.90E-03	1.6	down	4.55E-03	1.6	down		
17250740	6.16E-03	1.6	down	5.18E-04	2.0	down	Small nucleolar RNA, C/D box 49B	Snord49b
17278850	4.55E-04	1.6	up	2.30E-03	1.4	up	microRNA544	Mir544
17334147	1.05E-04	1.5	up	1.67E-03	1.5	up	microRNA5125	Mir5125
17499682	1.85E-02	1.5	up	2.07E-04	1.6	up		LOC100861653
17419411	4.50E-02	1.5	down	9.26E-04	2.1	down	Small nucleolar RNA, C/D box 99	Snord99
17278759	1.32E-02	1.5	up	4.78E-03	1.5	up		
17214043	8.53E-04	1.5	down	6.09E-03	1.4	down		
17254200	8.72E-04	1.5	down	5.93E-03	1.4	down		
17214041	8.67E-04	1.5	down	6.11E-03	1.4	down		
17214039	8.76E-04	1.5	down	6.12E-03	1.4	down		
17278810	1.30E-02	1.5	up	9.04E-03	1.4	up	microRNA380	Mir380
17266967	6.26E-03	1.5	up	6.25E-03	1.4	up	Chemokine (C-Cmotif) ligand 3	Ccl3
17538295	6.08E-03	1.5	down	5.63E-03	1.8	down	Heterogeneous nuclear ribonucleoprotein A1 pseudogene	Gm5643
17546586	1.36E-02	1.5	down	3.88E-02	1.3	down		
17491495	1.34E-02	1.5	up	9.77E-05	1.4	up	RIKEN cDNA A230073K19 gene | ubiquitin protein ligase E3A	A230073K19Rik | Ube3a
17214025	1.63E-03	1.5	down	1.86E-02	1.4	down		
17491741	2.43E-03	1.5	up	4.43E-03	1.4	up		
17382359	4.09E-03	1.5	up	2.87E-03	1.4	up		
17405817	5.94E-05	1.5	up	5.77E-05	1.5	up		
17524209	2.35E-03	1.5	up	2.28E-04	1.5	up		
17314679	3.06E-03	1.5	up	2.27E-03	1.6	up	Tubulin, alpha1B	Tuba1b
17248123	4.70E-03	1.5	up	3.91E-03	1.3	up		
17415971	7.77E-04	1.4	up	1.23E-05	1.5	up		
17509839	1.78E-02	1.4	up	2.84E-03	1.5	up		LOC100505125
17378896	4.41E-03	1.4	up	2.56E-03	1.4	up		
17397575	2.12E-02	1.4	up	8.58E-03	1.4	up	Periostin, osteoblast specific factor	Postn
17460933	6.06E-03	1.4	down	4.25E-03	1.3	down	LSM3 homolog, U6 small nuclear RNA associated (S.cerevisiae)	Lsm3
17417697	1.77E-03	1.4	up	1.65E-04	1.6	up		
17240987	1.70E-03	1.4	down	3.23E-04	1.5	down		
17535143	1.79E-03	1.4	up	6.19E-04	1.4	up	RIKEN cDNA C030023E24 gene	C030023E24Rik
17474268	3.31E-02	1.4	up	9.23E-03	1.4	up		
17330850	4.49E-03	1.4	up	1.24E-03	1.3	up		
17250742	3.60E-03	1.4	down	3.32E-03	1.9	down	Small nucleolar RNA, C/Dbox 49A	Snord49a
17499647	3.76E-02	1.4	up	1.63E-03	1.4	up		
17491568	1.68E-04	1.4	down	3.50E-06	1.7	down		
17547915	6.74E-03	1.4	up	3.98E-04	1.3	up	RIKEN cDNA9130004J05 gene	9130004J05Rik
17548738	2.55E-02	1.4	up	1.67E-04	1.4	up		LOC100503226
17548740	2.58E-02	1.4	up	1.58E-04	1.4	up		LOC100503226

17548742	2.52E-02	1.4	up	1.78E-04	1.4	up		LOC100503226
17467690	1.23E-02	1.4	down	9.53E-04	1.4	down	Pentatricopeptide repeat domain 3	Ptcd3
17510626	1.32E-03	1.4	up	2.94E-05	1.4	up		
17501311	4.27E-02	1.3	up	6.68E-03	1.5	up		
17451126	2.35E-03	1.3	down	2.21E-05	1.6	down	E1A binding protein p400	Ep400
17400375	1.19E-02	1.3	up	5.33E-04	1.4	up	CathepsinS	Ctss
17445236	3.91E-02	1.3	up	5.34E-03	1.3	up		
17499670	1.90E-02	1.3	up	9.03E-04	1.4	up	Cadherin11 pseudogene	2610005L07Rik | 6820431F20Rik
17278781	7.36E-04	1.3	up	2.57E-04	1.3	up		
17323320	3.14E-04	1.3	up	1.49E-03	1.4	up	Predicted gene 5481	Gm5481
17491541	2.76E-03	1.3	up	5.06E-04	1.4	up		
17406527	1.03E-04	1.3	up	1.18E-04	1.4	up	Predicted gene 6570	Gm6570
17322911	5.78E-04	1.3	up	5.29E-04	1.4	up		
17546668	1.53E-02	1.3	down	2.57E-02	1.3	down	Ssty1 family member | uncharacterized LOC 100040031 | uncharacterized LOC 100041256 | uncharacterized LOC 100039810 | uncharacterized LOC 100039614	LOC382133 | LOC100040031 | LOC100041256 | LOC100039810 | LOC100039614
17378858	1.07E-02	1.3	down	4.20E-03	1.4	down		
17293704	6.49E-03	1.3	up	1.27E-02	1.4	up		
17421413	3.40E-03	1.3	down	1.83E-02	1.3	down	Zinc finger protein 600 | zinc finger protein 160-like	Zfp600 | LOC100862458 | LOC673430
17278753	1.56E-03	1.3	up	8.67E-04	1.4	up		
17367652	2.06E-03	1.3	down	4.50E-05	1.4	down	Interleukin1 family, member9	Il1f9
17541776	4.08E-03	1.3	down	1.35E-03	1.3	down	CAAX box1 homolog A (human) | CAAX box1 homolog B (human)	Cxx1a | Cxx1b
17520599	1.71E-04	1.3	up	7.47E-04	1.4	up		
17242322	2.99E-02	1.3	down	3.74E-02	1.3	down	Predicted gene 9507 | predicted gene 2696 | predicted gene 9508 | predicted gene 7137 | predicted gene 10318 | predicted gene 9736	Gm9507 | Gm2696 | Gm9508 | Gm7137 | Gm10318 | Gm9736

significantly changed or by <1.2 fold change in the SOD1^{G93A}/Tg(*Prnp-Oxr1*) spinal cord (data not shown).

Therefore, we chose to use a less stringent analysis, a moderated t-test with no multiple testing correction, and we identified 85 genes that are altered in SOD1^{G93A} spinal cords, when compared to wild-type and Tg(*Prnp-Oxr1*) spinal cords (Table 5.3) (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Perrin *et al.*, 2005; Ferraiuolo *et al.*, 2007; Chen *et al.*, 2010; Lincecum *et al.*, 2010). Many of these differentially expressed genes, such as *Small proline-rich protein 1A* (*Sprr1a*) and *chemokine (C-C motif) ligand 3* (*Ccl3*), have been found in previous studies (Table 5.3) (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Perrin *et al.*, 2005; Ferraiuolo *et al.*, 2007; Chen *et al.*, 2010; Lincecum *et al.*, 2010). Interestingly, using this less stringent analysis, we identified 52 genes as being "rescued," including *Cathepsin S (Ctss)* and *Lysozyme 2 (Lyz2)* (Table 5.4; Figure 5.1B-C). Ingenuity Pathway Analysis demonstrated that a number of biological functions are "rescued" downstream of Oxr1 up-regulation, including cell cycle, post-translational modification, protein degradation, cell death and survival, and immune cell trafficking (Figure 5.1D). Immune system response, apoptosis, and protein synthesis and degradation have been previously shown to contribute to ALS disease progression (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Perrin *et al.*, 2005; Ferraiuolo *et al.*, 2007; Chen *et al.*, 2010; Lincecum *et al.*, 2010).

Furthermore, we also used a moderated t-test with no multiple testing corrections to examine whether there are subtle changes to the transcriptome under neuronal over-expression of Oxr1. We identified 48 genes that are significantly differentially expressed by >1.3 fold in Tg(*Prnp-Oxr1*) mice when compared with wild-type mice (Table 5.5). These genes include *Connective tissue growth factor* (*Ctgf*) and *Heat shock protein 1B* (*Hspa1b*) (Table 5.5). Using Ingenuity Pathway Analysis, we found that the most significant biological functions subtly altered by neuronal Oxr1 over-expression are cell

Table 5.4. Mutant SOD1-induced gene expression changes that are "rescued" by neuronal Oxr1 over-expression at P60 using a moderated t-test without testing correction

	SOD Vs. WT			SOD Vs. TG			SOD/TG Vs. WT			SOD/TG Vs. TG				
Transcripts Cluster Id	p-value (Corr)	Fold change	Up/ Down	p-value (Corr)	Fold change	Up/ Down	p-value (Corr)	Fold change	Up/ Down	p-value (Corr)	Fold change	Up/ Down	Gene Description	Gene Symbol
17514340	4.59E-02	5.1	down	1.74E-04	7.8	down	4.88E-01	1.3	up	8.28E-01	1.2	up		
17514333	2.72E-02	4.4	down	3.27E-05	6.5	down	4.19E-01	1.3	up	8.59E-01	1.1	up		
17514349	2.18E-02	4.4	down	2.52E-04	4.9	down	5.26E-01	1.3	up	8.46E-01	1.1	down		
17514352	3.72E-02	3.7	down	2.34E-04	4.6	down	3.54E-01	1.4	up	8.40E-01	1.1	down		
17514328	1.39E-02	3.6	down	1.09E-04	4.0	down	2.85E-01	1.4	up	6.75E-01	1.2	down		
17514337	1.44E-02	3.4	down	1.31E-04	3.9	down	2.94E-01	1.4	up	7.07E-01	1.2	down		
17514330	2.43E-02	3.2	down	4.22E-04	3.6	down	3.63E-01	1.4	up	6.86E-01	1.2	down		
17514346	2.71E-02	3.2	down	6.33E-04	3.6	down	3.82E-01	1.3	up	7.55E-01	1.2	down		
17388663	1.43E-02	3.2	down	6.26E-05	3.8	down	4.67E-01	1.1	up	8.84E-01	1.1	up		
17514343	4.72E-02	2.8	down	1.06E-04	3.7	down	1.38E-01	1.6	up	7.19E-01	1.2	down		
17514326	1.36E-02	2.1	down	3.46E-03	2.1	down	3.65E-01	1.3	up	4.46E-01	1.3	down		
17542034	4.43E-02	1.7	up	1.16E-03	2.4	up	8.67E-01	1.0	down	2.65E-01	1.4	down	Cerebellar degeneration related antigen 1	Cdr1
17514355	1.09E-02	1.7	down	5.11E-04	1.7	down	6.60E-01	1.1	up	7.99E-01	1.1	down		
17243953	4.45E-04	1.6	up	1.28E-04	1.7	up	1.46E-01	1.2	down	2.43E-01	1.1	up		
17240834	1.32E-02	1.6	up	1.27E-03	1.4	up	6.92E-01	1.0	down	3.47E-01	1.2	up		
17248348	4.12E-02	1.6	up	1.03E-03	1.5	up	8.56E-02	1.3	down	2.17E-01	1.4	up		
17245223	2.29E-02	1.6	up	9.27E-04	1.8	up	6.63E-01	1.0	down	5.73E-01	1.1	down	Lysozyme2	Lyz2
17373634	1.48E-02	1.6	down	3.11E-02	1.3	down	6.82E-01	1.1	up	2.65E-01	1.3	down		
17380392	3.90E-03	1.6	down	4.55E-03	1.6	down	9.22E-01	1.0	up	9.97E-01	1.0	down		
17499682	1.85E-02	1.5	up	2.07E-04	1.6	up	5.31E-02	1.2	down	4.53E-01	1.1	up		LOC100861653
17278759	1.32E-02	1.5	up	4.78E-03	1.5	up	4.85E-01	1.1	down	5.50E-01	1.1	up		
17278810	1.30E-02	1.5	up	9.04E-03	1.4	up	1.50E-01	1.2	down	1.09E-01	1.3	up	microRNA 380	Mir380
17538295	6.08E-03	1.5	down	5.63E-03	1.8	down	2.74E-01	1.3	up	7.79E-01	1.0	down	Heterogeneous nuclear ribonucleoprotein A1 pseudogene	Gm5643
17546586	1.36E-02	1.5	down	3.88E-02	1.3	down	3.35E-01	1.2	up	1.37E-01	1.3	down		
17491495	1.34E-02	1.5	up	9.77E-05	1.4	up	3.52E-02	1.2	down	1.88E-01	1.2	up	RIKENcDNAA230073K19gene | ubiquitin protein ligase E3A	A230073K19Rik | Ube3a
17314679	3.06E-03	1.5	up	2.27E-03	1.6	up	8.28E-02	1.3	down	1.22E-01	1.1	up	tubulin, alpha 1B	Tuba1b
17509839	1.78E-02	1.4	up	2.84E-03	1.5	up	5.39E-02	1.2	down	1.43E-01	1.2	up		LOC100505125
17378896	4.41E-03	1.4	up	2.56E-03	1.4	up	5.12E-02	1.2	down	5.33E-02	1.3	up		
17397575	2.12E-02	1.4	up	8.58E-03	1.4	up	2.90E-01	1.1	down	2.86E-01	1.2	up	periostin, osteoblast specific factor	Postn
17240987	1.70E-03	1.4	down	3.23E-04	1.5	down	7.12E-01	1.0	up	9.07E-01	1.0	up		
17535143	1.79E-03	1.4	up	6.19E-04	1.4	up	3.56E-01	1.1	down	1.55E-01	1.1	up	RIKEN cDNA C030023E24 gene	C030023E24Rik

17499647	3.76E-02	1.4	up	1.63E-03	1.4	up	2.31E-01	1.1	down	5.90E-01	1.1	up		
17547915	6.74E-03	1.4	up	3.98E-04	1.3	up	3.48E-01	1.1	down	3.57E-01	1.1	up	RIKEN cDNA 9130004J05 gene	9130004J05Rik
17548738	2.55E-02	1.4	up	1.67E-04	1.4	up	1.19E-01	1.2	down	3.48E-01	1.2	up		LOC100503226
17548740	2.58E-02	1.4	up	1.58E-04	1.4	up	1.16E-01	1.2	down	3.49E-01	1.2	up		LOC100503226
17548742	2.52E-02	1.4	up	1.78E-04	1.4	up	1.18E-01	1.2	down	3.45E-01	1.2	up		LOC100503226
17467690	1.23E-02	1.4	down	9.53E-04	1.4	down	4.42E-02	1.2	up	1.74E-01	1.2	down	Pentatricopeptide repeat domain 3	Ptcd3
17501311	4.27E-02	1.3	up	6.68E-03	1.5	up	9.14E-01	1.0	down	8.15E-02	1.1	down		
17400375	1.19E-02	1.3	up	5.33E-04	1.4	up	8.25E-01	1.0	up	4.83E-01	1.1	down	Cathepsin S	Ctss
17445236	3.91E-02	1.3	up	5.34E-03	1.3	up	1.65E-01	1.1	down	3.44E-01	1.1	up		
17499670	1.90E-02	1.3	up	9.03E-04	1.4	up	1.87E-01	1.1	down	5.96E-01	1.1	up	Cadherin 11 pseudogene	2610005L07Rik | 6820431F20Rik
17278781	7.36E-04	1.3	up	2.57E-04	1.3	up	2.52E-01	1.1	down	2.50E-01	1.1	up		
17323320	3.14E-04	1.3	up	1.49E-03	1.4	up	6.82E-01	1.0	up	3.77E-01	1.1	down	Predicted gene 5481	Gm5481
17491541	2.76E-03	1.3	up	5.06E-04	1.4	up	5.81E-01	1.1	down	9.80E-01	1.0	down		
17406527	1.03E-04	1.3	up	1.18E-04	1.4	up	4.72E-01	1.1	down	7.11E-01	1.0	up	Predicted gene 6570	Gm6570
17322911	5.78E-04	1.3	up	5.29E-04	1.4	up	4.09E-01	1.1	down	7.31E-01	1.0	up		
17546668	1.53E-02	1.3	down	2.57E-02	1.3	down	2.34E-01	1.2	up	1.96E-01	1.2	down	Ssty1familymember | uncharacterized LOC100040031 | uncharacterized LOC100041256 | uncharacterized LOC100039810 | uncharacterized LOC100039614	LOC382133 | LOC100040031 | LOC100041256 | LOC100039810 | LOC100039614
17293704	6.49E-03	1.3	up	1.27E-02	1.4	up	3.26E-01	1.1	down	4.53E-01	1.1	up		
17421413	3.40E-03	1.3	down	1.83E-02	1.3	down	9.43E-01	1.0	down	9.37E-01	1.0	up	Zinc finger protein 600 | zinc finger protein 160-like	Zfp600 | LOC100862458 | LOC673430
17541776	4.08E-03	1.3	down	1.35E-03	1.3	down	6.72E-01	1.0	up	7.90E-01	1.0	down	CAAX box 1 homolog A (human) | CAAX box 1 homolog B (human)	Cxx1a | Cxx1b
17520599	1.71E-04	1.3	up	7.47E-04	1.4	up	1.43E-01	1.1	down	1.99E-01	1.1	up		
17242322	2.99E-02	1.3	down	3.74E-02	1.3	down	8.42E-01	1.0	down	4.51E-01	1.0	up	Predicted gene 9507 | predicted gene 2696 | predicted gene 9508 | predicted gene 7137 | predicted gene 10318 | predicted gene 9736	Gm9507 | Gm2696 | Gm9508 | Gm7137 | Gm10318 | Gm9736

Table 5.5. Gene expression altered by Oxr1 over-expression in neurons at P60 using a moderated t-test without testing correction

Transcripts Cluster Id	p value	Fold change	Up / Down	Gene description	Gene symbol
17311286	4.30E-09	2.1	Up	Oxidation resistance 1	Oxr1
17376541	8.67E-09	1.7	Up	Prion protein | prion protein dublet	Prnp | Prnd
17386477	3.68E-03	1.6	Up		
17411319	2.03E-03	1.5	Up	RAB geranylgeranyl transferase, b subunit	Rabggtb
17239773	1.50E-02	1.4	Up	Connective tissue growth factor	Ctgf
17524206	1.53E-02	1.4	Up		
17225173	3.25E-02	1.4	Up	Small nucleolar RNA, C/D box 82	Snord82
17312079	1.38E-02	1.4	Up		
17284366	3.99E-02	1.4	Up		
17491896	2.19E-03	1.3	Up		
17372725	1.20E-02	1.3	Up	Apelin receptor	Aplnr
17261033	4.18E-02	1.3	Up		
17281400	2.67E-02	1.3	Up		
17458163	4.23E-02	1.3	Up	KRAB-A domain containing 1	Krba1
17330497	1.98E-02	1.3	Up		
17477362	7.07E-03	1.3	Up	Kallikrein1-related peptidase b24	Klk1b24
17513854	2.29E-02	1.3	Up		
17344126	4.98E-02	1.3	Up	Heat shock protein 1B	Hspa1b
17464713	5.99E-03	1.3	Up		
17312716	1.15E-02	1.3	Up	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	Csf2rb
17357648	1.93E-02	1.3	Up	Membrane-spanning 4-domains, subfamily A, member 4C	Ms4a4c
17472085	3.31E-04	1.3	Up	Phosphatidylethanolamine binding protein 2	Pbp2
17473988	3.32E-02	1.3	Up	Oocyte specific homeobox 5	Obox5
17465711	5.91E-03	1.3	Up	Aldo-keto reductase family 1, member B3 (aldosereductase)	Akr1b3
17541060	2.47E-03	1.3	Up	Reproductiv ehomeobox 3G, pseudogene	Rhox3g-ps
17444009	3.90E-03	1.3	Up	RIKEN cDNAC130050O18 gene	C130050O18Rik
17325261	2.00E-03	1.3	Up	Hspb associated protein 1	Hspbap1
17457116	9.08E-03	1.3	Up		
17439532	2.87E-02	1.3	Up		
17344122	1.24E-03	1.3	Up		
17520288	7.34E-03	1.3	Up		
17467421	5.63E-03	1.3	Up		
17376276	1.43E-02	1.3	Up	Small nucleolar RNA, C/D box 57 | NOP56 ribonucleoprotein homolog (yeast)	Snord57|Nop56
17532106	4.46E-02	1.3	Up	Solute carrier family 22 (organic cation transporter), member 13b	Slc22a13b
17485258	8.39E-04	1.3	Up	Mitochondrial ribosomal protein L23	Mrpl23
17421865	4.85E-02	1.3	Up	microRNA34a	Mir34a
17294034	1.04E-03	1.3	Up	Zinc finger protein 71, related sequence	Zfp71-rs1
17437565	1.42E-02	1.3	Up		
17484513	1.08E-03	1.3	Up	CD163 molecule-like 1	Cd163l1
17321241	9.34E-03	1.3	Up	Small nucleolar RNA, H/ACA box 34	Snora34
17275060	4.15E-02	1.3	Up	microRNA5099	Mir5099

17423049	3.07E-02	1.3	Up		
17440130	1.06E-02	1.3	Up	Ribosomal protein L5 | predicted gene 14217	Rpl5 | Gm14217
17478799	2.30E-02	1.3	Up		
17349713	2.84E-02	1.3	Up	Ankyrin repeat and KH domain containing 1	Ankhd1
17481135	1.01E-03	1.3	Up	Olfactory receptor 554	Olfr554
17254616	3.56E-02	1.3	Up		
17233507	3.75E-03	1.3	Up		BB019430

death and survival, cellular compromise, and cell morphology (Figure 5.2). These functions are unsurprising given that Oxr1 protects against OS-induced apoptosis (Volkert *et al.*, 2000; Oliver *et al.*, 2011).

5.2.2 Transcriptomic analysis reveals that over-expression of Oxr1 in neurons delays widespread gene expression changes in spinal cord of ALS mice at P90

We observed only subtle changes in gene expression at pre-symptomatic SOD1-mediated ALS as previously described (Olsen *et al.*, 2001; Fukada *et al.*, 2007), thus we chose to gain further insight into Oxr1 function by conducting microarray analysis on RNA extracted from lumbar spinal cord tissue at the early symptomatic stage P90. We found that the correlation plot of normalized gene expression does not show distinct clustering of $SOD1^{G93A}$ ALS mice, possibly due to varied levels of disease progression in the mice (Figure 5.3A). Interestingly, we found more striking changes to the transcriptome, and therefore used a more stringent analysis, limma. We found that only *Oxr1* is differentially expressed in the spinal cord of $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice when compared to that of wild-type mice (Table 5.6). This result further demonstrates that long-term over-expression of Oxr1 does not alter the transcriptome within the spinal cord.

Furthermore, we identified 65 genes are differentially expressed in the $SOD1^{G93A}$ spinal cord, when compared to wild-type and Tg(*Prnp-Oxr1*) spinal cords (Table 5.7). Of these 65 genes, 63 genes are "rescued" by over-expression of Oxr1 in neurons at P90 (Figure 5.3B-C; Table 5.8). This increase of gene expression changes from pre-symptomatic to symptomatic stage of mutant SOD1 mice has been previously noted (Olsen *et al.*, 2001; Fukada *et al.*, 2007), and correlates well with the onset of symptoms, such as motor dysfunction, (Chapter 4) and pathology, such as inflammatory response and activation of protein and lipid scavenger pathways (Olsen *et al.*, 2001; Fukada *et al.*,

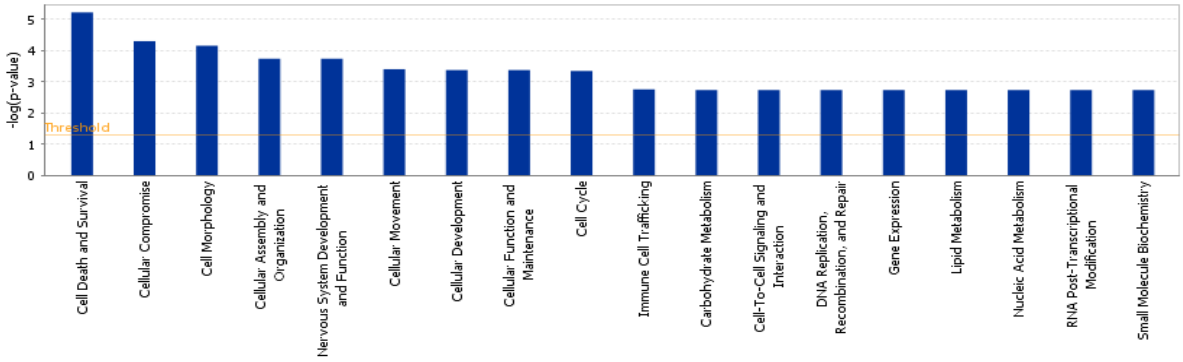

Figure 5.2. Over-expression of Oxr1 in neurons results in subtle transcriptome changes in the spinal cord at P60

Oxr1 over-expression in neurons induces subtle changes when using a moderated t-test with no multiple testing correction and pathway analysis demonstrates that neuronal Oxr1 over-expression alters gene expression changes in a number of different molecular pathways, particularly those involved in cell death and survival, cellular compromise, and cell morphology. Significance of identified pathways is presented as a –log(p-value), where $p \leq 0.05$ is $-\log(\text{p-value}) \geq 1.3$.

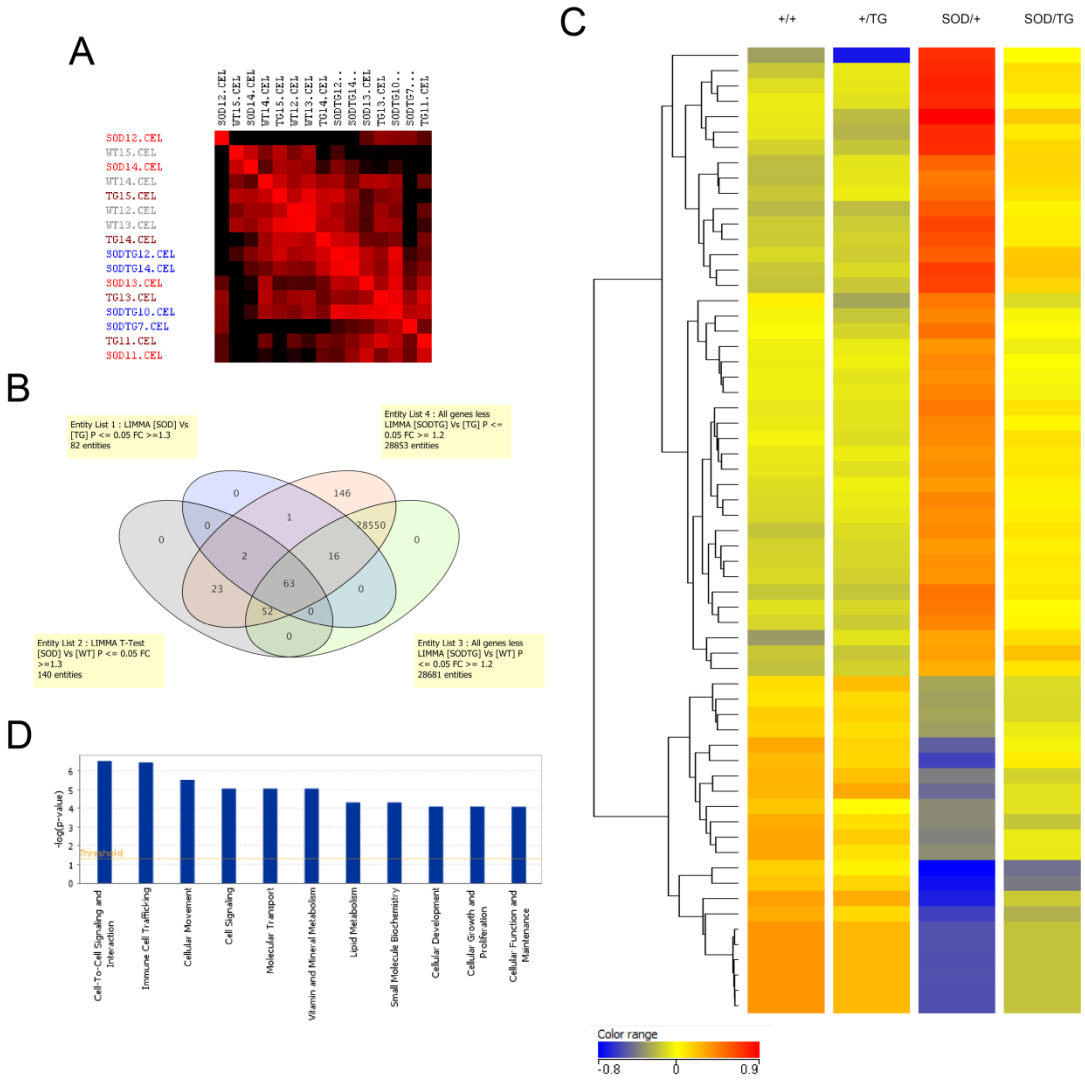

Figure 5.3. Over-expression of Oxr1 in neurons delays widespread transcriptome changes in the spinal cord of SOD1^{G93A} ALS mice at P90

(**A**) Correlation plot of RMA normalized gene expression of wild-type (WT), Tg(*Prnp-Oxr1*) (TG), SOD1 ALS (SOD), and SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SODTG) mice. (**B**) Venn diagram illustrates the common expression overlap from microarray analysis to identify 63 "rescued" genes, those that are significantly changed ($p \leq 0.05$) by >1.3 fold change in the SOD1^{G93A} (SOD) spinal cord, but not significantly changed ($p > 0.05$) or by <1.2 fold change in the SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SODTG) spinal cord. (**C**) Gene expression heat map for all genes identified as "rescued" by neuronal Oxr1 up-regulation from wild-type (+/+), Tg(Prnp-Oxr1) (+/TG), SOD1^{G93A} ALS (SOD/+), SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice. (**D**) Pathway analysis of 63 "rescued" genes demonstrates that Oxr1 over-expression in neurons delays SOD1^{G93A}-induced changes in a number of different molecular pathways. Significance of identified pathways is presented as a –log(p-value), where $p \leq 0.05$ is –log(p-value)≥ 1.3. RNA samples were prepared by me, microarray experiment was performed by Dr. Sheena Lee, and analysis was conducted together by Dr. Sheena Lee and me.

Table 5.6. Gene expression altered by Oxr1 over-expression in neurons at P90 using limma

Transcripts Cluster Id	p (Corr)	p-value	Fold change	Up / Down	Gene Symbol	Gene Description
10423921	9.81E-06	3.40E-10	2.5	up	Oxr1	Oxidation resistance 1

Table 5.7. Mutant SOD1-induced gene expression changes in lumbar spinal cord at P90 using limma

	SOD Vs. WT				SOD Vs. TG					
Transcripts Cluster Id	p (Corr)	p	Fold change	Up / Down	p (Corr)	p	Fold change	Up / Down	Gene description	Gene symbol
10499899	2.21E-05	2.30E-09	3.1	up	1.64E-04	6.33E-09	3.2	up	Small proline-rich protein 1A	Sprr1a
10589790	3.91E-02	1.06E-04	2.0	down	5.90E-03	1.01E-05	2.1	down		
10372648	1.15E-03	2.80E-07	2.7	up	2.30E-02	9.10E-05	2.0	up	Lysozyme2	Lyz2
10436623	3.45E-04	4.78E-08	1.8	down	1.64E-04	1.19E-08	2.0	down	Chondrolectin	Chodl
10517664	2.84E-03	1.38E-06	1.9	down	5.64E-04	1.76E-07	2.0	down		
10499189	4.43E-04	7.67E-08	2.1	up	1.64E-04	1.93E-08	1.9	up	Fc receptor-like S, scavenger receptor	Fcrls
10582578	2.12E-02	4.04E-05	1.7	down	1.13E-03	7.71E-07	1.9	down		
10582558	2.12E-02	4.40E-05	1.7	down	1.13E-03	8.62E-07	1.9	down		
10582574	2.12E-02	4.28E-05	1.7	down	1.13E-03	8.08E-07	1.9	down		
10582568	2.12E-02	4.16E-05	1.7	down	1.13E-03	8.06E-07	1.9	down		
10582556	2.12E-02	4.39E-05	1.7	down	1.13E-03	8.09E-07	1.9	down		
10582564	2.12E-02	4.21E-05	1.7	down	1.13E-03	8.09E-07	1.9	down		
10387536	1.61E-03	5.57E-07	1.7	up	1.64E-04	2.28E-08	1.9	up	CD68 antigen	Cd68
10602733	2.05E-03	9.24E-07	1.7	down	4.41E-04	1.22E-07	1.8	down		
10469322	5.74E-03	3.78E-06	1.9	up	9.14E-04	4.00E-07	1.8	up	Vimentin	Vim
10361091	6.31E-03	4.45E-06	1.8	up	9.14E-04	4.12E-07	1.8	up	Activating transcription factor 3	Atf3
10450242	1.37E-02	1.58E-05	1.8	up	4.35E-04	1.06E-07	1.8	up	Complement component 4B (Chido blood group)	C4b
10398075	1.53E-03	4.25E-07	1.7	up	1.99E-04	3.45E-08	1.8	up	Serine (or cysteine) peptidase inhibitor, clade A, member 3N	Serpina3n
10583056	1.72E-03	6.57E-07	1.8	up	4.35E-04	9.17E-08	1.8	up	Matrix metallopeptidase 12	Mmp12
10485357	4.52E-02	1.35E-04	1.7	down	2.96E-02	1.35E-04	1.8	down		
10474687	1.51E-02	2.09E-05	1.6	down	6.73E-03	1.28E-05	1.7	down		
10389231	1.57E-05	1.09E-09	1.9	up	9.81E-04	4.76E-07	1.7	up	Chemokine (C-Cmotif) ligand 3	Ccl3
10461721	9.73E-04	2.02E-07	1.7	up	1.19E-03	9.94E-07	1.7	up	Macrophage expressed gene 1	Mpeg1
10517165	3.71E-03	1.93E-06	1.7	up	2.06E-03	2.14E-06	1.7	up	CD52 antigen	Cd52
10548375	8.10E-03	7.46E-06	1.8	up	3.51E-03	3.77E-06	1.7	up	C-type lectin domain family 7, member a	Clec7a
10547657	4.17E-03	2.60E-06	1.7	up	1.13E-03	8.42E-07	1.7	up	Complement component 3a receptor 1	C3ar1
10404606	8.10E-03	7.58E-06	1.5	up	5.75E-04	1.99E-07	1.7	up	Lymphocyte antigen 86	Ly86
10547793	1.37E-02	1.55E-05	1.7	down	4.65E-02	2.97E-04	1.6	down	U7 small nuclear RNA | gene rich cluster, C10 gene	Rnu7|Grcc10
10425985	4.52E-02	1.41E-04	1.5	down	1.56E-03	1.57E-06	1.6	down	microRNA let7b	Mirlet7b
10360070	2.12E-02	3.97E-05	1.4	up	5.24E-03	7.71E-06	1.6	up	Fc receptor, IgE, high affinity I, gamma polypeptide	Fcer1g
10588037	1.61E-03	5.32E-07	1.6	up	6.25E-03	1.17E-05	1.6	up	Retinol binding protein 1, cellular	Rbp1
10517508	7.15E-03	5.45E-06	1.5	up	6.25E-04	2.38E-07	1.6	up	Complement component 1, q subcomponent, beta	C1qb

									polypeptide	
10459618	4.52E-02	1.43E-04	1.4	down	1.56E-03	1.53E-06	1.6	down		
10484735	3.49E-02	9.08E-05	1.5	down	1.95E-02	7.22E-05	1.6	down	Olfactory receptor 1175, pseudogene	Olfr1175-ps
10471878	1.51E-02	2.05E-05	1.4	down	1.94E-02	7.13E-05	1.6	down	microRNA 181a-2	Mir181a-2
10517513	6.31E-03	4.59E-06	1.6	up	8.05E-03	1.75E-05	1.6	up	Complement component 1, q subcomponent, C chain	C1qc
10393573	2.12E-02	3.65E-05	1.3	up	6.17E-03	1.13E-05	1.5	up	Lectin, galactoside-binding, soluble, 3 binding protein	Lgals3bp
10598976	2.63E-02	6.04E-05	1.4	up	5.24E-03	7.49E-06	1.5	up	Tissue inhibitor of metalloproteinase 1	Timp1
10578251	2.63E-02	6.39E-05	1.3	down	1.26E-02	3.56E-05	1.5	down		
10501063	2.04E-02	3.12E-05	1.5	up	1.26E-02	3.60E-05	1.5	up	CD53 antigen	Cd53
10541354	2.12E-02	3.84E-05	1.5	up	5.24E-03	7.83E-06	1.5	up	Alpha-2-macroglobulin	A2m
10500808	2.65E-02	6.52E-05	1.5	up	1.76E-02	6.35E-05	1.4	up	Olfactomedin-like3	Olfml3
10512949	1.37E-02	1.47E-05	1.4	up	5.90E-03	1.04E-05	1.4	up	ATP-binding cassette, sub-family A (ABC1), member 1	Abca1
10381588	1.24E-02	1.20E-05	1.4	up	1.56E-03	1.56E-06	1.4	up	Granulin	Grn
10563441	1.27E-02	1.28E-05	1.4	up	5.24E-03	8.17E-06	1.4	up	Epithelial membrane protein 3	Emp3
10536845	1.91E-02	2.78E-05	1.4	up	3.66E-03	4.45E-06	1.4	up	Filamin C, gamma	Flnc
10551883	4.11E-03	2.42E-06	1.5	up	1.15E-03	9.19E-07	1.4	up	TYRO protein tyrosine kinase binding protein	Tyrobp
10517517	1.51E-02	2.07E-05	1.4	up	1.91E-02	6.95E-05	1.4	up	Complement component 1, q subcomponent, alpha polypeptide	C1qa
10490212	7.21E-03	6.00E-06	1.4	up	1.56E-03	1.51E-06	1.4	up	CathepsinZ	Ctsz
10563344	4.52E-02	1.43E-04	1.3	up	1.34E-02	4.08E-05	1.4	up	Fibroblast growth factor 21	Fgf21
10494271	3.81E-03	2.11E-06	1.4	up	5.69E-03	9.47E-06	1.4	up	CathepsinS	Ctss
10424400	1.48E-02	1.79E-05	1.4	up	4.96E-03	6.71E-06	1.4	up	Myelocytomatosis oncogene	Myc
10522388	2.63E-02	6.27E-05	1.3	down	8.48E-03	1.88E-05	1.4	down	Solute carrier family 10 (sodium/bile acid cotransporter family), member 4	Slc10a4
10523451	2.05E-03	9.05E-07	1.4	up	8.05E-03	1.71E-05	1.4	up	Annexin A3	Anxa3
10439206	2.12E-02	4.07E-05	1.4	down	1.10E-02	2.71E-05	1.4	down		
10545101	4.74E-02	1.53E-04	1.4	up	8.05E-03	1.76E-05	1.4	up	Hematopoietic prostaglandin D synthase	Hpgds
10445781	2.63E-02	5.96E-05	1.3	up	3.60E-03	4.12E-06	1.4	up	Triggering receptor expressed on myeloid cells 2	Trem2
10360028	2.12E-02	3.66E-05	1.5	up	1.66E-02	5.87E-05	1.4	up	Fc receptor, IgG, low affinity IIb	Fcgr2b
10420155	8.10E-03	7.22E-06	1.3	up	5.24E-03	8.02E-06	1.3	up	Dehydrogenase/reductase (SDRfamily) member1	Dhrs1
10567580	1.51E-02	2.02E-05	1.4	up	1.54E-02	5.17E-05	1.3	up	Immunoglobulin super family, membe r6	Igsf6
10411373	1.27E-02	1.32E-05	1.4	up	3.18E-02	1.62E-04	1.3	up	Hexosaminidase B	Hexb
10542355	4.52E-02	1.44E-04	1.3	up	9.92E-03	2.37E-05	1.3	up	Epithelial membrane protein 1	Emp1
10351679	1.51E-02	2.12E-05	1.7	up	1.36E-02	4.19E-05	1.3	up	CD84 antigen	Cd84
10512653	3.49E-02	9.07E-05	1.4	down	5.50E-03	8.96E-06	1.3	down		
10433658	2.12E-02	4.48E-05	1.3	down	2.51E-02	1.03E-04	1.3	down	microRNA 365-1	Mir365-1

Table 5.8. Mutant SOD1-induced gene expression changes that are "rescued" by neuronal Oxr1 over-expression at P90 using limma

	SOD Vs. WT			SOD Vs. TG			SOD/TG Vs. WT			SOD/TG Vs. TG				
Transcripts Cluster Id	**p-value (Corr)**	**Fold change**	**Up/ Down**	**p-value (Corr)**	**Fold change**	**Up/ Down**	**p-value (Corr)**	**Fold change**	**Up/ Down**	**p-value (Corr)**	**Fold change**	**Up/ Down**	**Gene Description**	**Gene Symbol**
10589790	5.90E-03	2.1	down	3.91E-02	2.0	down	1.89E-01	1.4	down	6.75E-01	1.4	down		
10372648	2.30E-02	2.0	up	1.15E-03	2.7	up	4.93E-01	1.2	up	3.14E-01	1.7	up	Lysozyme 2	Lyz2
10436623	1.64E-04	2.0	down	3.45E-04	1.8	down	1.58E-01	1.5	down	5.73E-01	1.4	down	Chondrolectin	Chodl
10517664	5.64E-04	2.0	down	2.84E-03	1.9	down	1.75E-01	1.5	down	6.16E-01	1.5	down		
10499189	1.64E-04	1.9	up	4.43E-04	2.1	up	2.92E-01	1.2	up	6.10E-01	1.3	up	Fc receptor-like S, scavenger receptor	Fcrls
10582578	1.13E-03	1.9	down	2.12E-02	1.7	down	1.59E-01	1.5	down	6.30E-01	1.3	down		
10582558	1.13E-03	1.9	down	2.12E-02	1.7	down	1.58E-01	1.5	down	6.30E-01	1.3	down		
10582574	1.13E-03	1.9	down	2.12E-02	1.7	down	1.58E-01	1.5	down	6.30E-01	1.3	down		
10582568	1.13E-03	1.9	down	2.12E-02	1.7	down	1.58E-01	1.5	down	6.30E-01	1.3	down		
10582556	1.13E-03	1.9	down	2.12E-02	1.7	down	1.58E-01	1.5	down	6.30E-01	1.3	down		
10582564	1.13E-03	1.9	down	2.12E-02	1.7	down	1.58E-01	1.5	down	6.30E-01	1.3	down		
10387536	1.64E-04	1.9	up	1.61E-03	1.7	up	1.67E-01	1.2	up	7.14E-01	1.1	up	CD68 antigen	Cd68
10602733	4.41E-04	1.8	down	2.05E-03	1.7	down	1.75E-01	1.4	down	6.54E-01	1.3	down		
10469322	9.14E-04	1.8	up	5.74E-03	1.9	up	1.58E-01	1.2	up	6.03E-01	1.2	up	Vimentin	Vim
10450242	4.35E-04	1.8	up	1.37E-02	1.8	up	2.45E-01	1.1	up	8.02E-01	1.1	up	Complement component 4B (Chido blood group)	C4b
10398075	1.99E-04	1.8	up	1.53E-03	1.7	up	1.58E-01	1.3	up	6.03E-01	1.2	up	Serine (or cysteine) peptidase inhibitor, clade A, member 3N	Serpina3n
10583056	4.35E-04	1.8	up	1.72E-03	1.8	up	1.58E-01	1.3	up	5.02E-01	1.2	up	Matrix metallopeptidase 12	Mmp12
10485357	2.96E-02	1.8	down	4.52E-02	1.7	down	7.17E-01	1.1	down	9.51E-01	1.0	down		
10474687	6.73E-03	1.7	down	1.51E-02	1.6	down	2.78E-01	1.3	down	7.94E-01	1.1	down		
10389231	9.81E-04	1.7	up	1.57E-05	1.9	up	4.53E-01	1.1	up	2.18E-01	1.2	up	Chemokine (C-Cmotif) ligand 3	Ccl3
10461721	1.19E-03	1.7	up	9.73E-04	1.7	up	2.31E-01	1.2	up	5.25E-01	1.2	up	Macrophage expressed gene 1	Mpeg1
10517165	2.06E-03	1.7	up	3.71E-03	1.7	up	2.65E-01	1.2	up	6.50E-01	1.2	up	CD52 antigen	Cd52
10548375	3.51E-03	1.7	up	8.10E-03	1.8	up	4.67E-01	1.1	up	7.48E-01	1.1	up	C-type lectin domain family 7, member a	Clec7a
10547657	1.13E-03	1.7	up	4.17E-03	1.7	up	2.12E-01	1.2	up	6.51E-01	1.2	up	Complement component 3a receptor 1	C3ar1
10404606	5.75E-04	1.7	up	8.10E-03	1.5	up	1.58E-01	1.3	up	6.30E-01	1.2	up	Lymphocyte antigen 86	Ly86
10547793	4.65E-02	1.6	down	1.37E-02	1.7	down	5.03E-01	1.3	down	7.33E-01	1.3	down	U7 small nuclear RNA | gene rich cluster, C10 gene	Rnu7| Grcc10
10425985	1.56E-03	1.6	down	4.52E-02	1.5	down	2.94E-01	1.3	down	8.07E-01	1.2	down	microRNA let7b	Mirlet7b
10360070	5.24E-03	1.6	up	2.12E-02	1.4	up	1.58E-01	1.3	up	6.30E-01	1.2	up	Fc receptor, IgE, high affinity I, gammapolypeptide	Fcer1g
10588037	6.25E-03	1.6	up	1.61E-03	1.6	up	2.29E-01	1.2	up	4.90E-01	1.3	up	Retinol binding protein 1,cellular	Rbp1
10517508	6.25E-04	1.6	up	7.15E-03	1.5	up	1.58E-01	1.2	up	6.85E-01	1.1	up	Complement component 1, q subcomponent, beta polypeptide	C1qb
10459618	1.56E-03	1.6	down	4.52E-02	1.4	down	1.67E-01	1.4	down	6.94E-01	1.2	down		

10484735	1.95E-02	1.6	down	3.49E-02	1.5	down	3.25E-01	1.3	down	7.28E-01	1.3	down	Olfactory receptor 1175, pseudogene	Olfr1175-ps
10471878	1.94E-02	1.6	down	1.51E-02	1.4	down	2.55E-01	1.3	down	7.78E-01	1.1	down	microRNA 181a-2	Mir181a-2
10517513	8.05E-03	1.6	up	6.31E-03	1.6	up	2.60E-01	1.2	up	6.16E-01	1.2	up	Complement component 1,q subcomponent, C chain	C1qc
10393573	6.17E-03	1.5	up	2.12E-02	1.3	up	1.58E-01	1.4	up	6.11E-01	1.1	up	lectin, galactoside-binding, soluble, 3 binding protein	Lgals3bp
10598976	5.24E-03	1.5	up	2.63E-02	1.4	up	1.63E-01	1.2	up	6.44E-01	1.1	up	Tissue inhibitor of metalloproteinase 1	Timp1
10578251	1.26E-02	1.5	down	2.63E-02	1.3	down	3.66E-01	1.2	down	8.84E-01	1.1	down		
10501063	1.26E-02	1.5	up	2.04E-02	1.5	up	7.04E-01	1.1	up	8.45E-01	1.1	up	CD53 antigen	Cd53
10541354	5.24E-03	1.5	up	2.12E-02	1.5	up	4.40E-01	1.1	up	7.62E-01	1.1	up	Alpha-2-macroglobulin	A2m
10500808	1.76E-02	1.4	up	2.65E-02	1.5	up	3.17E-01	1.1	up	6.81E-01	1.1	up	Olfactomedin-like3	Olfml3
10512949	5.90E-03	1.4	up	1.37E-02	1.4	up	5.03E-01	1.1	up	8.56E-01	1.1	up	ATP-binding cassette, sub-family A (ABC1), member 1	Abca1
10381588	1.56E-03	1.4	up	1.24E-02	1.4	up	1.58E-01	1.2	up	6.05E-01	1.2	up	Granulin	Grn
10563441	5.24E-03	1.4	up	1.27E-02	1.4	up	2.92E-01	1.1	up	7.34E-01	1.1	up	Epithelial membrane protein 3	Emp3
10536845	3.66E-03	1.4	up	1.91E-02	1.4	up	2.33E-01	1.3	up	6.30E-01	1.3	up	Filamin C,gamma	Flnc
10551883	1.15E-03	1.4	up	4.11E-03	1.5	up	8.27E-01	1.0	up	7.02E-01	1.1	up	TYRO protein tyrosine kinase binding protein	Tyrobp
10517517	1.91E-02	1.4	up	1.51E-02	1.4	up	2.78E-01	1.1	up	6.03E-01	1.2	up	Complement component 1,q subcomponent, alpha polypeptide	C1qa
10490212	1.56E-03	1.4	up	7.21E-03	1.4	up	1.72E-01	1.1	up	6.16E-01	1.1	up	Cathepsin Z	Ctsz
10563344	1.34E-02	1.4	up	4.52E-02	1.3	up	1.58E-01	1.2	up	5.55E-01	1.2	up	Fibroblast growth factor 21	Fgf21
10494271	5.69E-03	1.4	up	3.81E-03	1.4	up	5.42E-01	1.1	up	7.14E-01	1.1	up	Cathepsin S	Ctss
10424400	4.96E-03	1.4	up	1.48E-02	1.4	up	2.22E-01	1.1	up	6.30E-01	1.1	up	Myelocytomatosis oncogene	Myc
10522388	8.48E-03	1.4	down	2.63E-02	1.3	down	3.14E-01	1.2	down	7.57E-01	1.1	down	Solute carrier family 10 (sodium/bile acid cotransporter family), member 4	Slc10a4
10523451	8.05E-03	1.4	up	2.05E-03	1.4	up	9.72E-01	1.0	up	9.46E-01	1.0	up	Annexin A3	Anxa3
10439206	1.10E-02	1.4	down	2.12E-02	1.4	down	1.63E-01	1.2	down	5.55E-01	1.2	down		
10545101	8.05E-03	1.4	up	4.74E-02	1.4	up	7.70E-01	1.0	up	9.42E-01	1.0	up	Hematopoietic prostaglandin D synthase	Hpgds
10445781	3.60E-03	1.4	up	2.63E-02	1.3	up	2.51E-01	1.1	up	7.95E-01	1.1	up	Triggering receptor expressed on myeloid cells 2	Trem2
10360028	1.66E-02	1.4	up	2.12E-02	1.5	up	9.84E-01	1.0	up	7.33E-01	1.1	up	Fc receptor, IgG, low affinity IIb	Fcgr2b
10420155	5.24E-03	1.3	up	8.10E-03	1.3	up	3.05E-01	1.1	up	6.93E-01	1.1	up	Dehydrogenase/reductase (SDRfamily) member 1	Dhrs1
10567580	1.54E-02	1.3	up	1.51E-02	1.4	up	3.29E-01	1.1	up	6.16E-01	1.2	up	Immunoglobulin super family, member 6	Igsf6
10411373	3.18E-02	1.3	up	1.27E-02	1.4	up	9.30E-01	1.0	up	8.85E-01	1.0	up	Hexosaminidase B	Hexb
10542355	9.92E-03	1.3	up	4.52E-02	1.3	up	9.83E-01	1.0	down	9.84E-01	1.0	up	Epithelial membrane protein 1	Emp1
10351679	1.36E-02	1.3	up	1.51E-02	1.7	up	3.02E-01	1.1	down	7.98E-01	1.1	up	CD84 antigen	Cd84
10512653	5.50E-03	1.3	down	3.49E-02	1.4	down	2.46E-01	1.2	down	6.30E-01	1.3	down		
10433658	2.51E-02	1.3	down	2.12E-02	1.3	down	2.77E-01	1.2	down	6.30E-01	1.2	down	microRNA 365-1	Mir365-1

2007). For example, *complement component 1, q subcomponent* (*C1q*) polypeptides, and *Hexosaminidase B* (*HexB*) were identified only in the P90 microarray as altered in the SOD1^{G93A} spinal cord, and rescued by over-expression of Oxr1 in neurons (Table 5.7-5.8). Next, using Ingenuity Pathway Analysis, we demonstrated a wide range of biological functions are rescued downstream of Oxr1 function, including immune cell trafficking, cell signalling, vitamin and mineral metabolism, and lipid metabolism (Figure 5.3D). Many of these pathways, particularly immune system response and lipid mobilization, have been previously identified as important regulators of ALS pathogenesis (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Perrin *et al.*, 2005; Ferraiuolo *et al.*, 2007; Chen *et al.*, 2010; Lincecum *et al.*, 2010). Finally, a recent study demonstrated that vitamin D has neuroprotective effects, and deficiency in vitamin D is widely observed in ALS patients, linking ALS with vitamin metabolism (Camu *et al.*, 2013).

5.2.3 Neuron-specific Oxr1 over-expression modulates neuroinflammatory response in ALS mice

Immune cell trafficking was identified as a common biological function significantly dysregulated in SOD1^{G93A} ALS mice, but not in that of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice at P60 and P90 (Figure 5.1D, 5.3D), thus, we next performed quantitative RT-PCR (qRT-PCR) to confirm the differential expression of selected gene candidates involved in neuroinflammation at pre-symptomatic P60, early symptomatic P90, and end-stage P135. We identified *Ctss* and *Lyz2*, two genes involved in lysosomal degradation and immune activation (Olsen *et al.*, 2001; Ferraiuolo *et al.*, 2007), as "rescued" in both the P60 and P90 arrays (Table 5.4, 5.8). Expression of *Ctss* and *Lyz2* is significantly up-regulated in the spinal cord of SOD1^{G93A} ALS mice, but not in that of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice by qRT-PCR (Figure 5.4A-B,D-E). Moreover, we observed similar significant

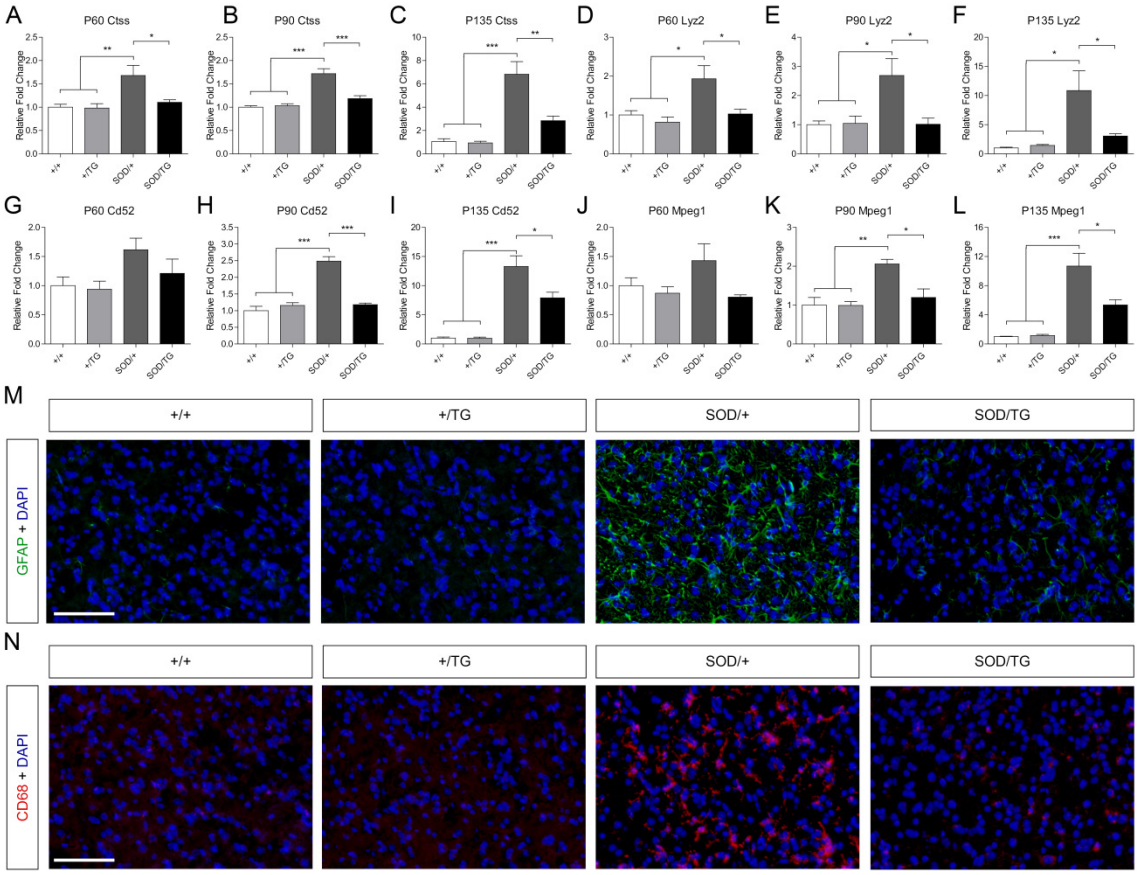

Figure 5.4. Over-expression of Oxr1 in neurons decreases neuroinflammation in the spinal cord of SOD1^{G93A} ALS mice

(**A-F**) SOD1^{G93A} (SOD/+) induced expression of *Ctss* and *Lyz2*, two markers of neuroinflammation, in the spinal cord is significantly reduced in SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice at (**A,D**) P60, (**B,E**) P90, and (**C,F**) P135. (**G-L**) Induced expression of *Cd52* and *Mpeg1*, two macrophage markers, in the SOD1^{G93A} spinal cord is significantly reduced by over-expression of Oxr1 at (**H,K**) P90, and (**I,L**) P135. (**A-L**) Values are the mean ± SEM (n=3-5 per genotype; *p<0.05, **p<0.01, ***p<0.001, 1-way ANOVA with Tukey's post hoc tests). (**M**) Immunocytochemical staining for GFAP, a marker of astrogliosis, on sections of lumbar spinal cord reveals markedly decreased astrogliosis in SOD/TG mice when compared to SOD/+ mice (n=3-4). (**N**) Immunocytochemical staining for CD68, a marker of microgliosis, on sections of lumbar spinal cord reveals strikingly decreased microgliosis in SOD/TG mice when compared to SOD/+ mice (n=3-4). (**M-N**) Scale bars: 100µm.

delay of up-regulated *Ctss* and *Lyz2* expression in P135 SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (Figure 5.4C,F). Furthermore, we selected *Cluster of differentiation 52 (Cd52)* and *Macrophage-expressing gene 1 (Mpeg1)*, two markers of macrophages (Elsner *et al.*, 1996; Nagler *et al.*, 2006; Ellett *et al.*, 2011), because they were only identified in the P90 array (Table 5.4, 5.8). We confirmed significantly increased expression of *Cd52* and *Mpeg1*, two markers of macrophages, at P90 and P135 in the spinal cord of SOD1^{G93A} ALS mice is rescued by neuronal over-expression of Oxr1 (Figure 5.4G-L).

To assess the neuroinflammatory response in more detail at the protein level, we conducted immunocytochemical analysis at P135, labelling for Glial fibrillary acidic protein (GFAP), a marker for astrogliosis, and Cluster of Differentiation 68 (CD68), a marker for microgliosis (Bignami *et al.*, 1972; Hatfield and Skoff, 1982; Hulette *et al.*, 1992; Muhleisen *et al.*, 1995). Levels of GFAP-positively stained activated astrocytes are dramatically reduced in spinal cord sections of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice when compared to that of SOD1^{G93A} ALS mice at P135, while little to no GFAP-staining was observed in spinal cord sections of wild-type and Tg(*Prnp-Oxr1*) mice (Figure 5.4M). Similarly, SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice have markedly decreased levels of CD68-postive activated microglia when compared to SOD1^{G93A} ALS mice at P135, and little to no CD68 expression was observed in spinal cord sections of wild-type and Tg(*Prnp-Oxr1*) mice (Figure 5.4N).

To better understand the mechanisms by which Oxr1 delays the inflammatory response in the SOD1^{G93A} spinal cord, we further examined activation pathways upstream of the immune response. In particular, the complement pathway was identified by Ingenuity Pathway analysis as significantly activated in SOD1^{G93A} transgenic ALS mice, but not in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (-log(p-value)=7.93) at P90 (Table 5.9). Genes encoding for C1q α, β, and γ polypeptides, *C1qa*, *C1qb*, and *C1qc*, respectively, were

Table 5.9. Ingenuity Canonical Pathways that are delayed in SOD1^{G93A} spinal cord after neuronal Oxr1 over-expression

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Molecules
Complement System	7.93E00	1.47E-01	C4A/C4B,C1QC,C1QA,C1QB,C3AR1
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	5.28E00	4.81E-02	CLEC7A,C1QC,C1QA,C1QB,C3AR1
Inhibition of Matrix Metalloproteases	3.8E00	7.69E-02	TIMP1,MMP12,A2M
Dendritic Cell Maturation	3.11E00	1.99E-02	TYROBP,FCER1G,FCGR2B,TREM2
Acute Phase Response Signaling	2.96E00	2.22E-02	C4A/C4B,SERPINA3,A2M,RBP1
Bladder Cancer Signaling	2.78E00	3.19E-02	MYC,FGF21,MMP12
Retinoate Biosynthesis II	2.07E00	1.11E-01	RBP1
TREM1 Signaling	2.04E00	2.82E-02	TYROBP,FCGR2B
ILK Signaling	1.9E00	1.51E-02	MYC,FLNC,VIM
Communication between Innate and Adaptive Immune Cells	1.89E00	1.96E-02	FCER1G,CCL3L1/CCL3L3
Crosstalk between Dendritic Cells and Natural Killer Cells	1.75E00	1.9E-02	TYROBP,TREM2
Prostanoid Biosynthesis	1.59E00	6.67E-02	HPGDS
Natural Killer Cell Signaling	1.53E00	1.71E-02	TYROBP,FCER1G
Chondroitin Sulfate Degradation (Metazoa)	1.47E00	4.76E-02	HEXB
Dermatan Sulfate Degradation (Metazoa)	1.44E00	4.76E-02	HEXB
LXR/RXR Activation	1.41E00	1.44E-02	C4A/C4B,ABCA1
The Visual Cycle	1.4E00	3.57E-02	RBP1
Lipid Antigen Presentation by CD1	1.35E00	3.57E-02	FCER1G

identified as "rescued" by neuronal Oxr1 over-expression (Table 5.8), and C1q activates the classic complement pathway, which subsequently modulates the immune system response (Lobsiger *et al.*, 2013). We confirmed that significant up-regulation of *C1qa*, *C1qb*, and *C1qc*, in the spinal cord of SOD1^{G93A} ALS mice is significantly reduced in that of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice at P90 and P135 (Figure 5.5A-I), suggesting that Oxr1 functions upstream of classic complement pathway activation. Furthermore, Ingenuity Pathway Analysis identified activated regulators, including STAT3, a transcription factor, that function upstream of the gene expression changes at P90 (Table 5.10). Interestingly, activation of STAT3 has been observed in ALS patients and SOD1^{G93A} ALS mice (Shibata *et al.*, 2009; Shibata *et al.*, 2010). Thus, we determined whether STAT3 is phosphorylated (p-STAT3) in SOD1^{G93A} ALS mice by immunoblotting. We found that while p-STAT3 is up-regulated at P90 in the spinal cord of SOD1^{G93A} ALS mice, p-STAT3 is markedly reduced in that of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (Figure 7J-K). Therefore, we examined the expression of two genes, *Vimentin (Vim)*, and *Serine (Or Cysteine) Proteinase Inhibitor, Clade A, Member 3 (Serpina3n)*, which were identified in the P90 microarray as being "rescued" and are downstream targets of the activated STAT3 pathway (Table 5.8, 5.10). At both P90 and P135, *Vim* and *Serpina3n* are significantly increased in SOD1^{G93A} ALS mice when compared to wild-type and Tg(*Prnp-Oxr1*) mice, and are reduced in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice when compared to SOD1^{G93A} ALS mice, further implicating Oxr1 as functioning upstream of the STAT3-activated immune response pathway (Figure 5.5L-Q). Together, these data demonstrate that neuronal over-expression of Oxr1 is sufficient to decrease pathological neuroinflammation by regulating activation of multiple pathways in the spinal cord of SOD1^{G93A} ALS mice.

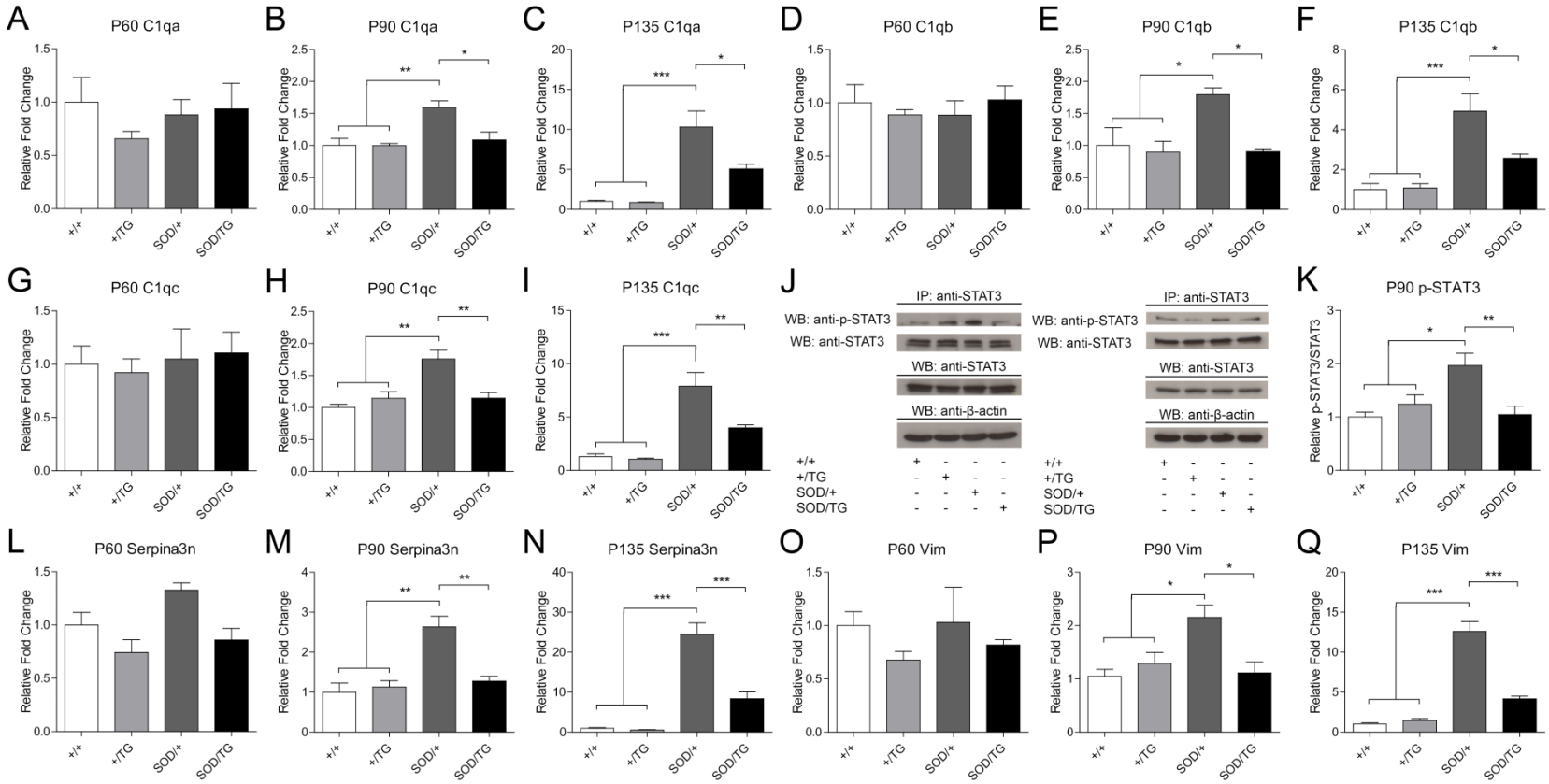

Figure 5.5. Neuronal Oxr1 over-expression delays activation of neuroinflammatory pathways in the spinal cord of SOD1^{G93A} ALS mice
(**A-I**) SOD1^{G93A} (SOD/+) induced activation of genes encoding C1q proteins, *C1qa, C1qb,* and *C1qb*, is significantly reduced in SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice at (**B,E,H**) P90, and (**C,F,I**) P135. (**J**) Induced expression of phosphorylated-STAT3 (p-STAT3) in the SOD1^{G93A} spinal cord is significantly reduced by over-expression of Oxr1 at P90. (**I**) Quantification of p-STAT3 normalized to total STAT3 (n=5 per genotype; *p<0.05, **p<0.01, 1-way ANOVA with Tukey's post hoc tests). (**L-Q**) SOD1^{G93A} induced activation of genes downstream of activated STAT3, *Serpina3n* and *Vim*, is significantly reduced in SOD/TG mice at (**M,P**) P90, and (**N,Q**) P135. (**A-I,L-Q**) Values are the mean ± SEM (n=3-5 per genotype; *p<0.05, **p<0.01, ***p<0.001, 1-way ANOVA with Tukey's post hoc tests).

Table 5.10. Ingenuity Predicted Activated Pathways that are delayed in SOD1^{G93A} spinal cord after neuronal Oxr1 over-expression

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset	Mechanistic Network
IL6	cytokine	Inhibited	-2.581	6.48E-07	A2M, ABCA1, CCL3L1/CCL3L3, LY86, MMP12, MYC, SERPINA3, TIMP1, VIM	IL6, NFκB (complex), SMAD3, SP1, STAT, STAT1, STAT3, Stat3-Stat3
STAT3	transcription regulator	Inhibited	-2.225	2.44E-05	A2M, FCER1G, MYC, SERPINA3, TIMP1, VIM	IL6, NFκB (complex), SMAD3, SP1, STAT, STAT1, STAT3, Stat3-Stat3
Vegf	group	Inhibited	-2.200	1.28E-03	A2M, EMP1, MYC, TIMP1, VIM	NFκB (complex), STAT, STAT1, STAT3, Vegf
HGF	growth factor	Inhibited	-2.200	3.97E-03	A2M, EMP1, MYC, TIMP1, VIM	EDN1, HGF, NFκB (complex), SP1, STAT, STAT1, STAT3
RAF1	kinase	Inhibited	-2.144	7.56E-05	EMP1, EMP3, GRN, MYC, TIMP1	
CSF1	cytokine		-1.949	2.15E-05	CD68, FCER1G, MMP12, MYC	
IL10	cytokine		-1.698	1.89E-05	CD68, CTSS, CTSZ, FCER1G, FCGR2B, TIMP1	
IL1B	cytokine		-1.686	1.04E-05	A2M, CCL3L1/CCL3L3, CTSS, HEXB, HPGDS, MMP12, SERPINA3, TIMP1	
TNF	cytokine		-1.435	1.52E-05	ABCA1, CTSS, FCGR2B, GRN, HEXB, HPGDS, MMP12, MYC, SERPINA3, TIMP1, TREM2	
ERBB2	kinase		-1.412	1.21E-03	EMP1, EMP3, MYC, SERPINA3, VIM	EGFR, ERBB2, NFκB (complex)
IL4	cytokine		-1.338	1.73E-03	FCER1G, FCGR2B, MMP12, MYC, TIMP1	
IFNG	cytokine		-1.266	1.93E-09	A2M, C1QA, C1QB, C1QC, C4A/C4B, CTSS, CTSZ, FCER1G, FCGR2B, LGALS3BP, MMP12, MYC, TIMP1	
EGFR	kinase		-1.000	1.55E-03	GRN, MYC, SERPINA3, VIM	EGFR, NFκB (complex), STAT3, Stat3-Stat3
OSM	cytokine		-0.765	9.20E-04	ABCA1, ANXA3, EMP3, MYC, SERPINA3, TIMP1	ERK1/2, LIF, OSM, SP1, STAT3
TGFB1	growth factor		-0.751	1.60E-02	ABCA1, HEXB, MMP12, MYC, SERPINA3, TIMP1, VIM	

5.2.4 Neuron-specific Oxr1 over-expression mediates lipid scavenge pathways and regulates metal ion homeostasis in ALS mice

In addition to studying genes identified by microarray analyses at P60 and P90, we further studied genes that have been previously found to be differentially expressed at end-stage SOD1^{G93A} ALS mice (Olsen *et al.*, 2001). Scavenge pathway genes for lipid metabolism have been implicated in a previous study (Olsen *et al.*, 2001), and in our microarray analysis at P90 (Figure 5.3D). *HexB* is an enzyme that targets degradation of glycosphingolipids, and was identified in the microarray as up-regulated in SOD1^{G93A} ALS mice, but not SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (Table 5.8). At both P90 and P135, expression of *HexB* is significantly elevated in SOD1^{G93A} ALS mice, and this induction is reduced in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice (Figure 5.6A-C). *Apolipoprotein E* (*ApoE*) has previously been found to be induced during ALS disease progression, and loss of *ApoE* significantly extends survival of SOD1^{G93A} ALS mice by 7.5% (Olsen *et al.*, 2001). We found that *ApoE* is increased at P135 in the spinal cord of SOD1^{G93A} ALS mice, and this induction is significantly reduced in that of SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice. We observed no difference in levels of *ApoE* between SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice, and wild-type and Tg(*Prnp-Oxr1*) mice (Figure 5.6D-F). Taken together, these results suggest that Oxr1 regulates glycolipid metabolism.

Next, we examined the expression of metallothioneins and ferritin, important regulators of metal ion homeostasis and OS (Olsen *et al.*, 2001). Increased expression of *ferritin heavy* (*FTH*) and *light* (*FTL*) subunits has been shown in a previous study (Olsen *et al.*, 2001). We confirmed significant up-regulation of *FTL*, but not *FTH*, at P135 in spinal cords of SOD1^{G93A} ALS mice (Figure 5.7A-F). Neuronal up-regulation of Oxr1 significantly reduces the induction of *FTL* at end-stage SOD1^{G93A} ALS spinal cords (Figure 5.7C). *Metallothionein-I* (*MT-I*) and *Metallothionein-III* (*MT-III*) were previously

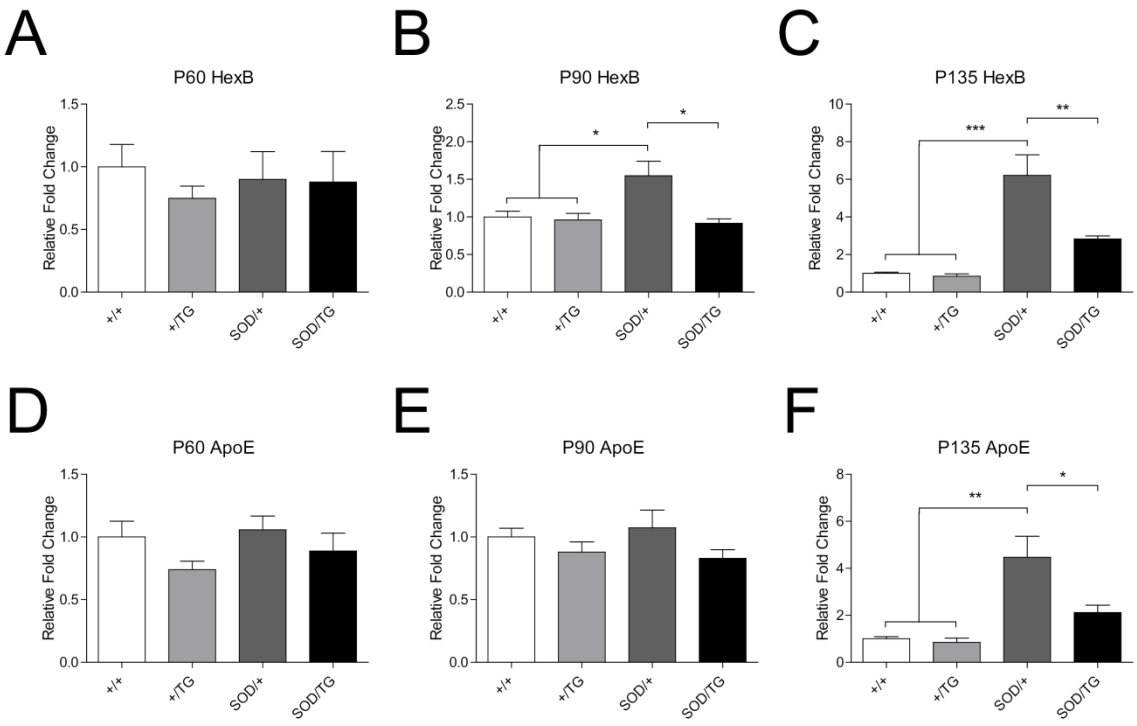

Figure 5.6. Neuronal Oxr1 over-expression delays dysregulation of lipid metabolism in the spinal cord of SOD1^{G93A} ALS mice

(**A-C**) SOD1^{G93A} (SOD/+) induced activation of *HexB* is significantly reduced in SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice at (**B**) P90, and (**C**) P135. (**D-F**) SOD1^{G93A} induced activation of *ApoE* is significantly reduced in SOD/TG mice at (**F**) P135. (**A-F**) Values are the mean ± SEM (n=3-5 per genotype; *p<0.05, **p<0.01, ***p<0.001, 1-way ANOVA with Tukey's post hoc tests).

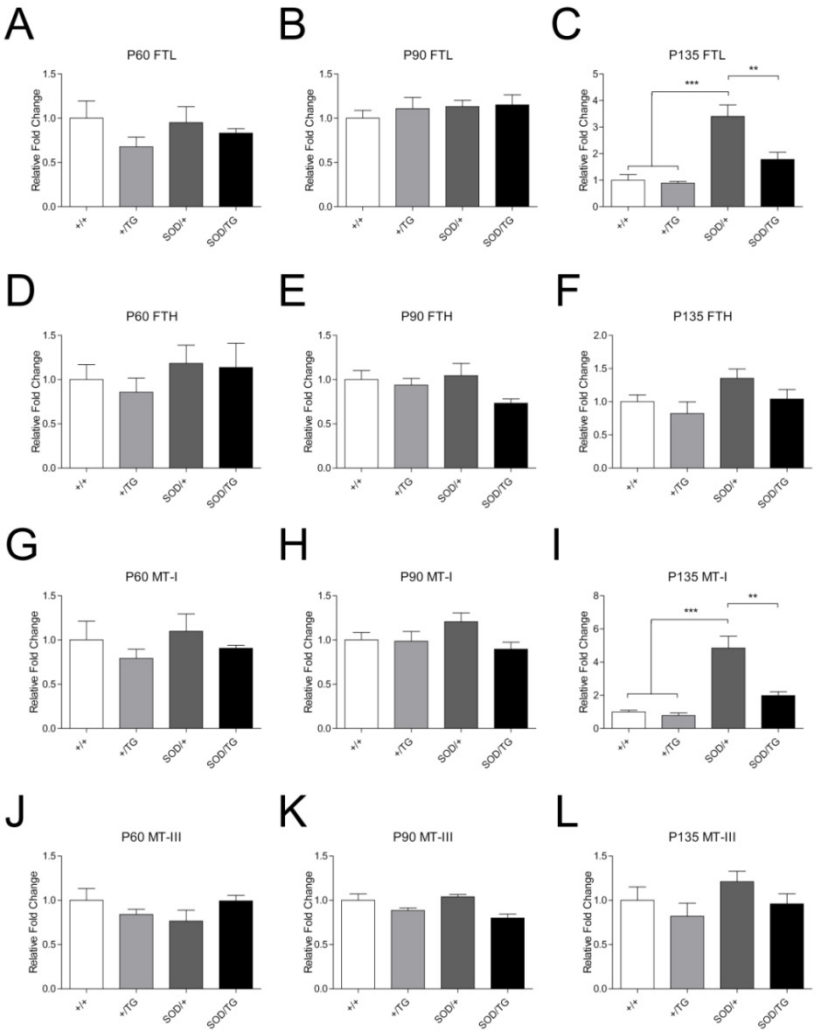

Figure 5.7. Neuronal Oxr1 over-expression delays dysregulation of metal ion homeostasis in the spinal cord of SOD1^{G93A} ALS mice

(**A-C**) SOD1^{G93A} (SOD/+) induced activation of *FTL* is significantly reduced in SOD1^{G93A}/Tg(*Prnp-Oxr1*) (SOD/TG) mice at (**C**) P135. (**D-F**) No difference in *FTH* expression was observed in wild-type (+/+), Tg(*Prnp-Oxr1*) (+/TG), SOD/+, SOD/TG mice at (**D**) P60, (**E**) P90, and (**F**) P135. (**G-I**) SOD1^{G93A} induced activation of *MT-I* is significantly reduced in SOD/TG mice at (**I**) P135. (**J-L**) No difference in *MT-III* expression was observed in +/+, +/TG, SOD/+, SOD/TG mice at (**J**) P60, (**K**) P90, and (**L**) P135. (**A-L**) Values are the mean ± SEM (n=3-5 per genotype; *p<0.05, **p<0.01, ***p<0.001, 1-way ANOVA with Tukey's post hoc tests).

demonstrated to be up-regulated at end-stage SOD1^{G93A} ALS mice (Olsen *et al.*, 2001). We confirmed that *MT-I*, but not *MT-III*, is significantly induced at end-stage in SOD1^{G93A} ALS spinal cords (Figure 5.7G-L). Interestingly, Oxr1 over-expression in neurons significantly decreases up-regulation of *MT-I* at P135 (Figure 5.6I). Together, these data demonstrate that Oxr1 functions upstream of metal ion homeostasis during ALS disease progression.

5.2.5 Oxr1 interacts with a wide range of proteins in the brain and spinal cord

Given little is known of Oxr1 function in modulating these diverse pathways during ALS disease pathogenesis, we next chose to identify novel Oxr1 binding partners *in vivo* by mass spectrometry. We immunoprecipitated binding partners of HA-tagged Oxr1 from the brain and spinal cord of Tg(*Prnp-Oxr1*) mice and SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice at three time points, presymptomatic P60, early symptomatic P90, and late symptomatic P135. Brain and spinal cord tissues from Tg(*Prnp-Oxr1*) mice and SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice were used to determine whether Oxr1 binds to different proteins under ALS disease conditions and in a tissue-specific manner, and the three time points were used to examine whether binding partners of HA-tagged Oxr1 full length (Oxr1-FL) isoform may change during the course of disease and to relate these findings to known ALS disease pathways.

Using the well-established Mascot algorithm using parameters described in Chapter 2 (Perkins *et al.*, 1999; Koenig *et al.*, 2008), we identified a number of putative binding partners that function in many different molecular pathways. Some putative binding partners, such as Band 4.1-like protein 2 (Epb41l2) and Band 4.1-like protein 3 (Epb41l3), were identified in the brain and spinal cord (Table 5.11, 5.12). Epb41l2 binds with FK506 binding protein 13 kDa (FKBP13), an immunophilin that binds to rapamycin, and is up-

Table 5.11. Putative binding partners of Oxr1 in the brain and ordered by number of detections in the 6 combinations of time-point and genotype

UniProt Accession	Protein Name	P60 TG	P60 SOD/TG	P90 TG	P90 SOD/TG	P135 TG	P135 SOD/TG
P18872	Guanine nucleotide-binding protein G(o) subunit alpha	✓	✓	✓	✓	✓	✓
Q4KMM3	Oxidation resistance protein 1	✓	✓	✓	✓	✓	✓
O70318	Band 4.1-like protein 2		✓	✓	✓	✓	✓
O08553	Dihydropyrimidinase-related protein 2	✓	✓	✓		✓	✓
A2ASS6	Titin	✓	✓	✓	✓		✓
P60710	Actin, cytoplasmic 1	✓	✓	✓	✓		
Q9WV92	Band 4.1-like protein 3	✓			✓	✓	✓
P62204	Calmodulin		✓	✓	✓	✓	
P05064	Fructose-bisphosphate aldolase A	✓	✓	✓		✓	
Q6GSS7	Histone H2A type 2-A	✓			✓	✓	✓
P08551	Neurofilament light polypeptide	✓		✓		✓	✓
Q60932	Voltage-dependent anion-selective channel protein 1	✓	✓			✓	✓
Q8BFZ3	Beta-actin-like protein 2			✓	✓		✓
Q91XV3	Brain acid soluble protein 1		✓	✓	✓		
Q3UHL1	CaM kinase-like vesicle-associated protein		✓	✓			✓
P00405	Cytochrome c oxidase subunit 2	✓		✓		✓	
Q62425	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	✓				✓	✓
P35486	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	✓		✓	✓		
Q62277	Synaptophysin		✓	✓			✓
P61982	14-3-3 protein gamma		✓	✓			
P68254	14-3-3 protein theta			✓		✓	
P46660	Alpha-internexin		✓				✓
Q78PG9	Coiled-coil domain-containing protein 25				✓		✓
Q8BMF4	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	✓		✓			
Q6PCX9	E3 ubiquitin-protein ligase TRIM37				✓		✓
P62874	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1			✓	✓		
P62880	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2		✓	✓			
Q61001	Laminin subunit alpha-5	✓					✓
Q3KNA1	Mas-related G-protein coupled receptor member B2	✓				✓	
Q99MV5	Putative helicase Mov10l1		✓		✓		
P38647	Stress-70 protein, mitochondrial		✓	✓			
Q8BRG8	Transmembrane protein 209		✓				✓
P35821	Tyrosine-protein phosphatase non-receptor type 1					✓	✓
P63024	Vesicle-associated membrane protein 3			✓		✓	
P50516	V-type proton ATPase catalytic subunit A	✓		✓			
P62259	14-3-3 protein epsilon			✓			
O70456	14-3-3 protein sigma				✓		
P63101	14-3-3 protein zeta/delta		✓				
O35083	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	✓					
Q8K4S1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1	✓					
P19253	60S ribosomal protein L13a	✓					
Q99P21	A/G-specific adenine DNA glycosylase				✓		
Q8CJ27	Abnormal spindle-like microcephaly-associated protein homolog	✓					
Q99KI0	Aconitate hydratase, mitochondrial	✓					
P68033	Actin, alpha cardiac muscle 1			✓			
A2AH22	Activating molecule in BECN1-regulated autophagy protein 1			✓			
Q8BK64	Activator of 90 kDa heat shock protein ATPase homolog 1	✓					
Q8VC66	Afadin- and alpha-actinin-binding protein	1✓					
Q02357	Ankyrin-1					✓	
P97384	Annexin A11			✓			
P05201	Aspartate aminotransferase, cytoplasmic	✓					

P05202	Aspartate aminotransferase, mitochondrial	✓					
Q9DB20	ATP synthase subunit O, mitochondrial			✓			
Q6PGC1	ATP-dependent RNA helicase Dhx29					✓	
Q80XK6	Autophagy-related protein 2 homolog B						✓
Q67FY2	B-cell CLL/lymphoma 9-like protein		✓				
O88597	Beclin-1			✓			
Q91ZZ3	Beta-synuclein			✓			
A0JNT9	Bicaudal D-related protein 1						✓
O35161	Cadherin EGF LAG seven-pass G-type receptor 1						✓
Q91ZI0	Cadherin EGF LAG seven-pass G-type receptor 3				✓		
Q8BH59	Calcium-binding mitochondrial carrier protein Aralar1					✓	
Q6IRU7	Centrosomal protein of 78 kDa		✓				
Q9D516	Coiled-coil domain-containing protein 130	✓					
P28481	Collagen alpha-1(II) chain			✓			
P08121	Collagen alpha-1(III) chain			✓			
Q07643	Collagen alpha-2(IX) chain		✓				
Q8BNX1	C-type lectin domain family 4 member G				✓		
P28659	CUGBP Elav-like family member 1				✓		
P26361	Cystic fibrosis transmembrane conductance regulator	✓					
Q9DB77	Cytochrome b-c1 complex subunit 2, mitochondrial		✓				
Q7TMB8	Cytoplasmic FMR1-interacting protein 1	✓					
Q8BGW4	DDB1- and CUL4-associated factor 12-like protein 2					✓	
Q8C147	Dedicator of cytokinesis protein 8	✓					
O88673	Diacylglycerol kinase alpha	✓					
Q9D2G2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	✓					
O88839	Disintegrin and metalloproteinase domain-containing protein 15	✓					
Q80Y75	DnaJ homolog subfamily B member 13		✓				
Q99MU3	Double-stranded RNA-specific adenosine deaminase		✓				
Q5U4C9	Dual specificity tyrosine-phosphorylation-regulated kinase 2				✓		
Q69Z23	Dynein heavy chain 17, axonemal					✓	
Q8VHE6	Dynein heavy chain 5, axonemal	✓					
Q9ESD7	Dysferlin						✓
Q4U2R1	E3 ubiquitin-protein ligase HERC2					✓	
Q9CY62	E3 ubiquitin-protein ligase RNF181			✓			
Q99K41	EMILIN-1				✓		
Q62448	Eukaryotic translation initiation factor 4 gamma 2						✓
P43006	Excitatory amino acid transporter 2		✓				
Q7TPM6	Fibronectin type III and SPRY domain-containing protein 1	✓					
Q6P9Q6	FK506-binding protein 15					✓	
Q6ZPF4	Formin-like protein 3						✓
O88537	Formyl peptide receptor-related sequence 3			✓			
Q91ZD6	Full=ELL-associated factor 2						✓
Q8R080	G2 and S phase-expressed protein 1	✓					
P51162	Gastrotropin						✓
P23818	Glutamate receptor 1						✓
P23819	Glutamate receptor 2					✓	
Q9Z2W8	Glutamate receptor 4			✓			
Q8K0U4	Heat shock 70 kDa protein 12A		✓				
P11499	Heat shock protein HSP 90-beta			✓			
Q9Z0Z4	Hephaestin				✓		
P15864	Histone H1.2				✓		
P10853	Histone H2B type 1-F/J/L				✓		
Q6LBE8	Histone H3.2					✓	
P02301	Histone H3.3C						✓
Q9JKY5	Huntingtin-interacting protein 1-related protein		✓				
P70312	Hyaluronan synthase 2				✓		
Q8R0N6	Hydroxyacid-oxoacid transhydrogenase, mitochondrial						✓
Q9R1Y5	Hypermethylated in cancer 1 protein						✓

Q3TNL8	Inositol 1,4,5-trisphosphate receptor-interacting protein	✓					
P59823	Interleukin-1 receptor accessory protein-like 1	✓					
P70380	Interleukin-18			✓			
Q99JN2	Kelch-like protein 22	✓					
Q61768	Kinesin-1 heavy chain		✓				
Q8R2U7	Leucine-rich repeat-containing protein 42				✓		
P16125	L-lactate dehydrogenase B chain	✓					
Q8CB67	Low-density lipoprotein receptor-related protein 11					✓	
Q8BVP6	Ly6/PLAUR domain-containing protein 4		✓				
Q9CYC6	m7GpppN-mRNA hydrolase					✓	
Q4VC33	Macrophage erythroblast attacher				✓		
P14152	Malate dehydrogenase, cytoplasmic		✓				
P08249	Malate dehydrogenase, mitochondrial	✓					
Q8K310	Matrin-3				✓		
P20357	Microtubule-associated protein 2		✓				
Q60592	Microtubule-associated serine/threonine-protein kinase 2	✓					
Q8CCM6	Mitochondrial import inner membrane translocase subunit Tim21	✓					
Q8BUR9	Mitotic-spindle organizing protein 1		✓				
Q9D2Y4	Mixed lineage kinase domain-like protein						✓
P59759	MKL/myocardin-like protein 2			✓			
Q9ERZ3	Muscarinic acetylcholine receptor M3	✓					
P26645	Myristoylated alanine-rich C-kinase substrate				✓		
Q8C3P7	N6-adenosine-methyltransferase 70 kDa subunit		✓				
Q9JIL4	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	✓					
Q9CXZ1	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	✓					
Q9QY36	N-alpha-acetyltransferase 10	✓					
Q8JZS6	NEDD4-binding protein 2-like 2	✓					
Q8C4Y3	Negative elongation factor B					✓	
O70340	Neuronal pentraxin-2		✓				
P29477	Nitric oxide synthase, inducible						✓
Q9CQ49	Nuclear cap-binding protein subunit 2					✓	
Q5NCR9	Nuclear speckle splicing regulatory protein 1						✓
Q3UA06	Pachytene checkpoint protein 2 homolog			✓			
Q99NH2	Partitioning defective 3 homolog			✓			
Q3URU2	Paternally-expressed gene 3 protein		✓				
O88593	Peptidoglycan recognition protein 1		✓				
Q9QZH3	Peptidyl-prolyl cis-trans isomerase E				✓		
Q3UQ28	Peroxidasin homolog						✓
B1AUE5	Peroxisome biogenesis factor 10		✓				
P70296	Phosphatidylethanolamine-binding protein 1			✓			
Q8BH04	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	✓					
Q64436	Potassium-transporting ATPase alpha chain 1	✓					
Q8VC90	Probable palmitoyltransferase ZDHHC12			✓			
Q91V51	Probable tubulin polyglutamylase TTLL1			✓			
Q9R0E2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1						✓
Q9D1C2	Protein chibby homolog 1		✓				
P20444	Protein kinase C alpha type			✓			
P56844	Protein TCL1B4		✓				
Q8BHK3	Proton-coupled amino acid transporter 2			✓			
Q8K183	Pyridoxal kinase						✓
Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	✓					
P52480	Pyruvate kinase isozymes M1/M2				✓		
P69566	Ran-binding protein 9			✓			
P62823	Ras-related protein Rab-3C			✓			
A2AWP8	Rho guanine nucleotide exchange factor 10-like protein	✓					
Q99M28	RNA-binding protein with serine-rich domain 1			✓			
Q8CAV0	rRNA maturation factor homolog					✓	
Q64131	Runt-related transcription factor 3				✓		
Q9EPB5	Serine hydrolase-like protein						✓
Q924X7	Serine/threonine-protein kinase 33						✓
O70405	Serine/threonine-protein kinase ULK1				✓		
Q62388	Serine-protein kinase ATM		✓				

Q8VDN2	Sodium/potassium-transporting ATPase subunit alpha-1		✓				
Q8R0Y8	Solute carrier family 25 member 42						✓
Q62261	Spectrin beta chain, non-erythrocytic 1					✓	
Q62252	Sperm surface protein Sp17		✓				
P55821	Stathmin-2			✓			
Q9CU62	Structural maintenance of chromosomes protein 1A			✓			
Q8R191	Synaptogyrin-3		✓				
P60879	Synaptosomal-associated protein 25			✓			
Q8R570	Synaptosomal-associated protein 47			✓			
Q7TN83	Synaptotagmin-16	✓					
Q8BYI9	Tenascin-R			✓			
Q3UTY6	Thrombospondin type-1 domain-containing protein 4				✓		
P01831	Thy-1 membrane glycoprotein		✓				
Q9Z0U1	Tight junction protein ZO-2		✓				
Q6PFR5	Transformer-2 protein homolog alpha			✓			
Q922P8	Transmembrane protein 132A	✓					
P17751	Triosephosphate isomerase			✓			
Q1PSW8	Tripartite motif-containing protein 71				✓		
P05214	Tubulin alpha-3 chain			✓			
Q922F4	Tubulin beta-6 chain						✓
Q9D777	Tumor necrosis factor ligand superfamily member 13						✓
Q8R1S0	Ubiquinone biosynthesis monooxygenase COQ6						✓
P62984	Ubiquitin-60S ribosomal protein L40			✓			
Q8C5R2	Uncharacterized protein C10orf47 homolog				✓		
Q8BT88	Uncharacterized protein C16orf90 homolog	✓					
Q8C1A9	Uncharacterized protein C4orf29 homolog		✓				
Q3UPC7	Uncharacterized protein KIAA0825 homolog			✓			
Q0VBV7	Uncharacterized protein KIAA1377			✓			
E9Q634	Unconventional myosin-Ie				✓		
F8VQB6	Unconventional myosin-X				✓		
Q91ZD4	Vang-like protein 2	✓					
P35917	Vascular endothelial growth factor receptor 3			✓			
P63044	Vesicle-associated membrane protein 2		✓				
Q99246	Voltage-dependent L-type calcium channel subunit alpha-1D		✓				
O55017	Voltage-dependent N-type calcium channel subunit alpha-1B					✓	
Q9D565	WD repeat-containing protein 64		✓				
Q9R0X5	X-linked retinitis pigmentosa GTPase regulator				✓		
P0CL69	Zinc finger protein 703					✓	
Q9CWL2	Zinc finger protein castor homolog 1				✓		

Mass spectrometry samples were prepared by me, the experiment performed by Dr. Holger Kramer, and the analysis conducted by me with guidance from Dr. Holger Kramer.

Table 5.12. Putative binding partners of Oxr1 in the spinal cord and ordered by number of detections in the 6 combinations of time-point and genotype

UniProt Accession	Protein Name	P60 TG	P60 SOD/TG	P90 TG	P90 SOD/TG	P135 TG	P135 SOD/TG
Q4KMM3	Oxidation resistance protein 1	✓	✓	✓	✓	✓	✓
O70318	Band 4.1-like protein 2	✓		✓	✓	✓	✓
A2ASS6	Titin	✓	✓	✓		✓	✓
O35955	Proteasome subunit beta type-10	✓	✓		✓	✓	
Q9WV92	Band 4.1-like protein 3	✓	✓			✓	
Q9EST3	Eukaryotic translation initiation factor 4E transporter				✓	✓	✓
Q9Z2W9	Glutamate receptor 3				✓	✓	✓
P27573	Myelin protein P0	✓	✓			✓	
P20029	78 kDa glucose-regulated protein		✓		✓		
Q9D3D9	ATP synthase subunit delta, mitochondrial	✓					✓
Q9CY62	E3 ubiquitin-protein ligase RNF181		✓		✓		
Q6P1I6	PH and SEC7 domain-containing protein 2				✓		✓
Q8BY46	Zinc finger protein 574	✓	✓				
P68254	14-3-3 protein theta	✓					
O35083	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha						✓
Q8CBW3	Abl interactor 1						✓
P51829	Adenylate cyclase type 7; EC=4.6.1.1	✓					
P51881	ADP/ATP translocase 2			✓			
O54931	A-kinase anchor protein 2			✓			
Q9D3D0	Alpha-tocopherol transfer protein-like					✓	
Q9DAX9	Amyloid protein-binding protein 2	✓					
Q06335	Amyloid-like protein 2				✓		
P07146	Anionic trypsin-2						✓
Q91ZT9	Ankyrin repeat and SOCS box protein 8						✓
Q3U829	AP-5 complex subunit zeta-1						✓
Q7TS75	APC membrane recruitment protein 1						✓
Q61324	Aryl hydrocarbon receptor nuclear translocator 2					✓	
Q61137	Astrotactin-1	✓					
Q9CQQ7	ATP synthase subunit b, mitochondrial						✓
P56480	ATP synthase subunit beta, mitochondrial			✓			
Q9DBJ3	Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1						✓
O70445	BRCA1-associated RING domain protein 1			✓			
P97929	Breast cancer type 2 susceptibility protein homolog						✓
P55284	Cadherin-5						✓
Q924I3	C-C chemokine receptor type 11				✓		
Q91ZW8	CD209 antigen-like protein D			✓			
P49452	Centromere protein C			✓			
Q811Q9	Choline-phosphate cytidylyltransferase B						✓
Q9Z0E2	Chordin					✓	
Q8BYH8	Chromodomain-helicase-DNA-binding protein 9						✓
P70375	Coagulation factor VII			✓			
Q9JIF7	Coatomer subunit beta				✓		
Q78PG9	Coiled-coil domain-containing protein 25					✓	
P11087	Collagen alpha-1(I) chain			✓			
Q02105	Complement C1q subcomponent subunit C						✓
Q8BH52	Cyclic AMP-responsive element-binding protein 3-like protein 2						✓
Q9JJZ9	Cyclic nucleotide-gated cation channel beta-3		✓				
P97314	Cysteine and glycine-rich protein 2				✓		
Q9CZ13	Cytochrome b-c1 complex subunit 1, mitochondrial					✓	
Q9JHU4	Cytoplasmic dynein 1 heavy chain 1					✓	
A2AGT5	Cytoskeleton-associated protein 5					✓	
A2AF47	Dedicator of cytokinesis protein 11						✓
Q9R0P5	Destrin					✓	
Q9D0A0	DET1 homolog	✓					
Q9D2G2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	✓					
Q99KK7	Dipeptidyl peptidase 3						✓
Q811D0	Disks large homolog 1						✓
P70388	DNA repair protein RAD50	✓					

P49718	DNA replication licensing factor MCM5					✓	
P54103	DnaJ homolog subfamily C member 2		✓				
P60904	DnaJ homolog subfamily C member 5	✓					
P60904	DnaJ homolog subfamily C member 5				✓		
Q05AA6	Dystrophin-related protein 2	✓					
Q8CH72	E3 ubiquitin-protein ligase TRIM32			✓			
Q80TP3	E3 ubiquitin-protein ligase UBR5				✓		
Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial	✓					
P10126	Elongation factor 1-alpha 1	✓					
Q99K41	EMILIN-1	✓					
Q63961	Endoglin				✓		
Q8R0W0	Epiplakin			✓			
Q80XI3	Eukaryotic translation initiation factor 4 gamma 3	✓					
P43006	Excitatory amino acid transporter 2					✓	
Q8BMI4	Flap endonuclease GEN homolog 1	✓					
Q8BWQ4	FtsJ methyltransferase domain-containing protein 1				✓		
Q3TCV3	Gamma-secretase-activating protein				✓		
Q8VHI3	GDP-fucose protein O-fucosyltransferase 2		✓				
Q8K0C9	GDP-mannose 4,6 dehydratase			✓			
P15105	Glutamine synthetase	✓					
Q9CQM9	Glutaredoxin-3				✓		
P16858	Glyceraldehyde-3-phosphate dehydrogenase			✓			
Q64467	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific						✓
Q91X51	Golgi reassembly-stacking protein 1						✓
Q8VHN7	G-protein coupled receptor 98	✓					
P20612	Guanine nucleotide-binding protein G(t) subunit alpha-1				✓		
P27601	Guanine nucleotide-binding protein subunit alpha-13		✓				
Q9WVD4	H(+)/Cl(-) exchange transporter 5						✓
Q8K0U4	Heat shock 70 kDa protein 12A						✓
Q6PCN7	Helicase-like transcription factor						✓
P02089	Hemoglobin subunit beta-2						✓
Q6GSS7	Histone H2A type 2-A			✓			
Q3KNY0	Immunoglobulin-like and fibronectin type III domain-containing protein 1				✓		
Q9R000	Integrin beta-1-binding protein 2				✓		
P09922	Interferon-induced GTP-binding protein Mx1				✓		
P70380	Interleukin-18	✓					
Q8R2V2	Intermediate filament family orphan 2	✓					
Q6PCQ0	IQ domain-containing protein E			✓			
B7ZNG0	Kinesin-like protein KIF7		✓				
O08584	Krueppel-like factor 6				✓		
Q8K1F9	Lactase-like protein		✓				
Q9JI18	Low-density lipoprotein receptor-related protein 1B	✓					
O70546	Lysine-specific demethylase 6A		✓				
P17897	Lysozyme C-1				✓		
A2A9G7	Membrane protein FAM159A						✓
Q68ED2	Metabotropic glutamate receptor 7				✓		
Q5HZI1	Microtubule-associated tumor suppressor 1 homolog	✓					
Q8R0Y8	Mitochondrial coenzyme A transporter SLC25A42				✓		
Q8CAQ8	Mitochondrial inner membrane protein			✓			
Q8K595	Mucolipin-2				✓		
O70468	Myosin-binding protein C, cardiac-type				✓		
Q80VA0	N-acetylgalactosaminyltransferase 7				✓		
Q62425	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4			✓			
Q9CQZ5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6			✓			
Q80XB4	Nebulin-related-anchoring protein	✓					
P19246	Neurofilament heavy polypeptide			✓			
O35516	Neurogenic locus notch homolog protein 2				✓		
Q9EQ80	NIF3-like protein 1		✓				
O09000	Nuclear receptor coactivator 3				✓		
Q9JL19	Nuclear receptor coactivator 6			✓			

Q5NCR9	Nuclear speckle splicing regulatory protein 1			✓			
Q3UHX0	Nucleolar protein 8		✓				
P00688	Pancreatic alpha-amylase	✓					
Q5DU28	Pecanex-like protein 2			✓			
Q9D787	Peptidyl-prolyl cis-trans isomerase-like 2				✓		
Q9Z211	Peroxisomal membrane protein 11A			✓			
Q7M6Y3	Phosphatidylinositol-binding clathrin assembly protein	✓					
Q8BH04	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial			✓			
Q9EQ32	Phosphoinositide 3-kinase adapter protein 1			✓			
Q8BUL6	Pleckstrin homology domain-containing family A member 1				✓		
B2RPU2	Pleckstrin homology domain-containing family D member 1					✓	
Q9JIY0	Pleckstrin homology domain-containing family O member 1		✓				
Q9ER47	Potassium voltage-gated channel subfamily H member 7			✓			
Q6PI62	Probable G-protein coupled receptor 173	✓					
Q9R1K6	Probable G-protein coupled receptor 34	✓					
P50580	Proliferation-associated protein 2G4	✓					
Q6W3F0	Prolyl 4-hydroxylase subunit alpha-3			✓			
Q8CIG8	Protein arginine N-methyltransferase 5					✓	
Q924A2	Protein capicua homolog			✓			
Q8CJF7	Protein ELYS				✓		
Q61115	Protein patched homolog 1				✓		
Q01405	Protein transport protein Sec23A				✓		
Q80TM6	R3H domain-containing protein 2			✓			
Q9QXG2	Rab proteins geranylgeranyltransferase component A 1	✓					
Q9D684	Ras and Rab interactor 2			✓			
Q640N3	Rho GTPase-activating protein 30					✓	
O89026	Roundabout homolog 1					✓	
E9Q401	Ryanodine receptor 2						✓
Q3URD3	Sarcolemmal membrane-associated protein						✓
P59222	Scavenger receptor class F member 2				✓		
Q60988	SCL-interrupting locus protein homolog			✓			
Q8BRF7	Sec1 family domain-containing protein 1	✓					
Q62028	Secretory phospholipase A2 receptor						✓
O09126	Semaphorin-4D					✓	
Q7TSJ6	Serine/threonine-protein kinase LATS2			✓			
Q9JK88	Serpin I2		✓				
O08641	SH3 domain-containing YSC84-like protein 1					✓	
Q9Z179	SHC SH2 domain-binding protein 1						✓
Q9WVC5	Short transient receptor potential channel 7				✓		
Q8BQM7	Single-pass membrane and coiled-coil domain-containing protein 3						✓
Q60665	Ski-like protein	✓					
P14094	Sodium/potassium-transporting ATPase subunit beta-1			✓			
Q0P5V9	Solute carrier family 45 member 4			✓			
Q3UMC0	Spermatogenesis-associated protein 5			✓			
P35175	Stefin-1		✓				
Q6P1D7	Structure-specific endonuclease subunit SLX4			✓			
Q80X82	Symplekin	✓					
Q62209	Synaptonemal complex protein 1	✓					
Q91YE8	Synaptopodin-2					✓	
Q9CXF4	TBC1 domain family member 15			✓			
Q8BYJ6	TBC1 domain family member 4			✓			
O70372	Telomerase reverse transcriptase						✓
P61406	Telomerase-binding protein EST1A					✓	
Q9WTS6	Teneurin-3			✓			
Q80VM3	Tetratricopeptide repeat protein 29		✓				
P01831	Thy-1 membrane glycoprotein	✓					
Q5HZG4	Transcription initiation factor TFIID subunit 3						✓
Q8VHE0	Translocation protein SEC63 homolog	✓					
Q8BRG8	Transmembrane protein 209;				✓		
Q9D328	Transmembrane protein 35						✓
Q99LG1	Transmembrane protein 51		✓				
Q9ERD7	Tubulin beta-3 chain;			✓			
Q8BWR4	Ubiquitin carboxyl-terminal hydrolase 40	✓					

Q8BUM9	Ubiquitin carboxyl-terminal hydrolase 43	✓					
Q7TQI3	Ubiquitin thioesterase OTUB1			✓			
Q3UCV8	Ubiquitin thioesterase otulin					✓	
Q8C753	Uncharacterized protein KIAA0556	✓					
Q6A000	Uncharacterized protein KIAA0753		✓				
Q68FF0	Uncharacterized protein KIAA1841				✓		
A2AFS3	UPF0577 protein KIAA1324				✓		
Q62463	Vasopressin V1a receptor						✓
Q9Z109	V-set and immunoglobulin domain-containing protein 2				✓		
Q3UGF1	WD repeat-containing protein 19						✓
P70315	Wiskott-Aldrich syndrome protein homolog						✓
Q9DAZ9	Zinc finger FYVE domain-containing protein 19		✓				
P47806	Zinc finger protein GLI1			✓			
O88799	Zonadhesin			✓			

Mass spectrometry samples were prepared by me, the experiment performed by Dr. Holger Kramer, and the analysis conducted by me with guidance from Dr. Holger Kramer.

regulated to heat shock and increase of misfolded proteins in the endoplasmic reticulum, and binds to AMPA receptor, Glutamate receptor 1 (GluR1) through a C-terminal domain (Jin *et al.*, 1991; Partaledis and Berlin, 1993; Bush *et al.*, 1994; Walensky *et al.*, 1998; Shen *et al.*, 2000). Epb41l3 inhibits Protein arginine N-methyltransferase 3 (PRMT3)-mediated methylation events, binds to cell adhesion-related Ezrin, Radixin, and Moesin proteins and cytoskeleton βII-spectrin, and suppresses tumour cell growth (Tran *et al.*, 1999; Gutmann *et al.*, 2000; Gutmann *et al.*, 2001; Charboneau *et al.*, 2002; Singh *et al.*, 2004). Furthermore, Epb41l3 also interacts with 14-3-3 protein gamma, which was also identified as a putative Oxr1 binding partner in the brain and along with other 14-3-3 proteins, modulates various apoptosis pathways (Yu *et al.*, 2002; Berg *et al.*, 2003)

Furthermore, we identified a number of Oxr1 binding candidates only in co-immunoprecipitation experiments from brain tissue, such as Dihydropyrimidinase-related protein 2 (Dpysl2) and Voltage-dependent anion-selective channel protein 1 (Vdac1) (Table 5.11). Dpysl2 regulates neurite outgrowth and neuronal polarity, and dysregulation of Dpysl2 function is linked to a number of neurological disorders, including ALS (Inagaki *et al.*, 2001; West *et al.*, 2004; Uchida *et al.*, 2005; Yoshimura *et al.*, 2005; Hensley *et al.*, 2010b; Hensley *et al.*, 2010a; Khanna *et al.*, 2012; Yamashita *et al.*, 2012). An outer mitochondrial membrane voltage-dependent anion channel, Vdac1 is an important regulator of mitochondrial permeability, which is responsible for metabolic transport and release of apoptotic factors, and mutant SOD1 inhibits Vdac1 conductance through aberrant complex formation with Bcl-2 (Israelson *et al.*, 2010; Tan *et al.*, 2013). In addition, some proteins, such as Coiled-coil domain-containing protein 25 (Ccdc25) and Putative helicase Mov10l1, were identified as putative binding partners of Oxr1 specifically in mutant SOD1 conditions in the brain. Little is known about Ccdc25, but Ccdc25 is enriched in layer V of the cortex, where CSMN, upper motor neurons that

degenerate in ALS, reside (Tandan and Bradley, 1985; Belgard *et al.*, 2011; Ozdinler *et al.*, 2011). Mov10l1 interacts with Piwi proteins and loss of Mov10l1 prevents formation of Piwi-interacting RNAs (piRNAs), resulting in reduced DNA methylation and impaired silencing of transposable elements (Zheng *et al.*, 2010).

Similarly, we identified various spinal cord-specific Oxr1 binding candidates, such as Proteasome subunit beta type-10 (Psmb10) and Eukaryotic translation initiation factor 4E (eIF4E) transporter (Eif4enif1) (Table 5.12). Psmb10, also known as LMP10 and MECL-1, is a proteasome-associated subunit that is induced by interferon-γ, and processes major histocompatibility complex (MHC) class I presenting antigens (Nandi *et al.*, 1996; Groettrup *et al.*, 1997; Schwarz *et al.*, 2000). Eif4enif1 is a nucleocytoplasmic shutting protein that regulates nuclear import of eIF4E, which participates in initiation of mRNA translation and is localised to cytoplasmic stress granules under cellular stress (Dostie *et al.*, 2000; Sonenberg and Hinnebusch, 2009; Suzuki *et al.*, 2009). Additionally, we identified some proteins, such as PH and SEC7 domain-containing protein 2 (Psd2) and E3 ubiquitin-protein ligase RNF181, as potential binding partners of Oxr1 specifically in the spinal cord under mutant SOD1 conditions. Little is known about the function of Psd2, but RNF181 suppresses tumour growth by inhibiting Extracellular signal-regulated kinase (ERK)/Mitogen-activated protein kinase (MAPK) signalling, and RNF181 function is modulated by NO-mediated S-nitrosylation (Wang *et al.*, 2011b; Lee *et al.*, 2014).

Given that Oxr1 delays neuroinflammation in the spinal cord during ALS pathogenesis and PSMB10 is dysregulated in the $SOD1^{G93A}$ ALS mice (Cheroni *et al.*, 2009), we chose to confirm Oxr1-FL binding with Psmb10. We found that Oxr1-FL interacts with Psmb10 in co-transfected HeLa cells (Figure 5.8), suggesting that the mass spectrometry experiments identified binding partners of Oxr1 and that the interaction between Oxr1-FL and Psmb10 may play role in modulating neuroinflammation in ALS.

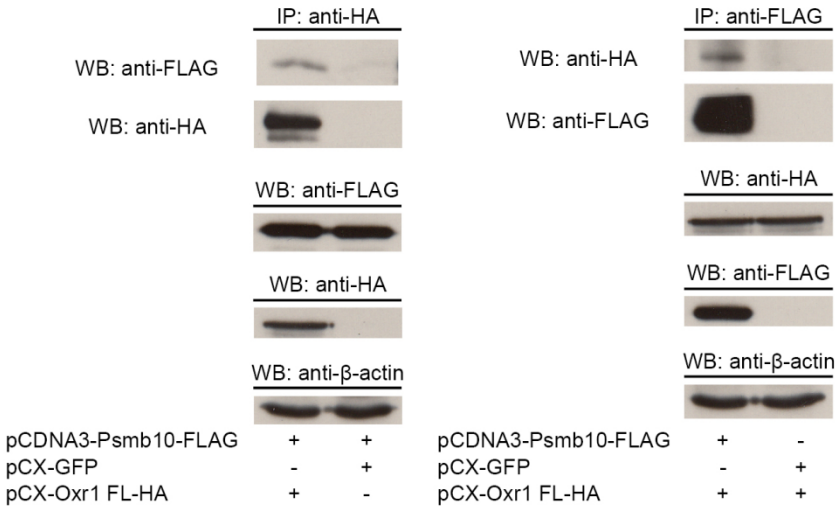

Figure 5.8. Oxr1-FL interacts with Psmb10
Co-immunoprecipitation of HA-tagged Oxr1-FL and FLAG-tagged Psmb10 in co-transfected HeLa cells demonstrates that Oxr1-FL interacts with Psmb10 *in vitro*.

Taken together, these results suggest that Oxr1 interacts with proteins regulating diverse molecular pathways, including OS and immune response.

5.3 Discussion

Here, we further demonstrated that Oxr1 modifies disease progression in a mouse model of ALS by using transcriptomics, and found that Oxr1 functions upstream of a number of molecular pathways. Very few studies examining genetic modifiers or possible treatments of ALS have further investigated the effect of these modifiers on global changes in spinal cord gene expression during ALS disease progression. Neuronal over-expression of ATF3 reduces the induction of neuroinflammatory and apoptosis pathways, and prevents alterations to genes associated with nerve transmission in early symptomatic $SOD1^{G93A}$ ALS mice (Seijffers *et al.*, 2014). Furthermore, a study testing the efficacy of a treatment using monoclonal antibody against Cluster of Differentiation 40 (CD40) ligand (CD40L), a T-cell surface ligand that activates the immune response by binding to CD40 receptors, conducted transcriptomics analysis to demonstrate anti-CD40L treatment decreased expression of co-stimulatory pathway genes (Lincecum *et al.*, 2010). Finally, exercise regimens alters gene expression in spinal cord and gastrocnemius muscle of $SOD1^{G93A}$ ALS mice, with motor neurons up-regulating neurotrophic factors, and altering electrophysiological properties and synaptic organization (Ferraiuolo *et al.*, 2009; Hashimoto *et al.*, 2009). In this study, we conducted microarray analysis to uncover the downstream pathways by which Oxr1 confers its neuroprotective benefits. We found that over-expression of Oxr1 in neurons significantly delays many molecular pathways involved in ALS disease progression, even at a pre-symptomatic stage, including many pathways identified in previous microarray studies of mutant SOD1 ALS mice (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Fukada *et al.*, 2007; Chen *et al.*, 2010).

5.3.1 Oxr1 and neuroinflammation

Of the gene expression changes in SOD1^{G93A} ALS mice rescued by neuronal over-expression of Oxr1, we observed a striking delay in the neuroinflammatory response, including microgliosis markers, and activation of classic complement and STAT3 pathways. Neuroinflamation has been intricately linked to ALS disease progression, and in particular, non-neuronal cell types, such as microglia and astrocytes, substantially contribute to motor neuron apoptosis (Ilieva *et al.*, 2009; Ferraiuolo *et al.*, 2011; Bowerman *et al.*, 2013). Specific deletion of ALS mutant SOD1 from either astrocytes or microglia significantly slows disease progression (Boillee *et al.*, 2006; Yamanaka *et al.*, 2008). Importantly, a recent study confirmed non-cell autonomous contributions of glial cells in ALS disease pathology by demonstrating that transplantation of SOD1^{G93A} glial progenitors into spinal cords of wild-type rats induces astrogliosis and microgliosis, motor dysfunction, and motor neuron death (Papadeas *et al.*, 2011). Moreover, anti-CD40L treatment at a presymptomatic stage of ALS delays onset of disease, extends life-span, and significantly reduces astrogliosis and microgliosis in the spinal cord of SOD1^{G93A} ALS mice (Lincecum *et al.*, 2010). Here, we demonstrated that at P90, over-expression of Oxr1 in neurons prevents increased expression of immune response genes, from cytokines, such as *Ccl3* and *Cd68*, to microglia-specific genes, such as *Cathepsin Z* (*Ctsz*) (Table 5.8), all of which have been previously identified as being altered during ALS disease progression (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Fukada *et al.*, 2007; Chen *et al.*, 2010; Lincecum *et al.*, 2010). The delay in inflammatory response is also strikingly evident in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice, when compared to SOD1^{G93A} ALS mice at P135 (Figure 5.4). We observed markedly reduced microgliosis and astrogliosis by immunocytochemistry in the lumbar spinal cord (Figure 5.4).

Furthermore, we identified that Oxr1 functions upstream of multiple pathways that function in concert with the immune system, including the complement system and STAT3-activated pathway. In particular, over-expression of Oxr1 rescues up-regulation of of *C1qa/b/c*, encoding for C1qα-, β-, γ-chains, which initiates the classic complement pathway, responsible for mediating the immune system response (Figure 5.5). Induction of C1q in motor neurons and the global complement pathway in the spinal cord of mutant SOD1 ALS mice has been reported in many studies (Olsen *et al.*, 2001; Perrin *et al.*, 2005; Bonifati and Kishore, 2007; Ferraiuolo *et al.*, 2007; Fukada *et al.*, 2007; Lobsiger *et al.*, 2007; Lee *et al.*, 2013). While deletion of C1q or C3, a protein central to both the classic and alternative complement pathway, does not affect onset of disease or survival, treatment with a selective C5a receptor antagonist or loss of C5a receptor 1 extends survival of mutant SOD1 ALS mice by about 6% (Woodruff *et al.*, 2008; Lobsiger *et al.*, 2013; Woodruff *et al.*, 2014). In addition, we found that the increase in p-STAT3 is rescued in SOD1^{G93A}/Tg(*Prnp-Oxr1*) mice, and showed that expression changes in downstream targets of activated STAT3, such as *Vim* and *Serpina3n*, are similarly rescued (Figure 5.5). In response to proinflammatory cytokines, STAT3, a transcription factor, becomes activated, translocates to the nucleus, and induces expression of additional immune response genes, and activation of STAT3 pathways have been observed in motor neurons and glia cells in the spinal cord of ALS patients and SOD1^{G93A} ALS mice (Shibata *et al.*, 2009; Shibata *et al.*, 2010). *Vim* is an intermediate filament that forms inclusions in SOD1 mutant ALS motor neurons and is an early pathological hallmark of ALS (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Perrin *et al.*, 2005; Ferraiuolo *et al.*, 2007; Fukada *et al.*, 2007). Similarly, *Serpina3n* has been found to accumulate along these neurofilamentous conglomerates, and the imbalance of serine proteases and their respective inhibitors during oxidative injury may contribute to the formation of ALS motor neuron inclusions (Chou *et*

al., 1998). Although ultimately unsuccessful during a clinical trial for ALS patients, treatment with antidiabetic drug pioglitazone reduces activation of STAT3 and neuroinflammatory response, and significantly extends survival in $SOD1^{G93A}$ ALS mice by approximately 10% (Kiaei *et al.*, 2005; Schutz *et al.*, 2005; Shibata *et al.*, 2010; Dupuis *et al.*, 2012). Together, these data suggest that Oxr1 functions upstream of the classic complement and STAT3 multiple pathways in ALS disease progression, particularly in response to neuroinflammation, and demonstrates that addressing multiple pathogenic mechanisms may be required due to compensatory pathways.

While the exact mechanisms by which Oxr1 regulates the immune response remain elusive, the link between neuroinflammation and oxidative stress has been previously hypothesized within ALS disease progression. (Barber and Shaw, 2010). In addition, co-cultures of primary astrocytes expressing ALS mutant SOD1 with primary motor neurons or embryonic mouse and human stem-cell-derived motor neurons reduce survival of motor neurons through the release of toxic trophic factors (Nagai *et al.*, 2007; Di Giorgio *et al.*, 2008; Marchetto *et al.*, 2008). Damaged motor neurons release ROS, inducing oxidation and reducing glutamate uptake in neighbouring astrocytes, which subsequently lead to a cyclical process by which increased extracellular glutamate levels and excitotoxicity further damage motor neurons (Rao *et al.*, 2003). Furthermore, ROS have been found to activate glial cells, which can subsequently generate more ROS, reactive nitrogen species, and proinflammatory mediators, and thereby activating more glial cells, eventually leading to motor neuron injury (Banati *et al.*, 1993; Koutsilieri *et al.*, 2002; Zhao *et al.*, 2004b). ALS mutant SOD1 in microglia disrupt regulation of NOX activity through persistent activation of Ras-related C3 botulinum toxin substrate 1 (Rac1), resulting in prolonged production of ROS (Harraz *et al.*, 2008). Moreover, we identified Psmb10 as a binding partner of Oxr1, thus Oxr1 may mediate processing of peptide ligands for MHC class I

presentation through Psmb10 (Table 5.12; Figure 5.8). Therefore, we hypothesize that Oxr1 mediates the crosstalk between damaged motor neurons and toxic mutant SOD1 microglia and astrocytes by 1) conferring protection against increasing intracellular levels of ROS, reducing the release of ROS from damaged neurons, and thereby decreasing glial cell activation; 2) conferring protection against extracellular levels of ROS released by activated glia, which subsequently further extends survival of motor neurons and slows activation of additional glia; and 3) regulating the generation of MHC class I antigens responsible for triggering astrogliosis and microgliosis.

5.3.2 Oxr1 and protein degradation

Furthermore, Oxr1 delays mutant SOD1-induced changes in protein degradation and synthesis, such as up-regulation of *Ubiquitin-protein ligase E3A* (*Ube3a*) and *Ctss* (Table 5.5, 5.8). Ubiquitin-proteasome pathway and lysosomal-autophagic degradation are important mechanisms that remove damaged proteins under cellular stress, and dysregulation of these pathways has been previously established in $SOD1^{G93A}$ ALS mice (Olsen *et al.*, 2001; Yoshihara *et al.*, 2002; Kabashi *et al.*, 2004; Perrin *et al.*, 2005; Ferraiuolo *et al.*, 2007; Ferraiuolo *et al.*, 2011). Aggregates of mutant SOD1 have been found in motor neurons of ALS patients and mouse models of ALS, and occur before the onset of symptoms (Shibata *et al.*, 1994; Rakhit *et al.*, 2007; Bosco *et al.*, 2010b; Ferraiuolo *et al.*, 2011). Interestingly, *Ube3a* encodes for E6AP ubiquitin-protein ligase, which interacts with SOD1 and promotes SOD1 degradation. Over-expression of E6AP along with Heat shock protein 70 (Hsp70) decreases aggregation of mutant SOD1 proteins, and mutant SOD1-mediated cell death (Mishra *et al.*, 2013). Therefore, up-regulation of *Ube3a* in presymptomatic $SOD1^{G93A}$ ALS mice may be a marker of misfolded mutant SOD1, and induction of *Ube3a* expression is decreased in $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice

may indicate a delay in mutant SOD1 aggregation. Moreover, although *Ctss* is predominantly expressed in glia (Olsen *et al.*, 2001; Wendt *et al.*, 2008) during disease progression in $SOD1^{G93A}$ ALS mice, up-regulation of *Ctss* has also been detected in motor neurons of pre-symptomatic $SOD1^{G93A}$ ALS mice (Ferraiuolo *et al.*, 2007). Interestingly, up-regulation of *Ctss* has been observed in cortical and hippocampal neurons of patients suffering from Alzheimer's disease and Down Syndrome (Lemere *et al.*, 1995). Our mass spectrometry experiments also identified a number of putative Oxr1 binding partners that function in the proteasome pathway, including E3 ubiquitin-protein ligases tripartite motif containing 37 (TRIM37) and RNF181 (Table 5.11-5.12). Future work should examine whether Oxr1 affects proteasome-mediated degradation through interactions with these E3 ubiquitin-protein ligases. Thus, our data suggests that Oxr1 ameliorates dysregulation of protein degradation pathways during ALS disease pathogenesis, and possibly through a proteasome-dependent pathway.

5.3.3 Oxr1 and lipid metabolism

Dysregulation of lipid metabolism, particularly hyperlipidaemia, has also been implicated in ALS (Cutler *et al.*, 2002; Dupuis *et al.*, 2008; Dorst *et al.*, 2011; Dupuis *et al.*, 2011; Dodge *et al.*, 2013), and our microarray analysis at P90 demonstrates that Oxr1 modulates lipid scavenge pathways. In particular, Oxr1 functions upstream of *HexB*, an enzyme that targets degradation of glycosphingolipid, and delays elevated *HexB* expression (Table 5.8, Figure 5.6), which has been previously reported in mutant SOD1 ALS motor neurons during disease pathogenesis (Olsen *et al.*, 2001; Perrin *et al.*, 2005; Ferraiuolo *et al.*, 2007; Lobsiger *et al.*, 2007). Interestingly, levels of ceramides, sphingomyelins, cholesterol esters, and phospholipids are significantly up-regulated in ALS patients, and these elevated levels are associated with increasing levels of OS (Cutler

et al., 2002). Furthermore, inhibition of sphingolipid synthesis protects motor neurons against exogenous peroxide- and glutamate-induced apoptosis *in vitro* (Cutler *et al.*, 2002). Elevated levels of ApoE have been found in ALS patients, and loss of ApoE significantly increases survival of $SOD1^{G93A}$ ALS mice (Olsen *et al.*, 2001; Lacomblez *et al.*, 2002). We demonstrated that over-expression of Oxr1 in neurons significantly decreases up-regulation of *ApoE* at P135 in the spinal cord of $SOD1^{G93A}$ ALS mice as the fold change of *ApoE* is increased by two-fold in $SOD1^{G93A}$ ALS mice when compared to $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice (Figure 5.6). Finally, other genes, such as *ATP-Binding Cassette Sub-Family A Member 1* (*Abca1*), have not been investigated in-depth with regards to ALS, but are associated to other neurodegenerative disorders. Abca1 regulates intracellular cholesterol efflux, and has been implicated in disease pathogenesis of Alzheimer's disease, particularly in modulating β-amyloid deposition *in vivo* (Koldamova *et al.*, 2010). A recent study of gene expression profile in blood of ALS patients found that *Abca1* was significantly down-regulated (Saris *et al.*, 2009). Identified as a potential binding partner of Oxr1 in the spinal cord, low-density lipoprotein receptor-related protein 1b (LRP1B) interacts with ligands that regulate lipid metabolism, and is required for C1q-dependent protection in neurons against amyloid-β toxicity (Table 5.12) (Haas *et al.*, 2011; Benoit *et al.*, 2013). Taken together, Oxr1 regulates lipid metabolism, and its function upstream of *HexB* and *Abca1* and in concert with LRP1B should be further examined and may present novel implications for ALS disease pathogenesis.

5.3.4 Oxr1 and metal ion homeostasis

Finally, aberrant regulation of metal ion homeostasis is a pathological feature of ALS and has been linked to increased ROS-induced damage (Olsen *et al.*, 2001; Carri *et al.*, 2003). Copper and iron are two transition metal ions that play an important role in

redox cycling during the formation of ROS (Carri *et al.*, 2003). Deletion of *MT-I*, a protein specific to glia, significantly reduces survival of $SOD1^{G93A}$ ALS mice, while over-expression of MT-I extends survival and decreases inflammation (Nagano *et al.*, 2001; Puttaparthi *et al.*, 2002; Tokuda *et al.*, 2014). These results suggest that MT-I has beneficial effects in glia by improving copper homeostasis during disease progression. On the other hand, treatment with copper chelator d-penicillamine or iron chelator salicylaldehyde isonicotinoyl hydrazine in mutant SOD1 ALS mice delays onset of disease and extends lifespan (Hottinger *et al.*, 1997; Jeong *et al.*, 2009). Interestingly, expression of *FTL* and *MT-I*, which modulate binding to iron and copper, respectively, are up-regulated in glia of end-stage $SOD1^{G93A}$ ALS mice (Nagano *et al.*, 2001; Olsen *et al.*, 2001; Puttaparthi *et al.*, 2002; Jeong *et al.*, 2009). We found that induction of *FTL* and *MT-I* is significantly reduced after Oxr1 over-expression in neurons at P135 (Figure 5.7). While Oxr1 may mediate metal ion homeostasis in glia cells directly through crosstalk between motor neurons and glia during ALS progression, this marked decrease in *FTL* and *MT-I* expression is more likely due to strikingly reduced astrogliosis and microgliosis in $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice. Nonetheless, to distinguish between the two hypotheses, future experiments should be conducted to demonstrate whether glia cells in $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice express less *FTL* and *MT-I* than those in $SOD1^{G93A}$ ALS mice. Together, these data demonstrate that Oxr1 functions upstream of metal ion homeostasis during ALS disease progression.

5.3.5 Conclusions and future work

Future studies will explore the role of Oxr1 as a regulator of neuronal survival and integrate Oxr1 in the global antioxidant defence network. As previously noted, the use of whole spinal cord tissue allows for a good screen of global ALS disease pathways, but

makes it difficult to dissect the precise mechanisms by which Oxr1 functions. In particular, the large proliferation of activated microglia and astrocytes due to neuroinflammation may mask motor neuron-specific changes as previously noted (Ferraiuolo *et al.*, 2007; Heath *et al.*, 2013). Although a previous study has shown that Oxr1 functions upstream of catalase and glutathione peroxidase in *Anopheles gambiae* (Jaramillo-Gutierrez *et al.*, 2010), we did not identify OS-related pathways (Figure 5.1-5.3). Therefore, it may be important to conduct microarray analysis on microdissected motor neurons from lumbar spinal cord of $SOD1^{G93A}$/Tg(*Prnp-Oxr1*) mice to determine which gene expression changes within motor neurons are delayed to confer the neuroprotective effects of Oxr1, and identify novel regulators downstream of Oxr1 that modulate OS and the immune response.

Furthermore, it will be necessary to confirm putative binding partners of Oxr1 *in vivo* and determine mechanisms by which Oxr1 functions in concert with these binding partners to regulate the various downstream molecular pathways. In particular, Psmb10 and Epb41l3, a putative binding partner of Oxr1, may be the most interesting candidates due to their role in the immune response, thus linking Oxr1 function with the decrease in neuroinflammatory response during ALS disease progression (Jin *et al.*, 1991; Nandi *et al.*, 1996; Groettrup *et al.*, 1997). Because we chose to retain proteins that weakly bind to Oxr1, we used a less stringent buffer and relatively low number of wash steps, thus some of the putative Oxr1 binding partners that we identified in the brain and spinal cord may be false positives (Table 5.11-5.12). More stringent buffers and wash steps can be used prior to mass spectrometry to eliminate false positives and narrow the candidates for future study. Because the current Oxr1 antibody developed in the laboratory is unable to immunoprecipitate Oxr1 and its binding partners (Oliver *et al.*, 2011), we used an HA antibody to pull-down over-expressed Oxr1 in the brain and spinal cord, thus our data may not be representative of endogenous Oxr1. Future studies should also generate novel Oxr1

antibodies that are capable of immunoprecipitation to better understand Oxr1 function in an endogenous environment.

Finally, a meta-analysis of therapeutic strategies found that drugs targeting inflammation prior to onset of symptoms and antioxidant pathways at symptom onset are most effective in prolonging survival in mutant SOD1 mice (Benatar, 2007). The particular function of Oxr1 in regulating neuronal sensitivity to OS (Oliver *et al.*, 2011) and immune cell trafficking within the spinal cord demonstrates that Oxr1 is capable of addressing at least two important molecular pathways underlying ALS disease pathogenesis. Moreover, transcriptomics analysis suggests that over-expression of Oxr1 in neurons causes no significant gene expression changes (Table 5.2;5.6), and Oxr1 acts upstream of many diverse cellular pathways governing the maintenance of a healthy environment for neuronal survival (Figure 5.2). Here, we presented the first line of evidence that Oxr1 over-expression in neurons modifies SOD1-mediated ALS, Oxr1 functions upstream of the inflammatory response, and a neuronal antioxidant modulates cross-talk between neuroinflammation and OS during neurodegenerative disease pathogenesis, demonstrating the potential for Oxr1 to serve as a therapeutic target for ALS patients.

6 Characterisation of conditional deletion of *Oxr1* in motor neurons

6.1. Introduction

Motor neuron diseases encompass a number of different disorders, in which selective loss of motor neurons comprise the principal clinical feature (Talbot, 2002; James and Talbot, 2006; McDermott and Shaw, 2008; Andersen and Al-Chalabi, 2011). Amyotrophic lateral sclerosis (ALS), the most prevalent of motor neuron diseases, has an incidence of 5-7 per 100,000, but riluzole remains the only drug approved for ALS patients (Talbot, 2002; McDermott and Shaw, 2008). Riluzole provides only a modest extension of survival, and the benefits of riluzole in non-ALS motor neuron diseases are unknown (Talbot, 2002; Hardiman *et al.*, 2011). Unfortunately, molecular mechanisms that underlie the selective vulnerability of motor neurons remain unclear, but tremendous progress have been made in understanding pathogenic pathways by identifying genetic causes and modifiers of ALS and other motor neuron diseases, including *Cu,Zn-superoxide dismutase* (*SOD1*), *Fused in sarcoma* (*FUS*), and *transactive response DNA binding protein 43 kDa* (*TDP-43*) (Talbot, 2002; James and Talbot, 2006; McDermott and Shaw, 2008; Turner and Talbot, 2008; Andersen and Al-Chalabi, 2011; Ferraiuolo *et al.*, 2011; Riboldi *et al.*, 2011; Van Hoecke *et al.*, 2012; Seijffers *et al.*, 2014). Oxidative stress (OS) plays a central role in ALS pathogenesis, and has also been reported in other forms of motor neuron disease (Hayashi *et al.*, 2002; Atorino *et al.*, 2003; McDermott and Shaw, 2008; Ranganathan *et al.*, 2009; Andersen and Al-Chalabi, 2011; Ferraiuolo *et al.*, 2011).

Oxidation resistance 1 (Oxr1) is a highly conserved protein that is expressed throughout the central nervous system, and confers protection in neurons against OS-mediated apoptosis (Volkert *et al.*, 2000; Elliott and Volkert, 2004; Durand *et al.*, 2007; Oliver *et al.*, 2011; Murphy and Volkert, 2012). Deletion of *Oxr1* in *N*-ethyl-*N*-nitrosourea (ENU)-generated Bella (*bel*) mice causes severe ataxic gait, cerebellar neurodegeneration,

and failure to survive beyond postnatal day 26 (P26) (Oliver *et al.*, 2011). Oxr1 regulates neuronal sensitivity to exogenous peroxide-induced apoptosis *in vitro*, and the highly conserved TLDc domain of Oxr1 is sufficient to provide protection against OS damage through an unknown mechanism (Oliver *et al.*, 2011). Taken together, these findings provide evidence that Oxr1 plays an important role in OS-mediated neurodegeneration.

Interestingly, recent work suggests that Oxr1 function may be linked to motor neuron diseases, and in particular, ALS. Intermediate isoforms of OXR1 expression are up-regulated in the spinal cord of ALS patients, and the full-length isoform of Oxr1 (Oxr1-FL) is up-regulated in pre-symptomatic low-copy $SOD1^{G93A}$ ALS mice (Oliver *et al.*, 2011). In addition, Oxr1 interacts with FUS and TDP-43 (Finelli, 2010), and in Chapter 3, I found that Oxr1 regulates localisation of FUS and TDP-43 under OS through a pathway dependent of the TLDc domain and Protein arginine N-methyltransferase 1 (PRMT1) function. Previous work in the laboratory (Oliver *et al.*, 2011) and work in Chapter 4 showed that *Oxr1* is endogenously expressed specifically in motor neurons in the spinal cord (Figure 4.1). Moreover, in Chapter 4 and 5, we demonstrated that neuronal over-expression of Oxr1 significantly increases lifespan of $SOD1^{G93A}$ ALS mice. In particular, we showed that Oxr1 over-expression in neurons delays neuroinflammatory response, muscle pathology, and spinal cord pathology, including motor neuron apoptosis, during ALS pathogenesis. Therefore, we hypothesize that Oxr1 plays an important role in regulating motor neuron survival *in vivo* and that loss of Oxr1 in motor neurons may cause a motor neuron degenerative phenotype.

6.1.1 Aims of chapter 6

Oxr1 is critical for survival of cerebellar granule neurons *in vivo*, and while cortical neurons do not degenerate in *bel* mice, Oxr1 protects cortical neurons against exogenous

H_2O_2 stress *in vitro* (Oliver *et al.*, 2011). Due to the early postnatal death of *bel* mice (Oliver *et al.*, 2011), whether Oxr1 also plays an important role in protecting other neuronal populations *in vivo* remains unknown. In Chapter 3, I showed that Oxr1 regulates localisation of FUS and TDP-43 under OS *in vitro*, and in Chapter 4 and 5, we demonstrated that over-expression of Oxr1 delays pathogenesis in and extends survival of $SOD1^{G93A}$ ALS mice. Thus, the goal of this chapter was to determine whether Oxr1 is essential for motor neuron survival *in vivo* by characterising a mouse model with specific deletion of *Oxr1* in motor neurons.

6.2. Results

6.2.1 Generation of mouse line with an *Oxr1* conditional allele

Mice expressing solely the short isoform of Oxr1 (Oxr1-C), which contains the highly conserved TLDc domain, exhibit no abnormalities (Oliver *et al.*, 2011); therefore, to study deletion of Oxr1 in specific populations of neurons, we generated a mouse model that could conditionally delete two essential exons, 15 and 16, in the TLDc domain to ablate expression of both *Oxr1-FL* and *Oxr1-C* (Figure 6.1A). In particular, we obtained $Oxr1^{tm1a(EUCOMM)Wtsi}$ knockout-first conditional allele (tm1a) mice ($Oxr1^{tm1a/+}$) from the Wellcome Trust Sanger Institute using the gene targeting method as previously described (Figure 6.1B) (Skarnes *et al.*, 2011). $Oxr1^{tm1a/+}$ mice have a modified truncated Oxr1 allele that is generated from splicing to a lacZ trapping element with the commonly used mouse *Engrailed 2* splice acceptor element and SV40 polyadenylation sequences (Figure 6.1B) (Skarnes *et al.*, 1992; Mitchell *et al.*, 2001; Testa *et al.*, 2004; Skarnes *et al.*, 2011). To confirm that the null allele was properly targeted, we examined mice homozygous for the tm1a allele, $Oxr1^{tm1a/tm1a}$ mice. We found that similar to *bel* mice, $Oxr1^{tm1a/tm1a}$ mice fail to gain weight beginning around 2 weeks of age, develop an ataxic gait, and fail to survive

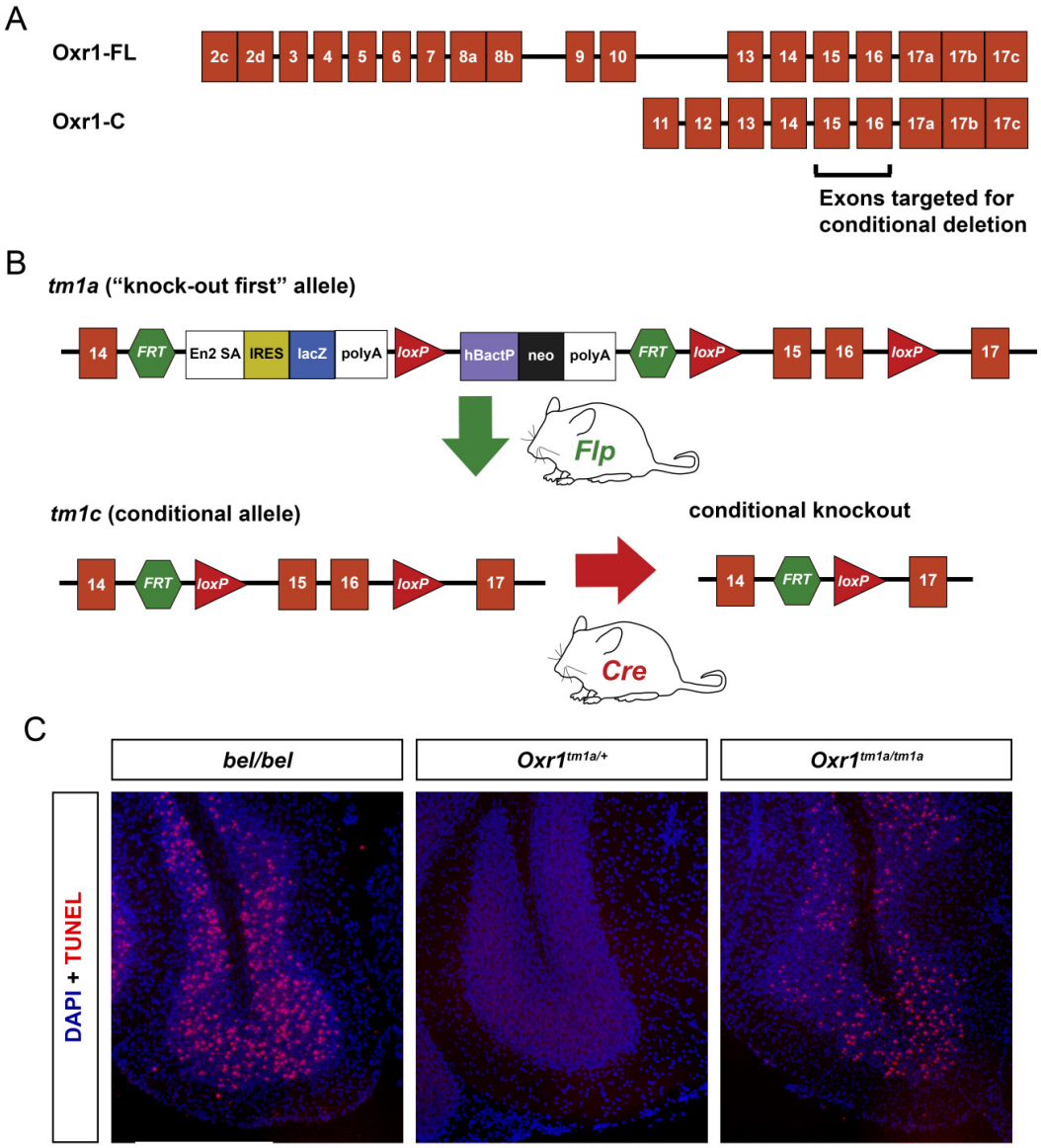

Figure 6.1. Generation of Oxr1 "knock-out first" conditional allele
(**A**) Schematic of Oxr1-FL and Oxr1-C isoforms, showing the locations of exons 15 and 16, which are targeted for the conditional construct to knock-out all isoforms of Oxr1. Note: not to scale. (**B**) Schematic of "knock-out first" allele (tm1a), which introduces a floxed promoter-driven neo cassette and an IRES-lacZ trapping cassette into the intronic region between exons 14 and 17 of Oxr1. Crossing the tm1a mouse line with a β-actin driven Flpe mouse line (Flp) converts the knock-out first allele into a conditional allele (tm1c). Mice expressing tm1c crossed with the appropriate promoter-driven Cre mouse line (Cre) will generate a conditional knock-mouse line, in which exons 15 and 16 of Oxr1 are deleted in a tissue-specific, cell-specific, or temporal-specific manner to generate a frameshift mutation that subsequently leads to nonsense mediated decay of the truncated transcript. FRT: flippase recognition target sites; En2 SA: mouse *engrailed 2* exon 2 splice acceptor; IRES: internal ribosome entry site; lacZ: *lacZ* reporter gene; polyA: SV40 poly-adenylation signal; LoxP: Locus of X-over P1 Cre recombinase target sites; hBactP: autonomous human β-actin promoter; and neo: neomycin resistance gene. (**C**) $Oxr1^{tm1a/tma1}$ mice show TUNEL-positive granule cells in the cerebellum when compared to $Oxr1^{tm1a/+}$, demonstrating that tm1a ablates Oxr1 function, and $Oxr1^{tm1a/tma1}$ mice exhibit the *bel* phenotype. (**B**) Scale bar: 400µm.

past the first month of age (data not shown) (Oliver *et al.*, 2011). Importantly, we confirmed that end-stage Oxr1$^{tm1a/tm1a}$ mice display cerebellar neurodegeneration in the granule cell layer, reminiscent of the *bel* phenotype (Figure 6.1C) (Oliver *et al.*, 2011). These data confirm loss of Oxr1 function in Oxr1$^{tm1a/tm1a}$ mice, and that deletion of *Muscle Activator of Rho Signalling* (*Abra*) is not responsible for the neuropathology observed in *bel* mice (Oliver *et al.*, 2011).

Next, we crossed Oxr1$^{tm1a/+}$ mice with Tg(ACTFLPe) mice, in which the human *β-actin* promoter drives FLPe variant recombinase to generate the conditional allele by removing the lacZ trapping and floxed promoter-driven neo cassette with flippase recombinase target sites (Figure 6.1B). The progeny of this cross have a conditional allele (tm1c), and are named Oxr1$^{tm1c/+}$ mice, which can be crossed with different Cre lines to excise exons 15 and 16, which are flanked by loxP sites, in a targeted manner (Figure 6.1B).

6.2.2 Generation of mouse model with specific deletion of *Oxr1* in motor neurons

To specifically delete *Oxr1* in motor neurons, we crossed Oxr1$^{tm1c/tm1c}$ mice with Choline acetyltransferase (ChAT)-Cre transgenic mice, in which Cre recombinase is conditionally expressed in motor neurons under the control of the bacterial artificial chromosome (BAC) containing the endogenous *Chat*-promoter (Gong *et al.*, 2007). The Cre recombinase transgene was always donated from the maternal lineage. First, we characterised ChAT-Cre transgenic mice to confirm specific Cre expression in ChAT-positive motor neurons by crossing ChAT-Cre transgenic mice with Rosa26 locus lacZ reporter (R26RlacZ) mice. The progeny of this cross, R26RlacZ/ChAT-Cre mice, express lacZ in neurons that express the *Cre* transgene. We found that Cre recombinase is specifically expressed in ChAT-postive neurons in the spinal cord, and facial nerve motor

nucleus, pedunculopontine tegmental nucleus, and medial habenula in the brain (Figure 6.2A-D). Next, we examined expression of *Oxr1* in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice by *in situ* hybridisation, and confirmed that *Oxr1* expression in spinal motor neurons is ablated in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice (Figure 6.2E-F). Taken together, these data establish a novel mouse model in which Oxr1 is specifically deleted in ChAT-positive motor neurons.

6.2.3 Specific deletion of *Oxr1* in motor neurons may affect rotarod performance in male mice

Given that loss of Oxr1 in *bel* mice leads to severe ataxic gait and failure to gain weight, we analysed weight, motor function, and muscle strength in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice. We observed no differences in survival for Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice when compared to +/ChAT-Cre mice and Oxr1$^{tm1c/tm1c}$/+ mice up to 1 year of age. In addition, we found no differences in weight for Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice when compared to +/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice at P365 (Figure 6.3A-B). To assess motor function, we used the accelerating rotarod to measure latency to fall for +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice. We found no significant differences in latency to fall for female Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice when compared to female +/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice at P365 (Figure 6.3C). Male Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice, on the other hand, have significantly reduced latency to fall when compared to male +/ChAT-Cre mice, but no difference was observed between male Oxr1$^{tm1c/tm1c}$/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice at P365 (Figure 6.3D). We then assessed muscle strength with grip strength monitor, and found no significant differences in muscle strength for +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice at P365 (Figure 6.3E-F). These results suggest that loss of Oxr1 specifically in motor neurons does not affect weight or muscle strength, but may affect motor function in male mice.

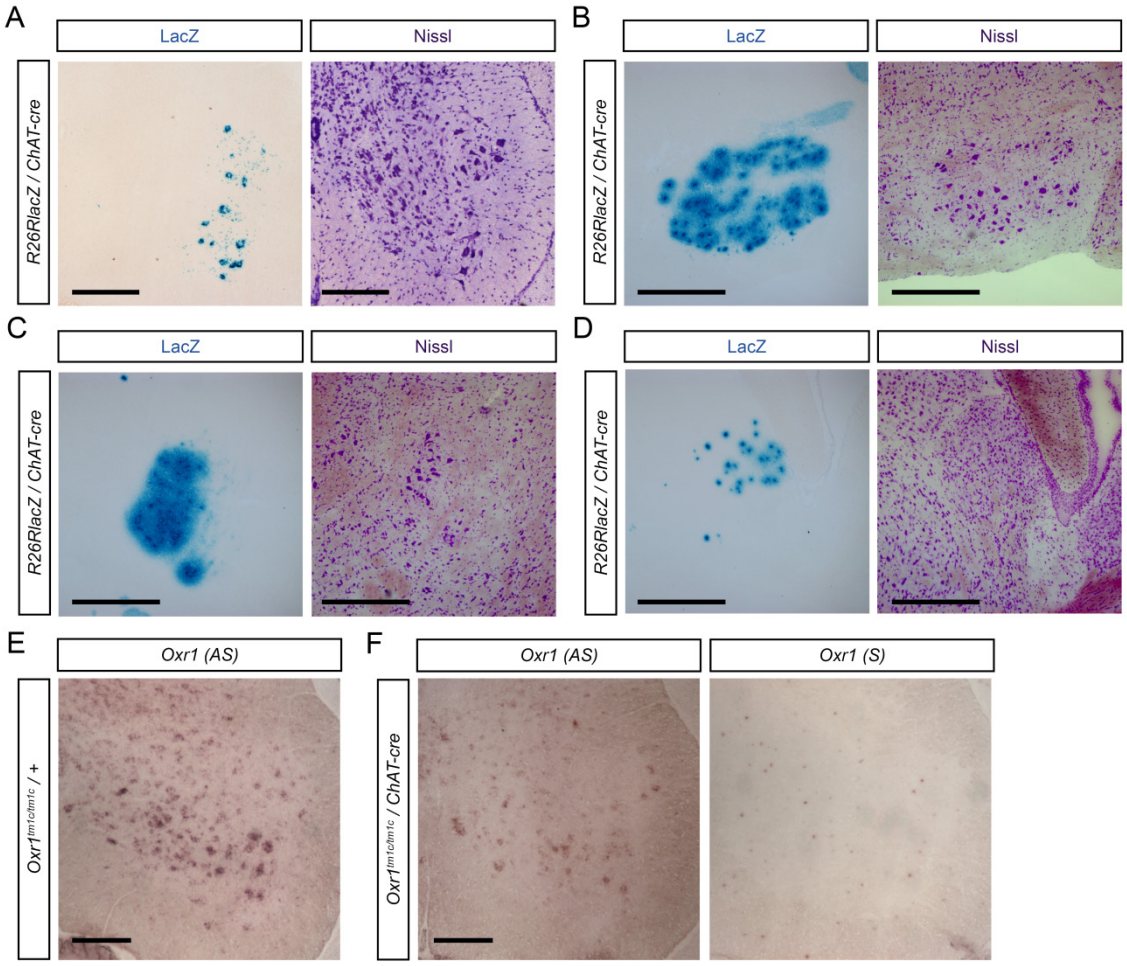

Figure 6.2. *Oxr1* expression in ChAT-positive motor neurons is specifically deleted in Oxr1$^{tm1c/tm1c}$/ChAT-cre mice

LacZ staining to show specific Cre activity in ChAT-positive motor neurons and in an adjacent section, nissl staining of motor neurons in the (**A**) spinal cord, (**B**) facial nerve motor nucleus, (**C**) pedunculopontine tegmental nucleus, and (**D**) medial habenula. (**A-D**) Scale bars: 500µm. (**E**) *In situ* hybridization with anti-sense labelling showing *Oxr1* mRNA expression in spinal motor neurons of Oxr1$^{tm1c/tm1c}$/+ mice. (**F**) *In situ* hybridization with anti-sense labelling showing loss of *Oxr1* expression in spinal motor neurons in Oxr1$^{tm1c/tm1c}$/ChAT-cre mice. (**E-F**) Scale bars: 250µm. LacZ staining was performed by Arran Babbs, nissl staining for (**A**) was conducted by Dr. Peter Oliver, nissl staining for (**B-D**) was conducted by me, and *in situ* hybridisation was performed by Dr. Peter Oliver.

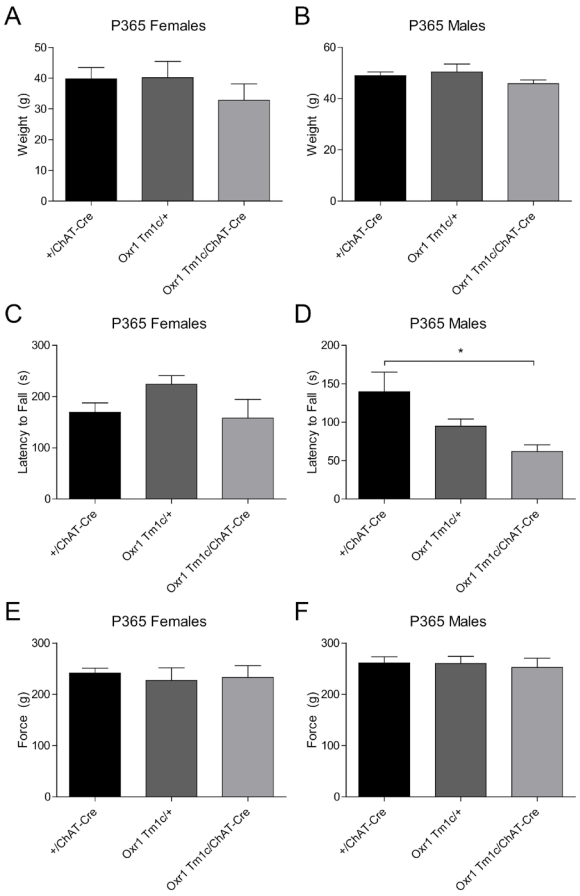

Figure 6.3. Specific loss of *Oxr1* in motor neurons may affect motor function in male mice at P365

No differences in weight of (**A**) female and (**B**) male +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+ (Oxr1 Tm1c/+), and Oxr1$^{tm1c/tm1c}$/ChAT-cre (Oxr1 Tm1c/ChAT-Cre) was observed. Rotarod analysis shows no difference in motor function for (**C**) female and (**D**) male Oxr1 Tm1c/ChAT-Cre mice when compared with +/ChAT-Cre or Oxr1 Tm1c/+ mice. Grip strength shows no difference in muscle strength for (**E**) female and (**F**) male Oxr1 Tm1c/ChAT-Cre mice when compared with +/ChAT-Cre or Oxr1 Tm1c/+ mice. (**A-F**) Values are mean ± SEM (n=3-6 for each genotype; Kruskal-Wallis 1-way ANOVA with Dunn's multiple comparison test).

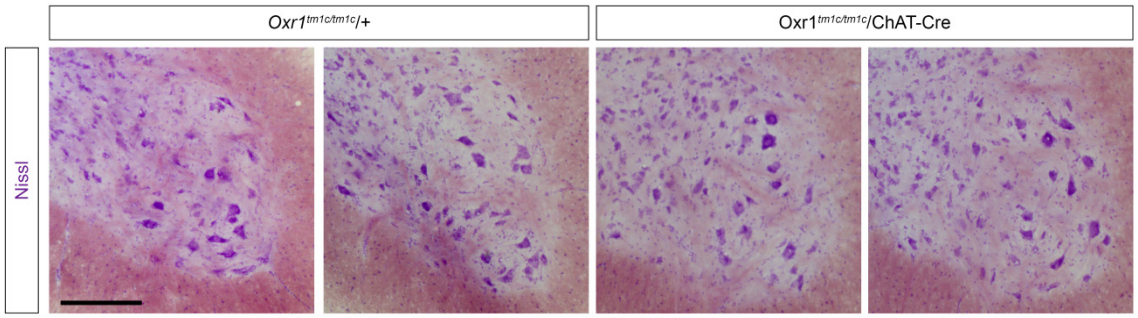

Figure 6.4. Specific deletion of *Oxr1* in motor neurons does not cause overt motor neuron degeneration at P365

No overt differences in motor neuron survival was observed by nissl staining of motor neurons in the ventral horn of matching lumbar spinal cord sections of Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-cre mice. Scale bar: 250µm.

6.2.4 Specific deletion of *Oxr1* in motor neurons does not cause overt neurodegeneration in the spinal cord

Deletion of *Oxr1* in *bel* mice causes cerebellar neurodegeneration, thus we assessed whether specific deletion of *Oxr1* in motor neurons affects survival of motor neurons in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice. We conducted nissl staining of matching lumbar spinal cord sections and observed no overt differences in motor neuron survival in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice when compared with Oxr1$^{tm1c/tm1c}$/+ mice at P365 (Figure 6.4). Furthermore, we observed no TUNEL staining in the lumbar spinal cord of Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice (Data not shown). Taken together, these findings suggest that selective loss of Oxr1 in motor neurons does not cause neurodgeneration in the spinal cord by P365.

6.2.5 Specific deletion of *Oxr1* in motor neurons may cause neuromuscular junction degeneration in soleus muscles of male mice

We also carried out histopathological analysis of muscle groups in +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice at P365. We found no significant differences in neuromuscular junction (NMJ) morphology of diaphragm, extensor digitorum longus (EDL), gastrocnemius, and tibialis anterior (TA) muscles of Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice when compared to +/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice (Figure 6.5A-C,E-H,J). Interestingly, while no significant differences in NMJ morphology of soleus muscles of female +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice were observed, NMJ morphology of soleus muscles in male Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice is significantly affected when compared to that of male +/ChAT-Cre mice (Figure 6.5D,I). No significant difference was observed between soleus NMJ morphology in Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice (Figure 6.5I). Furthermore, we found no

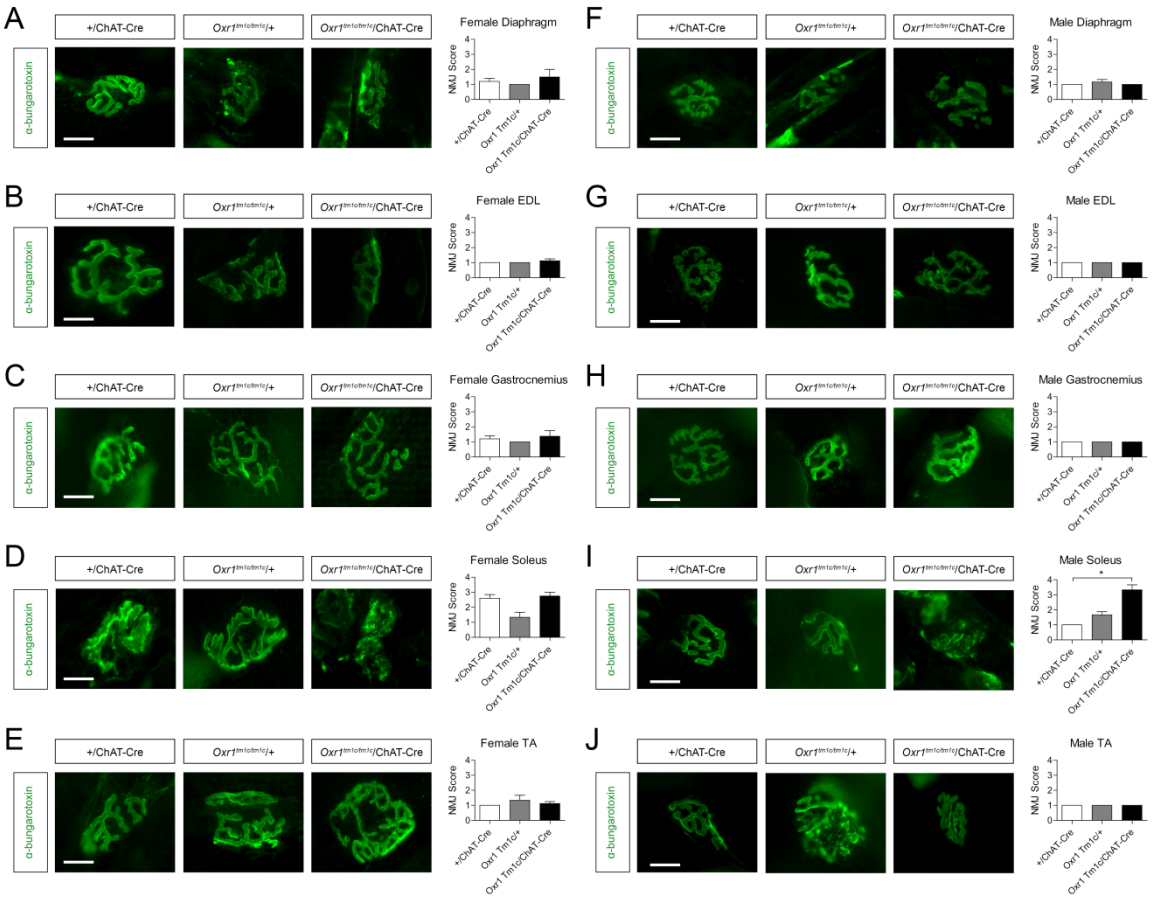

Figure 6.5. Specific loss of *Oxr1* in motor neurons may affect soleus neuromuscular junction morphology in male mice at P365

No differences in neuromuscular junction (NMJ) morphology were observed in female +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+ (Oxr1 Tm1c/+), and Oxr1$^{tm1c/tm1c}$/ChAT-cre (Oxr1 Tm1c/ChAT-Cre) mice for (**A**) diaphragm, (**B**) EDL, (**C**) gastrocnemius, (**D**) soleus, and (**E**) TA muscles. No differences in NMJ morphology were observed in male +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-cre mice for (**F**) diaphragm, (**G**) EDL, (**H**) gastrocnemius, and (**J**) TA muscles. (**I**) Soleus NMJ morphology for Oxr1$^{tm1c/tm1c}$/ChAT-cre mice is significantly worse than that of male +/ChAT-Cre controls, but no differences in soleus NMJ morphology were observed between male Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-cre mice. (**A-J**) Values are mean ± SEM (n=3-4 for each genotype; Kruskal-Wallis 1-way ANOVA with Dunn's multiple comparison test). (**A-J**) Scale bars: 25µm. NMJ processing was conducted together with Ben Edwards and NMJ scoring was conducted by Ben Edwards.

muscle atrophy in diaphragm, EDL, gastrocnemius, soleus, and TA muscles of male Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice when compared to +/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice (Figure 6.6). Finally, we also observed no significant differences in muscle fibre composition in diaphragm, EDL, gastrocnemius, soleus, and TA muscles of male Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice when compared to +/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice (Figure 6.7). Interestingly, soleus muscles of male Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice exhibit grouping of muscle fibres when compared to +/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice, suggesting that muscle denervation and subsequent reinnervation have occurred (Figure 6.7A) (Goebel *et al.*). Taken together, these data suggest that loss of Oxr1 in motor neurons may specifically affect soleus muscle NMJ morphology, and cause denervation and reinnervation of soleus muscles.

6.2.6 Loss of *Oxr1* causes neuroinflammation

Given that we found that Oxr1 delays neuroinflammation during ALS pathogenesis in Chapter 5, we chose to investigate whether deletion of *Oxr1* also leads to inflammatory response by immunocytochemical labelling for Glial fibrillary acidic protein (GFAP), a marker for astrogliosis, and Cluster of differentiation 68 (CD68), a marker for microgliosis (Bignami *et al.*, 1972; Hatfield and Skoff, 1982; Hulette *et al.*, 1992; Muhleisen *et al.*, 1995). We observed astrogliosis in the lumbar spinal cord of both Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice, but levels of activated astrocytes did not appear to be different between Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice (Figure 6.8A). In addition, we observed a small number of CD68-positive activated microglia in the lumbar spinal cord of Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice, and no activated microglia in the lumbar spinal cord of Oxr1$^{tm1c/tm1c}$/+ mice.

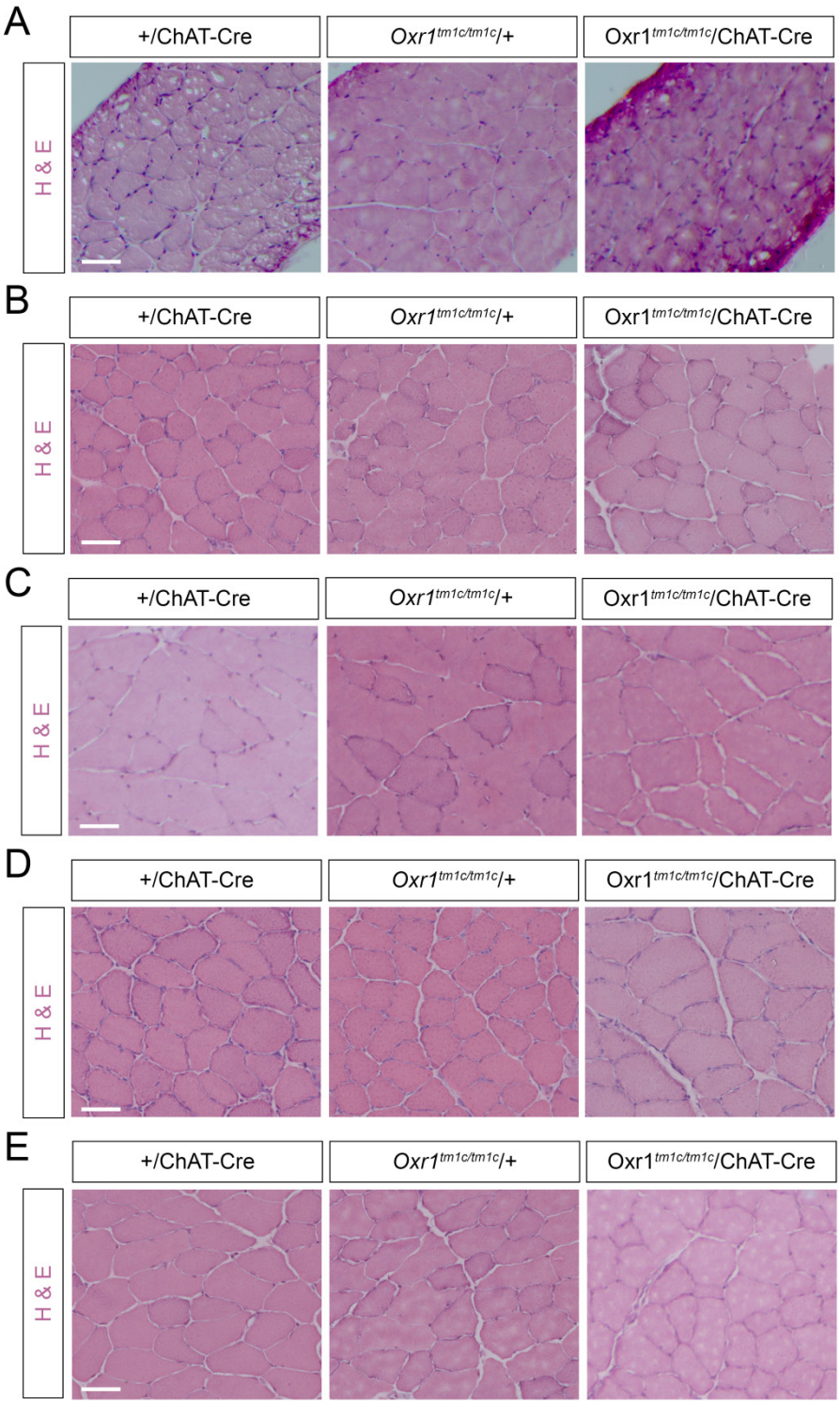

Figure 6.6. Specific loss of *Oxr1* in motor neurons does not cause muscle group atrophy at P365

Representative images of H&E stained muscles in male +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-cre mice showing no muscle atrophy for (**A**) diaphragm, (**B**) EDL, (**C**) gastrocnemius, (**D**) soleus, and (**E**) TA muscles. (**A-E**) Scale bars: 50µm. Muscle processing was conducted together with Ben Edwards and Muscle images were taken by Ben Edwards.

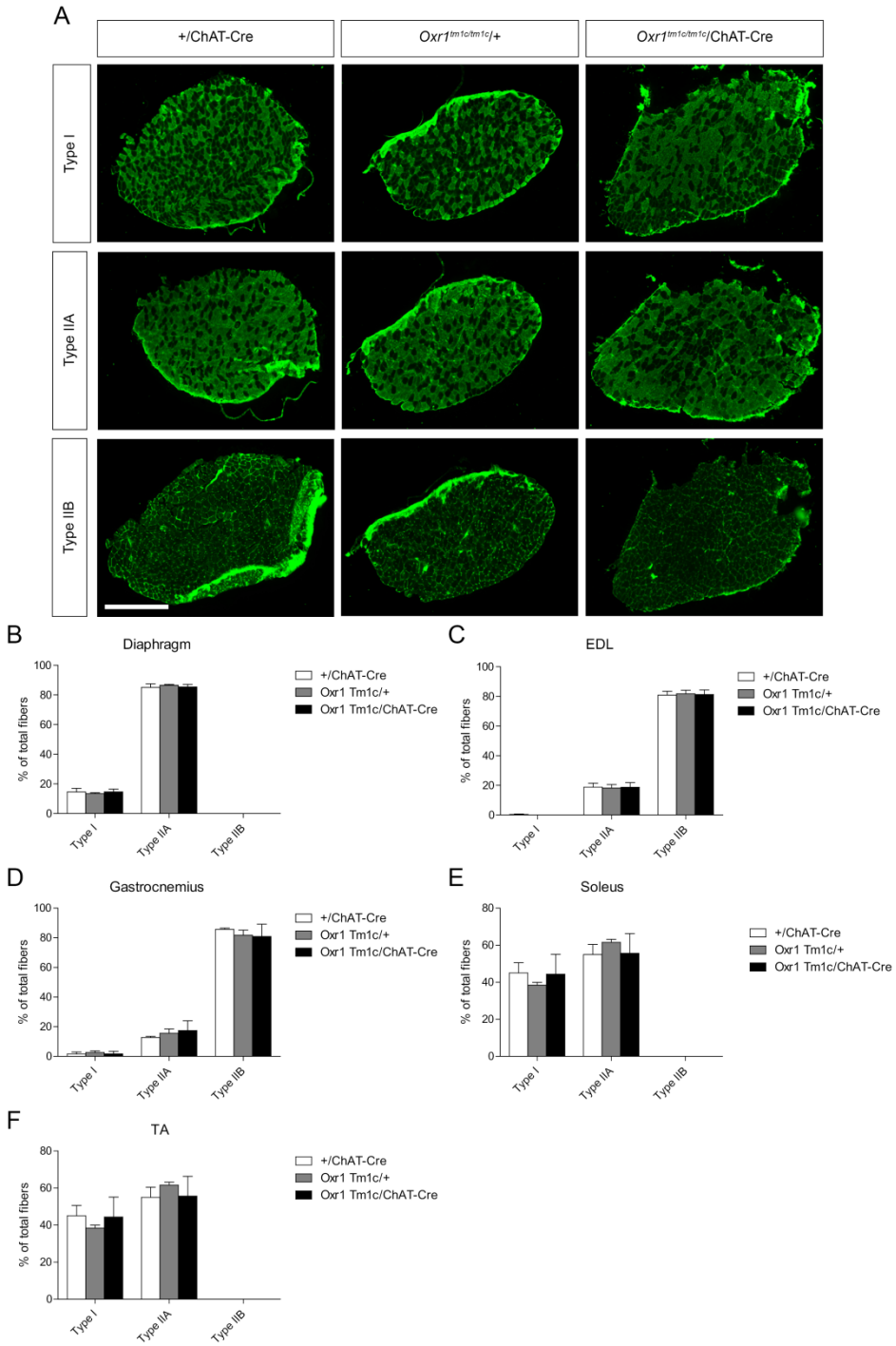

Figure 6.7. Specific loss of *Oxr1* in motor neurons does not alter muscle fiber composition at P365

(**A**) Representative images of Type I, IIA, and IIB muscle fibres in the soleus muscle of male +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-cre mice. Scale bar: 500µm. Quantification of muscle fiber type composition for (**B**) diaphragm, (**C**) EDL, (**D**) gastrocnemius, (**E**) soleus, and (**F**) TA muscles showing no differences between male +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+ (Oxr1 Tm1c/+), and Oxr1$^{tm1c/tm1c}$/ChAT-cre (Oxr1 Tm1c/ChAT-Cre) mice. (**B-F**) Values are mean ± SEM (n=3 for each genotype; 1-way ANOVA with Tukey's post-hoc test).

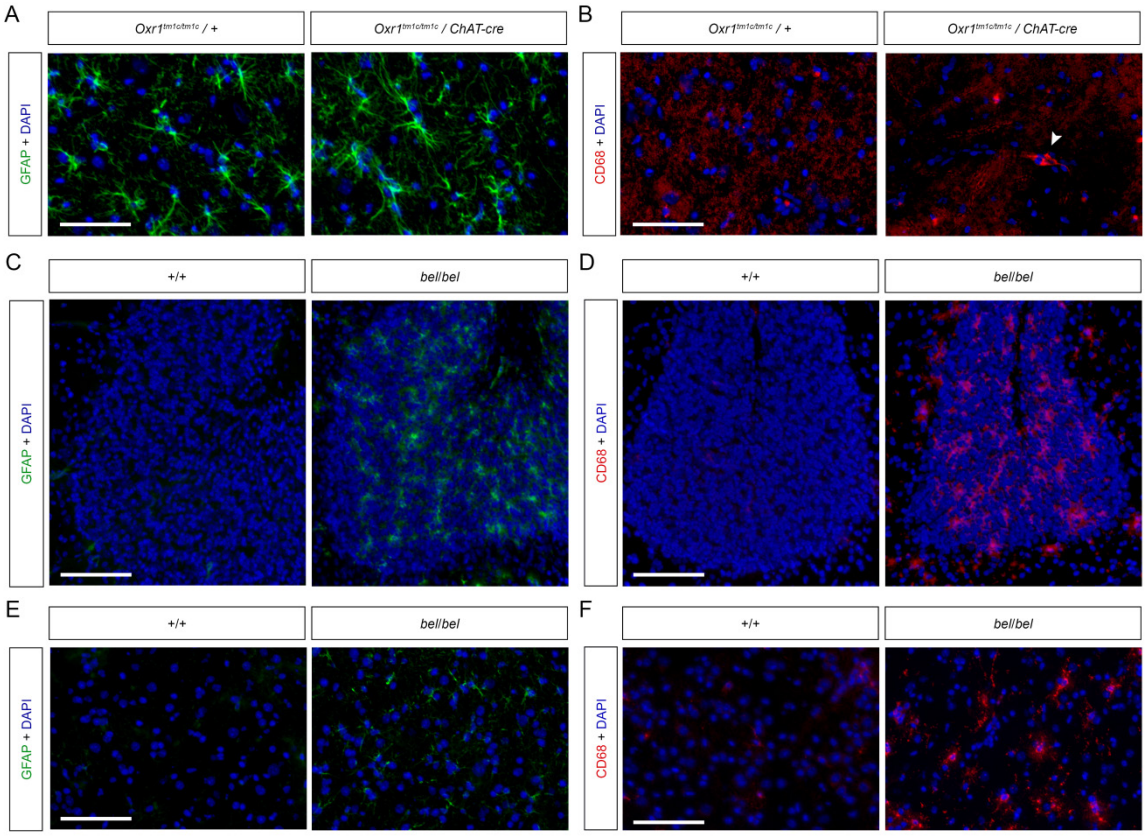

Figure 6.8. Loss of *Oxr1* results in neuroinflammation
(**A**) Immunocytochemical staining for GFAP on sections of lumbar spinal cord reveals astrogliosis in both Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-cre mice at P365. (**B**) Immunocytochemical staining for CD68 on sections of lumbar spinal cord reveals some activated microglia (arrow) in P365 Oxr1$^{tm1c/tm1c}$/ChAT-cre mice. (**C**) Immunocytochemical staining for GFAP on sections of spinal cord reveals astrogliosis in end-stage *bella* mice. (**D**) Immunocytochemical staining for CD68 on sections of spinal cord reveals microgliosis in end-stage *bella* mice. (**C**) Immunocytochemical staining for GFAP on sections of cerebellum reveals astrogliosis in end-stage *bella* mice (*bel*/*bel*). (**D**) Immunocytochemical staining for CD68 on sections of cerebellum reveals microgliosis in end-stage *bella* mice. (**E**) Immunocytochemical staining for GFAP on sections of spinal cord reveals astrogliosis in end-stage *bella* mice. (**F**) Immunocytochemical staining for CD68 on sections of spinal cord reveals microgliosis in end-stage *bella* mice. (**A-F**) Scale bars: 100µm.

Because these findings in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice may be masked by age-related changes and by selective deletion of *Oxr1* from a limited neuronal population, we also examined whether neuroinflammation occurs in constitutive Oxr1 null tissue from end-stage *bel* mice. Interestingly, we found a marked increase in GFAP-positive activated astrocytes and CD-68-positive activated microglia in the cerebellum and in the spinal cord of *bel* mice (Figure 6.8E-F). Taken together, these results demonstrate that Oxr1 plays a central role in the neuroinflammatory response.

6.3 Discussion

Here, we characterised a mouse model in which *Oxr1* is specifically deleted in motor neurons, and found that specific loss of Oxr1 in ChAT-positive motor neurons is not sufficient to cause neurodegeneration by P365. In particular, Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice exhibit no overt pathology, limited muscle pathology, and no differences in survival up to 1 year of age when compared to +/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice. Interestingly, we found that constitutive loss of Oxr1 leads to neuroinflammation with astrogliosis and microgliosis in the cerebellum and spinal cord of end-stage *bel* mice.

Genetic causes have been discovered for motor neuron diseases, including ALS, through genetic sequencing of human patients and study of various mouse models (James and Talbot, 2006; Wroe *et al.*, 2008; Lemmens *et al.*, 2010; Andersen and Al-Chalabi, 2011; Van Den Bosch, 2011; Rademakers and van Blitterswijk, 2013). The genes associated with ALS and other lower motor neuron diseases encode proteins that can be grouped into several functional categories, including antioxidant defence (Heat shock protein 27 (Hsp27), heat shock 22kDa protein 8 (HSPB8), and SOD1)); axonal transport (kinesin-associated protein 3 (KIFAP3), dynactin 1 (DCTN1), and neurofilament, heavy polypeptide)); RNA processing (FUS, TDP-43, angiogenin, senataxin, TATA-binding

protein-associated factor 2N (TAF15), elongator acetyltransferase complex subunit 3 (ELP3), glycyl-tRNA synthetase (GARS), immunoglobulin mu binding protein 2 (IGHMBP2), survival motor neuron (SMN), exosome component 3 (EXOSC3), and chromosome 9 open reading frame 72 (C9ORF72)); ubiquitin-proteasome system (Ubiquilin 2 and valosin-containing protein (VCP)), and vesicle trafficking (Unc-13 Homolog A (C. Elegans) (UNC13A), Polyphosphoinositide phosphatase (FIG4), and Vesicle-Associated Membrane Protein-Associated Protein B And C (VAPB)) (James and Talbot, 2006; Lemmens *et al.*, 2010; Andersen and Al-Chalabi, 2011; Wan *et al.*, 2012). Interestingly, Oxr1 function has been associated with many of these pathways, such as antioxidant defence, axonal transport, RNA processing, and ubiquitin-proteasome system (Figure 5.1;5.3) (Finelli, 2010; Oliver *et al.*, 2011). No mutations in *OXR1* have been associated with motor neuron diseases; however, given Oxr1's role in modifying $SOD1^{G93A}$ disease progression *in vivo*, future studies should examine whether mutations in *OXR1* affect survival of ALS patients and lead to motor neuron diseases (Figure 4.4)

Many mouse models of motor neuron diseases also harbour mutations in human disease-associated genes or genes involved in motor neuron diseases-associated pathways. For example, many ALS mouse models have been developed by introducing disease-associated mutations in DCTN1, FUS, SOD1, TDP-43, VAPB, and VCP (Van Den Bosch, 2011). Mutant DCTN1, FUS, SOD1, TDP-43, and VCP mice all recapitulate many phenotypes of ALS patients, including degeneration of motor neurons and motor deficits (Gurney *et al.*, 1994; Ripps *et al.*, 1995; Bruijn *et al.*, 1997; Liu *et al.*, 2005; Laird *et al.*, 2008; Johnson *et al.*, 2010; Acevedo-Arozena *et al.*, 2011; Kariya *et al.*, 2012; Yin *et al.*, 2012; Arnold *et al.*, 2013; Qiu *et al.*, 2014). Interestingly, expression of ALS-associated $VAPB^{P56S}$ mutant *in vivo* does not alter survival, and causes a corticospinal motor neuron-mediated motor phenotype when mutant $VAPB^{P56S}$ expressed approximately 8-fold (Tudor

et al., 2010; Aliaga *et al.*, 2013; Qiu *et al.*, 2013). In addition, TDP-43 cytoplasmic aggregation was observed in motor neurons at 18 months of age in one $VAPB^{P56S}$ ALS model (Tudor *et al.*, 2010). Furthermore, motor neuron degeneration, muscle atrophy, and motor deficits are phenotypes of several spontaneous or induced mouse models of motor neuron diseases. These mouse models include: the wobbler mouse, which has an autosomal recessive mutation in vacuolar protein sorting 54 (VSP54), a protein involved in vesicle trafficking (Duchen and Strich, 1968; Ishiyama *et al.*, 2004; Schmitt-John *et al.*, 2005); the neuromuscular degeneration (nmd) mouse, which has two independent mutations in *Ighmbp2*, an RNA-binding protein (Cook *et al.*, 1995; Cox *et al.*, 1998; Grohmann *et al.*, 2004); the progressive motor neuronopathy (pmn) mouse, which is homozygous for a missense mutation in *tubulin folding cofactor E* (*TBCE*), which encodes a tubulin-specific chaperone protein that is important for axonal transport and maintenance (Schmalbruch *et al.*, 1991; Martin *et al.*, 2002; Ferri *et al.*, 2003); the wasted (wst) mutant, which harbours a spontaneous autosomal recessive mutation that ablates expression of *eukaryotic translation elongation factor 1 alpha 2* (*eEF1A2*), an important translation factor during RNA processing (Chambers *et al.*, 1998; Newbery *et al.*, 2005); and the legs at odd angles (Loa) and Cramping 1 (Cra1) mutants, which harbour missense mutations in *cytoplasmic dynein heavy chain 1* (Rogers *et al.*, 2001; Hafezparast *et al.*, 2003). Interestingly, mutations in Vascular endothelial growth factor (VEGF), loss of ciliary neurotrophic factor receptor (CNTFR), and impaired function of DNA repair protein excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC-1) also cause a motor neuron degenerative phenotype in mice (DeChiara *et al.*, 1995; Oosthuyse *et al.*, 2001; Lee *et al.*, 2008; de Waard *et al.*, 2010).

In contrast to these studies, we found that $Oxr1^{tm1c/tm1c}$/ChAT-Cre mice present very little pathology. No changes in weight loss, muscle strength, motor function deficits

and lifespan were observed (Figure 6.3). Male Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice have a shorter latency to fall than male +/ChAT-Cre mice, but the difference between male Oxr1$^{tm1c/tm1c}$/ChAT-Cre and Oxr1$^{tm1c/tm1c}$/+ mice is not statistically significant (Figure 6.3D). This result may be due to the small number of mice analysed, thus, future studies should examine more Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice at 1 year and at time points beyond 1 year to confirm the lack of behavioural pathology. In addition, more detailed gait analysis might be necessary to identify possible subtle motor behavioural deficits, thus use of a system such as the Catwalk quantitative gait analysis test could provide important insight (Hamers *et al.*, 2006; Vandeputte *et al.*, 2010).

Moreover, we observed no motor neuron degeneration (Figure 6.4), suggesting not enough of Oxr1$^{tm1c/tm1c}$/ChAT-Cre motor neurons may have accumulated OS damage in the absence of Oxr1 by 1 year of age to exhibit any pathology or render behavioural deficits. Thus, Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice may require further aging to reach a threshold for abnormal phenotypes to be apparent. Furthermore, given that we demonstrated in Chapter 3 that Oxr1 regulates cytoplasmic localisation of FUS and TDP-43 *in vitro*, future work should investigate FUS and TDP-43 localisation in Oxr1$^{tm1c/tm1c}$/ChAT-Cre motor neurons. In addition, work is still needed to confirm that specific deletion of *Oxr1* in motor neurons does not result in neurodegeneration, such as counting neurons in the brain and spinal cord of +/ChAT-Cre mice to control for any differences due to the ChAT promoter-driven *Cre* transgene expression. Interestingly, recent research also identified whisking behaviours that are associated with changes in facial nuclei in SOD1^{G93A} ALS mice, and pathology and degeneration of motor neurons of facial nuclei has been observed in many models of motor neuron diseases (DeChiara *et al.*, 1995; Bordet *et al.*, 1999; Clowry and McHanwell, 2004; Lee *et al.*, 2008; Grant *et al.*, 2014), thus future work should examine

pathology of motor neurons in other areas of Oxr1$^{tm1c/tm1c}$/ChAT-Cre brains and whether whisking behaviours are perturbed in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice.

Interestingly, we identified abnormal soleus NMJ morphology and fibre type grouping, activated microglia in Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice, and increased astrogliosis and microgliosis in the spinal cord of *bel* mice (Figure 6.6;6.7-6.8). NMJ morphology of soleus muscles in male Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice is significantly affected when compared to that of male +/ChAT-Cre mice, but not when compared with Oxr1$^{tm1c/tm1c}$/+ mice (Figure 6.5I). This finding may be due to the small number of biological replicates, thus examining soleus NMJ morphology in more +/ChAT-Cre, Oxr1$^{tm1c/tm1c}$/+, and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice is necessary. Nonetheless, the abnormal soleus NMJ morphology in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice may explain the changes in muscle fibre grouping in the soleus muscle; however, the abnormal NMJ morphology does not affect soleus muscle atrophy (Figure 6.5-6.7). These results suggest that loss of Oxr1 may modulate, but is not essential to, soleus muscle function. Future studies should examine whether physiological properties of soleus muscles in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice are altered by assessing for tetanic force, specific force, and work (Dobrowolny *et al.*, 2008). In addition, we should examine whether Oxr1 is expressed even at low amounts within different muscle groups, which may explain the lack of pathology.

Furthermore, neuroinflammation in the spinal cord of *bel* mice and infiltration of activated microglia in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice are evidence of neuronal dysfunction (Figure 6.8), and further support evidence from Chapter 5 that Oxr1 plays an important role in regulating the immune response. Neuroinflammation is a central component of ALS, and has also been observed in the wobbler and wasted mutants (Hantaz-Ambroise *et al.*, 2001; Newbery *et al.*, 2005; Bowerman *et al.*, 2013). Astrogliosis in both Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice is likely to be age-related as previously

noted (Yin *et al.*, 2012); however, these findings may mask subtle changes of astrogliosis in Oxr1$^{tm1c/tm1c}$/+ and Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice as only small numbers of activated microglia were found in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice (figure 6.8A-B). Furthermore, aging Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice past P365 may induce greater levels of astrogliosis and microgliosis as age-related damage might be necessary to induce a phenotype (Yin *et al.*, 2012). Unfortunately, the mechanisms by which Oxr1 modulates the immune response remain unclear. Quantitative mass spectrometry-based proteomics approach could glean insight into this particular function of Oxr1 by examining differential protein expression between neurons with or without *Oxr1* to identify important signalling proteins that are essential to the immune response and regulated by Oxr1.

The lack of overt pathology in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice is not unsurprising since loss of ALS-associated Alsin also causes no motor neuron degeneration and no differences in survival (Cai *et al.*, 2005; Devon *et al.*, 2006; Hadano *et al.*, 2006; Yamanaka *et al.*, 2006; Tudor *et al.*, 2010; Aliaga *et al.*, 2013). Interestingly, loss of Alsin renders neurons more sensitivity to OS-induced apoptosis, and causes alterations of vesicle trafficking, and decreased Rab5-dependent endosome fusion activity *in vitro* (Cai *et al.*, 2005; Devon *et al.*, 2006; Hadano *et al.*, 2006). In addition, *in vitro* experiments demonstrated that in the absence of *Oxr1*, cortical neurons are significantly more susceptible to exogenous peroxide-induced apoptosis even though no loss of cortical neurons were observed in *bel* mice (Oliver *et al.*, 2011). Thus, primary culture of Oxr1-deficient motor neurons may provide additional understanding of Oxr1 function by assessing whether motor neurons without *Oxr1* are more susceptible to exogenous stresses, such as H_2O_2-mediated OS, tunicamycin-mediated unfolded protein response, and AMPA-mediated excitotoxicity (Kieran *et al.*, 2007; Oliver *et al.*, 2011). Furthermore, loss of Oxr1 may not be sufficient to cause a neurodegenerative phenotype, but loss of Oxr1 could

still render motor neurons more susceptible to apoptosis or damage during disease pathogenesis. To test this hypothesis, future studies should cross Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice with SOD1^{G93A} ALS mice or other models of motor neuron diseases, and examine whether survival is significantly shortened in the absence of *Oxr1*. SOD1-deficient mice develop an adult-onset progressive motor axonopathy, but fail to exhibit motor neuron degeneration, further corroborating the theory that ALS mutant SOD1 injures motor neurons through a toxic gain-of-function (Reaume *et al.*, 1996; Flood *et al.*, 1999; Muller *et al.*, 2006; Fischer *et al.*, 2012). Similar to SOD1, loss of Oxr1 may not provide detrimental effects, but a toxic gain-of-function mutation in *Oxr1* could cause deficits in motor neuron survival, thus future work to screen ENU mutagenesis archives for mice harbouring mutations in *Oxr1* to investigate this alternative hypothesis.

Here, we presented a preliminary characterisation of Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice to examine the effect of *Oxr1* deletion specifically in motor neurons. While we only identified abnormal soleus muscle NMJ morphology, soleus muscle fibre grouping, and some activated microglia in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice, more detailed analysis in the future will continue to elucidate mechanisms of Oxr1 function, particularly in regulating neuronal survival. Given that over-expression of Oxr1 delays pathogenesis in SOD1^{G93A} ALS mice, Oxr1 may serve as a therapeutic target. Thus, understanding Oxr1 function *in vivo* and particularly, in motor neurons, will prove crucial.

7 General Discussion

7.1 Summary and Future Directions

In this thesis, I described molecular mechanisms of the antioxidant Oxidation Resistance 1 (Oxr1) in conferring protection against oxidative stress, particularly focusing on the role of Oxr1 in modulating neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS). In Chapter 3, I found that Oxr1 regulates localisation of ALS-associated proteins Fused in Sarcoma (FUS) and transactive response DNA binding protein 43 kDa (TDP-43) to stress granules through function of the TLDc domain and a Protein arginine N-methyltransferase 1 (PRMT1)-dependent manner under oxidative stress (OS). In Chapter 4, I demonstrated that neuronal up-regulation of Oxr1 is protective *in vivo*, and extends survival of and ameliorates pathology in $SOD1^{G93A}$ ALS mice. In Chapter 5, I investigated downstream pathways of Oxr1 during ALS disease progression, revealing a role of Oxr1 in regulating neuroinflammation, and identifying putative binding partners of Oxr1 in the brain and spinal cord. In Chapter 6, I characterised a mouse model in which *Oxr1* is specifically knocked-out in Choline acetyltransferase (ChAT)-positive motor neurons. Together, this work established Oxr1 as a critical regulator of neuronal survival *in vivo*, and a genetic modifier of disease progression in an ALS mouse model, demonstrating the potential for OXR1 to serve as a therapeutic target in ALS and possibly other neurodegenerative diseases.

7.2 Function of Oxr1 and the TLDc domain

Because OS plays a central role in neurodegenerative disorders, identifying novel antioxidants is important in furthering our understanding of the complex antioxidant defence network and informing the development of future therapies for these diseases. Highly conserved among eukaryotes, Oxr1 is expressed throughout the central nervous

system (CNS) and contains a highly conserved TLDc domain (Volkert *et al.*, 2000; Oliver *et al.*, 2011). Induced by OS, Oxr1 is localised to the mitochondria and protects cells from ROS-induced damage; however, Oxr1 has also been shown to translocate to the cytoplasm after extended periods of stress (Volkert *et al.*, 2000; Elliott and Volkert, 2004; Finelli, 2010; Oliver *et al.*, 2011; Murphy and Volkert, 2012). My work in Chapter 4 revealed that *Oxr1* is expressed specifically in neurons within the CNS (Figure 4.1). In addition, I demonstrated in Chapter 3 and 4 that over-expression of full-length Oxr1 isoform (Oxr1-FL) and Oxr1 short isoform (Oxr1-C) *in vitro* and *in vivo* leads to Oxr1 localisation in the cytoplasm (Figure 3.6; 4.1). Taken together, these results suggest that Oxr1 is a neuron-specific antioxidant and the protective function of Oxr1 may occur primarily in the cytoplasm. Function of Oxr1 in mitochondria is currently being studied within the laboratory, and it will be important to determine whether Oxr1 tagged to the mitochondria similarly protects against OS-induced cell death both *in vitro* and *in vivo*.

OXR1 is induced by OS, and protects cells from reactive oxygen species (ROS)-induced damage through the function of its TLDc domain (Volkert *et al.*, 2000; Elliott and Volkert, 2004; Oliver *et al.*, 2011; Li *et al.*, 2012; Murphy and Volkert, 2012). Loss of *Oxr1* in an *N*-ethyl-*N*-nitrosourea (ENU)-generated Bella (*bel*) mouse mutant results in severe ataxia, growth retardation, and early postnatal death (Oliver *et al.*, 2011). Furthermore, deletion of *Oxr1* results in significant oxidative damage and decreased survival in the granule cell layer of the cerebellum (Oliver *et al.*, 2011). Interestingly, the phenotype of *bel* mice is rescued by expression of Oxr1-C, which only consists of the TLDc domain, *in vivo* (Oliver *et al.*, 2011). In Chapter 6, I showed that selective deletion of *Oxr1* in motor neurons is not sufficient to lead to neurodegeneration in the spinal cord, but may cause some abnormalities in fibre grouping and neuromuscular junction (NMJ) morphology of the soleus muscle. While more work is needed to fully characterise

Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice, these findings suggest that Oxr1 may be necessary for some neuronal populations, such as cerebellar granule cells, and not for others, such as ChAT-positive motor neurons in the spinal cord. Cerebellar granule cells have previously been identified as more susceptible to OS-mediated damage than cortical or hippocampal neurons (Wang and Michaelis, 2010), which may explain the lack of neurodegeneration in Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice. In the future, it may be important to age Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice longer to determine whether greater accumulation of age-related damage may induce a phenotype or introduce OS *in vivo* by crossing Oxr1$^{tm1c/tm1c}$/ChAT-Cre mice with ALS mutant mice to assess whether loss of Oxr1 renders motor neurons more susceptible to OS-mediated apoptosis. Finally, the laboratory is currently investigating whether *Oxr1* expression is necessary for dopamingeric neuron survival by crossing Oxr1$^{tm1c/tm1c}$/+ mice with a dopamine transporter-Cre mouse line.

Furthermore, Oxr1 has been suggested to regulate downstream pathways, particularly involving antioxidant pathways, to protect cells from ROS-induced damage. Oxr1 directly reacts with H_2O_2 through oxidation of Cys753 in the TLDc domain similar to that of DJ-1, but does not act like an ROS enzymatic scavenger (Andres-Mateos *et al.*, 2007; Lev *et al.*, 2009; Morimoto *et al.*, 2010; Oliver *et al.*, 2011; Wilson, 2011). While few differences in the expression of known antioxidants were observed in *bel* mice (Oliver *et al.*, 2011), recent research suggests that Oxr1 participates in antioxidant pathways. In *Anopheles gambiae*, Oxr1 functions downstream of Jun N-terminal kinase, and upstream of catalase and glutathione peroxidase (Jaramillo-Gutierrez *et al.*, 2010). Similarly, transplantation of mesenchymal stem cells over-expression OXR1 into the kidney of a mouse model of systemic lupus erythematosus down-regulated nitric oxide in the serum and urine, decreased tubular cell apoptosis, and increased catalase and glutathione peroxidase 1 (Gpx1) (Li *et al.*, 2013). In Chapter 5, however, we found no significant gene

expression changes under Oxr1 over-expression in neurons during ALS disease progression. These findings are likely due to the use of whole lumbar spinal cord for the microarray analysis, which may mask gene changes in a subset of the cells, which in this case are neurons. Future studies using micro-dissected spinal motor neurons may discern whether Oxr1 over-expression increases expression of any genes in the antioxidant pathway, such as *catalase* or *Gpx1*.

Interestingly, recent studies have also implicated Oxr1 in regulating immune pathways. A gain of function mutation in mustard, ortholog of Oxr1 in *Drosophila*, regulates aspects of the immune deficiency pathway to provide protection against *Vibrio cholera* infection (Wang *et al.*, 2012). In addition, over-expression of OXR1 in transplanted mesenchymal stem cells in kidneys of a mouse model of lupus nephritis significantly reduced inflammation and expression of inflammatory markers, such as Interleukin-1β, Interleukin 6, and nuclear factor-κB (NFκB) (Li *et al.*, 2013). Similarly, lentiviral over-expression of OXR1 into kidney of anti–glomerular basement membrane antibody-induced nephritis decreased inflammation, and expression of Interleukin-1β and Interleukin 6 (Li *et al.*, 2012). Corroborating these findings, we demonstrated, in Chapter 5, that over-expression of Oxr1 in neurons delays induction of neuroinflammatory pathways in ALS disease pathogenesis, including expression of cytokines, such as *chemokine (C-C motif) ligand 3* (*CCL3*), and genes involved in the complement and STAT3 immune pathways. Conversely, in Chapter 6, we found increased astrogliosis and microgliosis in the *bel* cerebellum and spinal cord, which demonstrates that loss of Oxr1 leads to neuroinflammation. Furthermore, other downstream pathways of Oxr1 over-expression, such as proteasome degradation, identified in Chapter 5 have also been linked to inflammation. Ubiquitin-proteasome system generates majority of the major histocompatibility complex (MHC) class I peptides for presentation during the immune

response (Goldberg *et al.*, 2002), and given that Psmb10 is a binding partner of Oxr1-FL *in vitro* (Figure 5.8), and is induced in motor neurons during ALS pathogenesis (Cheroni *et al.*, 2005; Puttaparthi and Elliott, 2005; Cheroni *et al.*, 2009), the proteasome degradation pathway may link Oxr1 function with regulation of inflammation. Therefore, in the future, it will be important to confirm Oxr1 interaction with Psmb10 *in vivo*, and examine whether Oxr1 binding with Psmb10 regulates Psmb10 expression and MHC class I antigen presentation during ALS pathogenesis.

To further understand the function of Oxr1, a number of putative interacting partners in addition to Psmb10 and others presented in Chapter 5 have been identified (Finelli, 2010). In particular, ALS-associated proteins, FUS and TDP-43 interact with Oxr1-C in the brain and cerebellum, while FUS also binds to Oxr1-FL (Figure 1.2) (Finelli, 2010). Furthermore, some ALS-linked mutations in FUS and TDP-43, specifically FUS^{P517L}, TDP-43^{A321G}, and TDP-43^{D169G}, lead to reduced binding with Oxr1-C (Figure 1.3) (Finelli, 2010). In Chapter 3, I demonstrated that Oxr1 regulates the localisation of FUS and TDP-43 to stress granules under OS in a manner dependent on both the TLDc domain and downstream PRMT1 function *in vitro* (Figure 3.8-3.9). Furthermore, over-expression of Oxr1 decreases mutant FUS- and TDP-43-positive stress granules for mutations that do not alter Oxr1 binding with FUS and TDP-43 (Figure 3.6), which suggests that Oxr1 may protect against mutant FUS- and TDP-43-mediated ALS pathology. The function of Oxr1 interaction with FUS and TDP-43 remains elusive, but recent studies have detailed roles of FUS and TDP-43 in splicing, mitochondrial function, and neurite outgrowth *in vitro* and *in vivo* (Shan *et al.*, 2010; Kino *et al.*, 2011; Fallini *et al.*, 2012; Lu *et al.*, 2012; Arnold *et al.*, 2013; Groen *et al.*, 2013; van Blitterswijk *et al.*, 2013; Wang *et al.*, 2013; Qiu *et al.*, 2014). Oxr1 has been previously found to interact with other proteins involved in RNA processing, and localise to the mitochondria under OS, and TBC1

domain family, member 24 (TBC1D24), another TLDc domain protein, has been shown to modulate neurite outgrowth (Elliott and Volkert, 2004; Corbett *et al.*, 2010; Falace *et al.*, 2010; Finelli, 2010; Oliver *et al.*, 2011; Milh *et al.*, 2013; Falace *et al.*, 2014). Thus, it will be important to explore whether over-expression of Oxr1 rescues any of these aberrant functions in mutant FUS and TDP-43. Furthermore, a number of recent studies have established mouse mutants expressing ALS mutant FUS and TDP-43, especially those that do not affect binding with Oxr1, such as FUS^{R513C}, $TDP\text{-}43^{Q331K}$, and $TDP\text{-}43^{M337V}$ (Arnold *et al.*, 2013; Qiu *et al.*, 2014). Therefore, in the future, we should cross the Tg(*Prnp-Oxr1*) line generated in Chapter 4 with these mutant FUS and TDP-43 ALS transgenic mouse models to determine whether Oxr1 similarly delays FUS- and TDP-43-mediated ALS disease progression.

Studies on other TLDc domain family proteins, Chromosome 20 open reading frame 118 (C20ORF118), KIAA1609, nuclear receptor coactivator 7 (NCOA7), and TBC1D24, have thus far revealed little about the function of this highly conserved domain (Durand *et al.*, 2007; Shkolnik *et al.*, 2008; Falace *et al.*, 2010; Oliver *et al.*, 2011). NCOA7 protects cells from oxidative damage through a TLDc domain-dependent mechanism, but the crystallization of the NCOA7 TLDc domain has uncovered no structural resemblance of the domain to any other known protein families (Durand *et al.*, 2007; Finelli, 2010; Blaise *et al.*, 2012). Recent studies have provided great insight into function of TBC1D24, though the function of the TLDc domain in TBC1D24 remains unknown. TBC1D24 modulates dendritic arborisation, radial migration, and multipolar-bipolar transition of cortical neurons in the intermediate zone *in vivo* through an ADP-ribosylation factor 6 (ARF6) dependent membrane-trafficking pathway (Corbett *et al.*, 2010; Falace *et al.*, 2010; Falace *et al.*, 2014). A similar pathway has been identified for Skywalker, the *Drosophila* ortholog of TBC1D24; Skywalker regulates trafficking of

synaptic vesicles through an endosomal compartment by inhibiting RAB35 GTPase activity (Uytterhoeven *et al.*, 2011). Interestingly, epilepsy-associated D147H, F229S, and F251L mutations in the TBC domain of TBC1D24 reduce neurite outgrowth when compared to that of wild-type TBC1D24 *in vitro*, and over-expression of TBC1D24^{D147H} or TBC1D24^{F251L} is unable to rescue the delay in radial migration caused by knock-down of TBC1D24, thus suggesting that these missense mutants of TBC1D24 lead to deficits in ARF6-dependent radial migration (Corbett *et al.*, 2010; Falace *et al.*, 2010; Milh *et al.*, 2013; Falace *et al.*, 2014). One epilepsy-linked mutation, A509V, occurs in the TLDc domain, and affects the structure of the TLDc domain by creating steric hindrance with Cys530, which was previously found to react directly with ROS (Falace *et al.*, 2010; Oliver *et al.*, 2011; Blaise *et al.*, 2012). Given that TBC1D24 interaction with ARF6 is not altered by the A509V mutation, and the A509V mutation in the TLDc domain also significantly impairs neurite outgrowth *in vitro*, the TLDc domain likely also functions in concert with other proteins to regulate neurite outgrowth (Falace *et al.*, 2010).

Therefore, understanding the binding partners of the TLDc domain will be crucial in understanding its role in conferring cellular sensitivity to OS, and in particular, during pathogenesis of neurological conditions given the role of TBC1D24. In Chapter 3, I demonstrated that function of the TLDc domain in Oxr1 is necessary for regulating recruitment of FUS and TDP-43 to stress granules. Furthermore, FUS binds with both Oxr1-FL and Oxr1-C (Finelli, 2010), thus, deletion constructs of *Oxr1* can determine whether FUS interacts Oxr1 in the TLDc domain. In the future, it will be important to determine whether other TLDc domain family proteins similarly regulate localisation of FUS and TDP-43 to stress granules under OS. Furthermore, to identify binding partners of the TLDc domain, mass spectrometry experiments should be conducted with other TLDc domain family members, and the common targets of these experiments compared with the

list of putative binding partners of Oxr1 in both the brain and spinal cord that we present in Chapter 5 should present putative binding partners of the TLDc domain. Taken together, further exploration of the binding partners of TLDc domain will provide insight into the function of the domain and the importance of TLDc domain proteins in modulating neuronal function and survival in various neurological disorders, including ALS and epilepsy.

7.3 Role of Oxr1 in human disease

While mutations in *OXR1* have not yet been found in human disease, changes in OXR1 expression are associated with a number of different neurodegenerative diseases. Intermediate OXR1 isoforms are up-regulated in the spinal cord biopsy samples of ALS patients, and full-length Oxr1 is up-regulated in pre-symptomatic $SOD1^{G93A}$ ALS mice. In the cortex of PD patients, *OXR1* is significantly down-regulated (Stamper *et al.*, 2008). In Chapter 4 and 5, I showed that over-expression of Oxr1 in neurons modifies ALS disease progression in $SOD1^{G93A}$ ALS mice, increasing lifespan and motor neuron survival, improving motor and muscle function, decreasing muscle pathology, and delaying transcriptomic changes, such as neuroinflammation (Figure 4.3-4.10; 5.1-5.7). Interestingly, I also found that Oxr1-FL is up-regulated approximately 4-fold in the striatum of pre-symptomatic N171-82Q HD mice (Figure 7.1). Recent studies have also identified up-regulation of Oxr1 in the mouse kidney after OS-induced renal damage, and decreased Oxr1 expression in kidneys of mice with spontaneous mutations that cause lupus nephritis (Li *et al.*, 2012; Li *et al.*, 2013). Furthermore, a genome-wide CpG island methylation analysis identified *OXR1* as a potential gene that is associated with renal cell carcinoma (RCC) (Ricketts *et al.*, 2012). Taken together, these data demonstrate that Oxr1

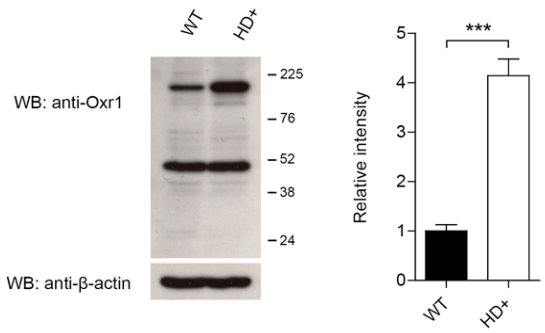

Figure 7.1. Oxr1 is up-regulated in a pre-symptomatic HD mouse model
Oxr1-FL is increased approximately 4-fold in the striatum of pre-symptomatic N171-82Q HD mice (HD+) compared to wild-type (WT) mice. Values are the mean ± SEM (n=3 per genotype; ***$p<0.001$, two-tailed student's t-test).

expression is altered during pathogenesis of diverse human diseases, and suggests that up-regulation of Oxr1 may serve as novel marker of OS.

Interestingly, other TLDc domain family proteins have also been linked to a number of different human diseases. Expression of *C20ORF118* has been linked to a number of different cancers. *C20ORF118* expression has been found to be down-regulated in the presence of $p53^{R175H}$, which is the most frequent mutant identified in colon cancer (Soussi *et al.*, 2006; Stambolsky *et al.*, 2010). Mutations in p53 are the most common genetic aberrations in cancers, and inhibit tumour suppressing function of p53 while inducing gain-of-function oncogenic activity (Zalcenstein *et al.*, 2003). In particular, mutant $p53^{R175H}$ inhibits apoptosis and interacts with regulatory elements to repress the CD95 promoter, thus interfering with chemotherapy treatment to kill tumour cells (Friesen *et al.*, 1996; Muller *et al.*, 1997; O'Connor *et al.*, 2000; Shao *et al.*, 2001; Zalcenstein *et al.*, 2003). Derived from a primary small cell ovarian carcinoma of the hypercalcemic type, an aggressive form of ovarian cancer, the BIN-67 cell line exhibits a number of genomic aberrations, including loss of heterozygosity of an approximately 16.7 Mb chromosome 20 contig, which contains *C20ORF118* along with 186 other genes (Upchurch *et al.*, 1986; Gamwell *et al.*, 2013).

Recent studies have begun to uncover the potential role of KIAA1609 in human disease. Loss of *KIAA1609* along with *ATPase, Ca^{2+} transporting, type 2C, member 2* (*ATP2C2*) was identified in a patient with Asperger syndrome with a hemizygous 86-kb deletion on chromosome 16 (Nava *et al.*, 2014). Furthermore, in rare cases, metastatic RCC can be successfully treated with interferon-α or interleukin-2 treatment, demonstrating that complete cytokine-driven immunity is possible (Motzer and Russo, 2000; Belldegrun *et al.*, 2008; Akaza *et al.*, 2010; Naito *et al.*, 2010). KIAA1609 was identified as a potential cancer antigen that induces cytotoxic T lymphocytes and may

serve as an immunotherapy target of RCC (Kawashima *et al.*, 2014). Transcriptomic analysis of tumour endothelial cells (TECs) from human ovarian cancers found that *KIAA1609* is significantly up-regulated in TECs from tumours with the absence of CD3+ tumour-infiltrating lymphocytes (Buckanovich *et al.*, 2008). *KIAA1609* is also up-regulated in hepatocellular carcinoma, human tumour liver cell lines, and in lymph node metastasis-positive primary breast tumours (Riou *et al.*, 2002; Ellsworth *et al.*, 2011).

NCOA7 is the only protein of the TLDc domain family that binds to and enhances transcriptional activities of nuclear receptors through the ERα-binding domain (Shao *et al.*, 2002; Arai *et al.*, 2008), and, in recent years, has also been linked to a number of different human diseases (Shao *et al.*, 2002; Arai *et al.*, 2008). NCOA7 is induced by all-trans-retinoic acid, a drug used to treat acute promyelocytic leukemia, in neuroblastoma-derived RTBM1 cells, and expression of *NCOA7* significantly correlates with better prognosis of patients with neuroblastomas (Arai *et al.*, 2008). Furthermore, in prostate cancer and glioblastoma tissue samples, expression of *NCOA7* is significantly negatively correlated with that of Phosphatase and tensin homolog (PTEN), a tumour suppressor gene that inhibits the Phosphoinositide 3-kinase/Akt-signaling pathway and whose inactivation can lead to oncogenesis (Trotman *et al.*, 2003; Alimonti *et al.*, 2010; Hollander *et al.*, 2011; Tay *et al.*, 2011). In addition, evaluation of associations between breast cancer and single-nucleotide polymorphisms of *NCOA7* found that one *NCOA7* haplotype, spanning the initial protein-coding exon and its flanking introns, has a significant inverse association with breast cancer (Higginbotham *et al.*, 2011). A unique short isoform of NCOA7 has been found to be induced in multiple sclerosis patients treated with interferon beta-1b (IFNB-1b) (Yu *et al.*, 2012). The exact mechanisms of IFNB-1b action in multiple sclerosis remain elusive, but IFNB-1b is an important immunomodulator, reducing

proinflammatory cytokines and regulating leukocyte trafficking into the CNS through the blood brain barrier (Stone *et al.*, 1995; Yong *et al.*, 1998; Sega *et al.*, 2004; Kieseier, 2011).

Interestingly, recessive mutations in *TBC1D24*, suggesting loss or partial loss of TBC1D24 function, have been found to cause a range of different neurological conditions, particularly those associated with epilepsy (Table 1.1). In particular, two missense mutations, including one in the TLDc domain, was recently discovered in Familial Infantile Myoclonic Epilepsy; a missense mutation *TBC1D24* is responsible for an autosomal-recessive syndrome of focal epilepsy, dysarthria, and intellectual disability with cortical thickening; and novel compound heterozygous mutations in *TBC1D24* cause familial Malignant Migrating Partial Seizures of Infancy (Corbett *et al.*, 2010; Falace *et al.*, 2010; Milh *et al.*, 2013). Furthermore, a 2bp deletion in exon 3 of *TBC1D24*, which truncates the protein such that the TLDc domain is missing, results in early onset progressive encephalopathy, myoclonic epilepsy with dystonia, and developmental and neurological retardation, and these patients die before 10 years of age (Duru *et al.*, 2010; Guven and Tolun, 2013). Exome sequencing identified a number of *TBC1D24* mutations in patients suffering from deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome, and some of the mutations disrupted stability of *TBC1D24* mRNA (Campeau *et al.*, 2014). A recent study also identified missense *TBC1D24* mutations that cause autosomal-recessive nonsyndromic deafness DFNB86, and are not associated with epilepsy (Rehman *et al.*, 2014).

Taken together, my work and recent studies suggest that Oxr1 along with the other TLDc domain family proteins modulate cellular survival during disease pathogenesis, and loss of these proteins may lead to different human diseases. Interestingly, oxidative stress and inflammation are common pathologies of auto-immune disorders, cancer, epilepsy, multiple sclerosis and neurodegenerative disorders (Beal, 1995; Kuroiwa and Lee, 1998;

Perez de Lema *et al.*, 2001; Coussens and Werb, 2002; Gilgun-Sherki *et al.*, 2004; Patel, 2004; Hauser and Oksenberg, 2006; Lin and Beal, 2006; Ozben, 2007; Gonsette, 2008; Demaria *et al.*, 2010; Fernandez-Checa *et al.*, 2010; Moroni *et al.*, 2010; Reuter *et al.*, 2010; Vezzani *et al.*, 2011). From my work in chapters 5, I showed that Oxr1 mediates neuroinflammation, particularly delaying activation of the complement and STAT3 pathways during ALS disease progression, and placed Oxr1 as a modulator of both cellular sensitivity to OS, and activation of inflammatory pathways (Figure 5.4-5.5). Complement and STAT3 pathways also induce inflammation that promotes tumour cell proliferation, survival, and metastasis, regulate inflammation during epilepsy, are activated in lupus nephritis, and are associated with multiple sclerosis (Passwell *et al.*, 1988; Niculescu *et al.*, 1992; Storch *et al.*, 1998; Liang *et al.*, 2006; Aronica *et al.*, 2007; Yu *et al.*, 2009; Jakkula *et al.*, 2010; Apostolidis *et al.*, 2011; Vezzani *et al.*, 2011; Xu *et al.*, 2011b). These findings suggest that Oxr1 may also modulate disease progression in cancer, epilepsy, lupus nephritis, and multiple sclerosis, thus future studies should examine whether OXR1 expression is altered in patients suffering from other neurodegenerative disorders, cancer, epilepsy, lupus nephritis, and multiple sclerosis. In addition, it will be important to explore whether over-expression of Oxr1 *in vivo* delays disease pathogenesis in other models of human disease by for example, breeding the Tg(*Prnp-Oxr1*) mice with models of AD, HD, and PD. Finally, because Oxr1 modifies disease progression in an ALS mouse model, investigation of *de novo* mutations in *OXR1* may identify mutations that alter disease progression and survival of ALS patients; however, these findings may be rare as homozygous or compound heterozygous mutations are necessary to render loss of Oxr1 function.

7.4 Up-regulation of Oxr1 as an antioxidant therapy

Increased levels of ROS, leading to OS, form a central component of pathophysiology of many human diseases, including neurodegenerative diseases (Hayashi *et al.*, 2002; Andersen, 2004; Patel, 2004; Lin and Beal, 2006; Ozben, 2007; Moroni *et al.*, 2010; Firuzi *et al.*, 2011). Unsurprisingly, a number of studies have examined whether antioxidants can serve as a therapeutic intervention to these various diseases. Unfortunately, while many studies have shown efficacy of antioxidants in preclinical testing, very few have been approved for clinical use (Firuzi *et al.*, 2011). Edaravone, a drug compound that functions as a free radical scavenger, is used to prevent neuron death after acute ischemic stroke (Yamamoto *et al.*, 1997; Watanabe *et al.*, 2008). Recent studies have shown that edaravone may also have some functional benefits in an ALS mouse model and ALS patients (Yoshino and Kimura, 2006; Ito *et al.*, 2008). α-Lipoic acid, an endogenous antioxidant with free radical scavenging properties, is approved for diabetic polyneuropathy, and decreases OS *in vivo* (Borcea *et al.*, 1999; Marangon *et al.*, 1999; Moini *et al.*, 2002; Ziegler *et al.*, 2004; Tesfaye, 2009). Flavonoids are natural antioxidant compounds, and some have been approved for clinical use (Firuzi *et al.*, 2011). A micronized purified flavonoid fraction, which consists of 90% diosmin and 10% flavonoids, is used to treat venous ulcers, and decreases OS-induced damage (Lyseng-Williamson and Perry, 2003; Smith, 2003; Unlu *et al.*, 2003; Coleridge-Smith *et al.*, 2005; Gohel and Davies, 2009). A semi-synthetic flavonoid, hydroxyethylrutoside, has also been used to manage venous diseases (Frick, 2000; Incandela *et al.*, 2002; Petruzzellis *et al.*, 2002; Belcaro *et al.*, 2008; Gohel and Davies, 2009). Silibinin is a natural flavonoid and is approved as a hepatoprotective agent, but the efficacy of Silibinin treatment for liver disease remains unknown (Jacobs *et al.*, 2002; Saller *et al.*, 2008). Finally, approved as a treatment for knee osteoarthritis, flavocoxid, a mixture of baicalein and catechin, inhibits

cyclooxygenase and 5-lipoxygenase enzymes, and has anti-inflammatory properties (Altavilla *et al.*, 2009; Levy *et al.*, 2009; Morgan *et al.*, 2009). Clinical trials of many other antioxidant therapies have failed to show efficacy due to a number of different reasons ranging from OS not being the only or primary cause of the disease to suboptimal timing or duration of therapy (Firuzi *et al.*, 2011).

Very few treatments exist for neurodegenerative diseases, and for the therapies that exist, none are disease-modifying. As the prevalence of neurodegenerative diseases increases, novel therapeutic strategies will be increasingly important to alleviate this public health burden. With increased susceptibility of the brain to OS due to higher metabolic rates and limited antioxidant defences, and the increasing body of evidence that demonstrate the involvement of OS in neurodegenerative disease pathogenesis, antioxidant therapies have been explored to treat neurodegenerative diseases (Andersen, 2004; Lin and Beal, 2006; Barber and Shaw, 2010; Ghosh *et al.*, 2011; Johri and Beal, 2012; Koppula *et al.*, 2012; Teixeira *et al.*, 2013). In Chapter 4 and 5, I presented evidence that over-expression of antioxidant Oxr1 in neurons modifies SOD1-mediated ALS and decreases neuroinflammation. Furthermore, we found no significant changes to the transcriptome of the lumbar spinal cord and no overt pathology from neuronal Oxr1 over-expression *in vivo* (Figure 4.2; Table 5.1;5.6). Similarly, over-expression of OXR1 also ameliorates renal pathology in models of nephritis. Transplantation of mesenchymal stem cells over-expression OXR1 in a mouse model of lupus nephritis, significantly improves renal pathology, reduces macrophage and T lymphocyte inflammation, and decreases OS (Li *et al.*, 2013). Lentiviral delivery of OXR1 into a mouse model of immune-mediated nephritis also decreased inflammation, and renal cell apoptosis (Li *et al.*, 2012). Taken together, these data suggest that up-regulation of Oxr1 is a promising therapeutic target for different neurodegenerative diseases.

In the future, it will be important to characterise the regulatory elements of Oxr1, such as transcription factors that bind upstream of Oxr1, and microRNAs that interact with the 3' *Oxr1* UTR to modulate mRNA stability. In particular, I conducted a preliminary screen that demonstrates mutations in pre-B-cell leukemia homeobox (PBX), GATA binding protein 3 (GATA3), and NK2 homeobox 5 (Nkx2-5) binding sites upstream of *Oxr1-C* significantly alter *Oxr1-C* promoter activity through the use of luciferase constructs (Figure 7.2-7.3). Interestingly, a ChIP-seq experiment in ovarian cancer cell line OVCAR3 identified PBX1 binding in the promoter of *Oxr1* (Thiaville *et al.*, 2012). In the future, chromatin immunoprecipitation (ChIP) experiments will be necessary to confirm PBX1, GATA3, and Nkx2-5 binding to the promoter of *Oxr1-C*. A recent study identified microRNA (miR)-200b as a novel repressor of *Oxr1* expression in a retinal cell line through binding to the *Oxr1* 3' UTR (Murray *et al.*, 2013). In addition, we identified a binding site for a highly conserved miR-137 in the 3' UTR of *Oxr1*, and found that over-expression of miR-137 strikingly up-regulates Oxr1-C *in vitro* (Figure 7.4). miR-137 is expressed in the CNS, plays an important role in neuronal maturation, and has been associated with glioblastoma (Silber *et al.*, 2008; Smrt *et al.*, 2010; Chen *et al.*, 2012a). microRNAs generally target mRNAs for degradation or repress translation, but some studies have found that they also activate translation in some circumstances (Vasudevan *et al.*, 2007; Wu and Belasco, 2008; Vasudevan, 2012). Alternatively, it is possible that miR-137 regulates mRNA expression of other genes, which subsequently affect Oxr1-C expression. Thus, to discern whether miR-137 directly activates translation of *Oxr1-C*, we will mutate the *Oxr1* 3'UTR luciferase construct at the predicted miR-137 binding site, and determine whether over-expression of miR-137 increases luciferase activity in the wild-type, but not in the mutant luciferase construct. Finally, we can conduct a high-throughput small-molecular screen using the *Oxr1-C* promoter construct to identify novel

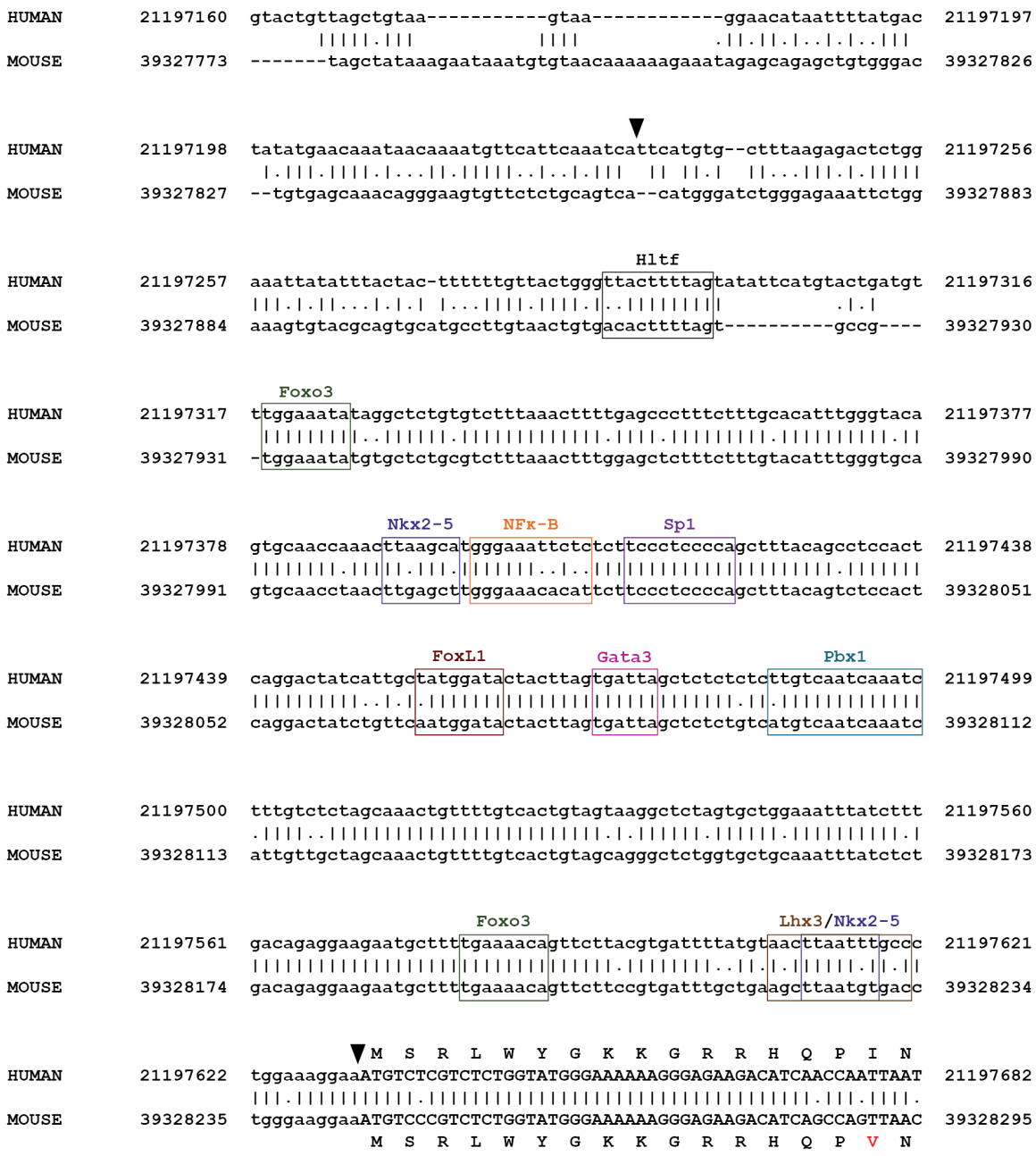

Figure 7.2. Sequence alignment of human and mouse promoter regions of *Oxr1-C*
Sequence conservation between human (top: GenBank accession number NW_004078037.1) and mouse (bottom: accession number CM000223.3) of the region upstream of *Oxr1-C*. The translated exon is shown and triangles define the cloned region of the promoter for further analysis. All putative transcription factor binding motifs are shown in boxes with the predicted transcription factor shown above.

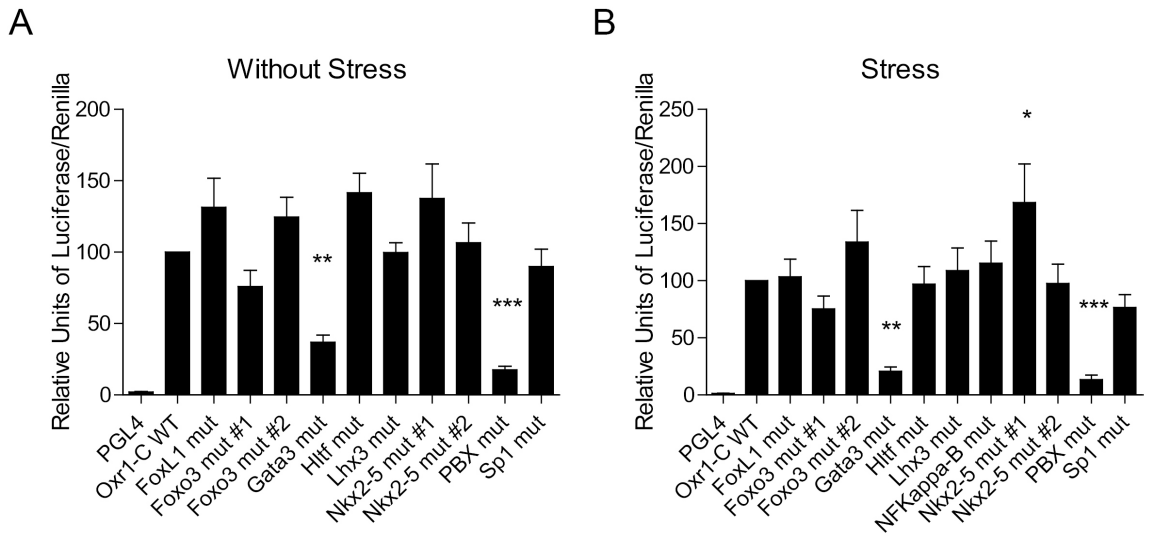

Figure 7.3. Oxr1-C promoter activity is enhanced at the predicted PBX and Gata3 consensus binding site, and repressed at more 5' upstream predicted Nkx2-5 consensus binding site under stress

(**A**) Dual Luciferase Assay demonstrates that Oxr1-C promoter activity is significantly reduced to approximately 17.5% when the PBX consensus binding site is mutated, and to approximately 37% when the Gata3 consensus binding site is mutated. (**B**) Under arsenite stress treatment, Oxr1-C promoter activity is similarly altered by mutations in the PBX and Gata3 consensus binding site, and is significantly increased approximately 68.5% when the more upstream Nkx2-5 consensus binding site is mutated. (**A-B**) Values are the mean ± SEM (n=15; *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA, followed by Dunnett's Multiple Comparison Test to the wild-type).

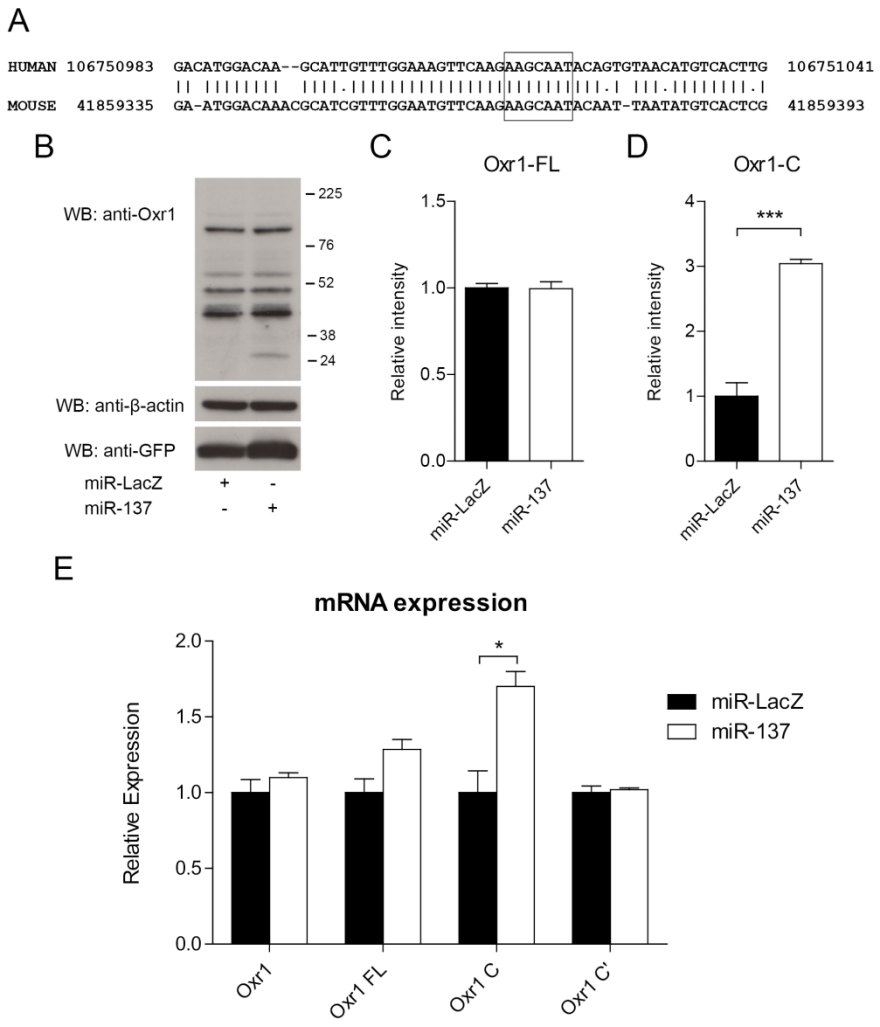

Figure 7.4. miR-137 increases expression of Oxr1-C

(**A**) Sequence conservation between human (top: NCBI Reference Sequence: NC_000008.11) and mouse (bottom: NCBI Reference Sequence: NC_000081.6) of the region in the 3'UTR of *Oxr1*, which contains a conserved binding motif (box) for miR-137. (**B**) Representative image of Oxr1 expression after miR-137 over-expression in N2A cells. (**C**) Quantification of Oxr1-FL after miR-137 over-expression by ImageJ showing no difference. (**D**) Quantification of Oxr1-C after miR-137 over-expression by Image J showing a three-fold increase. (**C-D**) Values are the mean ± SEM (n=3; ***p<0.001, two-tailed student's t-test). (**E**) Quantification of mRNA expression of Oxr1 isoforms after up-regulation of miR-137 demonstrates only Oxr1-C is significantly increased. Values are the mean ± SEM (n=4; *p<0.05, two-tailed student's t-test).

drug compounds that up-regulate Oxr1-C, thereby informing possible repurposing and repositioning efforts (Huang *et al.*, 2011b; Swamidass, 2011; Oprea and Mestres, 2012). Together, future studies that elucidate these upstream regulatory elements of *Oxr1* will provide crucial information for the development of drug therapies that up-regulate Oxr1.

The work that I presented in this thesis, in combination with the growing body of evidence in understanding the complex antioxidant defence network, begins to provide insight into the identification of novel genes, such as *Oxr1*, which are responsible for the crosstalk between oxidative stress and other pathways, such as neuroinflammation. Moreover, further understanding of the molecular mechanisms underlying various neurodegenerative diseases, particularly pathways that modify disease pathogenesis, provides a framework from which novel therapeutic strategies, such as up-regulation of Oxr1, can be developed to treat patients suffering from these debilitating disorders.

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