THE ROLE OF RASSF1A IN HUMAN EMBRYONIC AND NEURAL STEM CELLS DURING DIFFERENTIATION

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Thesis submitted to the University of Oxford
for the degree of Doctor of Philosophy

Trinity Term 2017

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The work presented in this thesis was performed at the Cancer Research UK/ Medical Research Council Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford. This study was funded by Cancer Research UK Oxford Centre Prize Studentship.

To my amazing family

Table of Contents

Declaration viii

Nomenclature ix

Abstract x

Acknowledgement xi

List of Tables xii

List of Figures xv

List of Abbreviations xvi

I Introduction 1

1 Embryonic Development 2

1.1 Human Embryogenesis 2

1.2 Stem Cells and Differentiation 4

1.2.1 Embryonic Stem Cells 4

1.2.2 Adult Stem Cells 7

1.2.3 Neural Stem Cells 7

2 Cancer 12

2.1 Cancer Stem Cell Hypothesis 12

2.2 Central Nervous System Tumours 13

2.3 Cancer Stem Cells in the Brain 16

3 Epigenetics 19

3.1 Epigenetic Mechanisms 21

3.2 The Histone Code 24

3.3 Histone modifications during Differentiation 24
3.4 SUV39H1 in Neural Differentiation 26

4 The Hippo Signalling Pathway 28
4.1 Hippo Signalling in Cancer Stem Cells 30
4.2 Ras association domain-containing protein 1 31
4.3 Aim of Study 33

II Materials and Methods 35

5 Materials 36
5.1 General Laboratory Reagents 36
5.2 Cell Culture Reagents 38
5.3 Antibodies 39
5.4 RNAi 42
5.5 Plasmids 43
5.6 TaqMan assays 43

6 Cell biology 44
6.1 Cell Lines and Culture 44
6.2 Differentiation Assays 46
6.3 Transient Transfection 47
6.4 Drug Treatments 47
6.5 Molecular Biology 48
6.5.1 Transformation of Bacteria 48
6.5.2 Isolation of Plasmid DNA 49
6.6 Molecular Biochemistry 49
6.6.1 Western Blotting 49
6.6.2 Immunoprecipitation 50
6.6.3 RNA Isolation and Reverse Transcriptase PCR 51
6.6.4 Quantitative Real Time Polymerase Chain Reaction 51
6.6.5 Methylation-specific Polymerase Chain Reaction 53
6.6.6 Immunofluorescence 54
6.6.7 CRISPR/Cas9 55

III Results 57

7 Human Embryonic Stem Cells 58

7.1 Introduction . . . 58
7.2 RASSF1A in Human Embryonic Stem Cells . . . 60
7.2.1 RASSF1A is upregulated in differentiated hESCs . . . 61
7.2.2 RASSF1A during Neuroectodermal Differentiation . . . 67
7.2.3 Epigenetic changes during Differentiation . . . 71
7.3 Discussion . . . 76

8 Human Neural Progenitor Cells 81
8.1 Introduction . . . 81
8.2 Characterisation of Human Neural Stem Cells . . . 83
8.3 RASSF1A during Neural Differentiation of hNSCs . . . 87
8.4 Discussion . . . 93

9 Epigenetics 95
9.1 Introduction . . . 95
9.2 Epigenetic Regulation of Neural Differentiation . . . 96
9.3 RASSF1A and Chromatin marks . . . 98
9.4 Discussion . . . 113

IV Discussion 116

10 RASSF1A and SUV39H1 in Differentiation 117

11 Remaining Aspects 122
11.1 Localisation of SUV39H1 . . . 123
11.2 SUV39H1 regulation . . . 124
11.3 RASSF1A dependent changes of chromatin marks . . . 124

12 Conclusion 126

References 127

Appendices 149
I RASSF1A Transcription levels . . . 149
II Additional NSC Marker . . . 151
III Optimisation of hNSC Transfection . . . 152
IV RASSF1A Knock-down at Day 7 . . . 155
V Regulation of SIRT1 with Neural Differentiation . . . 156
VI Generation of CRISPR Cell Lines . . . 157

VII Copyright licences 159

Declaration

I declare that this thesis was composed by myself and the work presented in this thesis is wholly my own, except where explicitly stated otherwise. No portion of this work has been submitted previously for any other degree or qualification at this or any other University.

Nomenclature

Human gene symbols are denoted in upper case italics (e.g. *RASSF1A*) whilst mouse genes are italicised with the first letter in uppercase and the remaining letters in lowercase (e.g. *Rassf1A*). Human and mouse protein symbols are denoted in upper case lettering (e.g. RASSF1A). Species are denoted in italic lettering (e.g. *Drosophila Melanogaster*).

Abstract

Stem cells are characterised by their ability to continuously self-renew and differentiate into various lineages. This balance is tightly controlled by intrinsic and extrinsic factors that maintain normal development and tissue homoeostasis. The dysregulation of these factors is associated with oncogenesis and is extensively studied in the context of treatment resistance and tumour reoccurrence. Stem and cancer cells share the ability to indefinitely self-renew and therefore it stands to reason that these cells harbour similar underlying regulatory processes. Stem cells that are unable to differentiate are widely described as cancer initiating (or cancer stem) cells and their inability to differentiate is therefore heavily investigated as a therapeutic target. A crucial signalling pathway regulating pluripotency and proliferation in stem and cancer cells is the Hippo tumour suppressor pathway. RASSF1A, a key regulator of the Hippo pathway, is frequently methylated in numerous brain/central nervous system (CNS) malignancies, indicating that a potential deregulation of stem cells may drive the association with poorer prognosis. In this work we demonstrate that RASSF1A mediates the localisation of suppressor of variegation 3-9 homologue 1 (SUV39H1), a histone methyltransferase that catalyses the trimethylation of histone 3 lysine 9 (H3K9me3). H3K9me3, a heterochromatin mark that stably represses genes during differentiation, is reduced with loss of RASSF1A, leading to impaired neural differentiation of stem cells. Our findings suggest that the epigenetic loss of RASSF1A therefore contributes to a dedifferentiated phenotype in cancer.

Acknowledgement

This thesis is dedicated to my wonderful parents, Mutti und Vati, my lovely sisters, Manni and Darja and the best grand parents in the world Oma und Opa Klötze and Oma und Opa Kusey. Tausend Dank für all Eure bedingungslose Unterstützung! Ohne Eure Hilfe wäre das alles nicht möglich gewesen. Mutti und Vati, danke für all die Ironie und den Sarkasmus, der uns drei Könnigs in die Wiege gelegt wurde. Wir haben immer eine Menge Spaß damit! Manni und Darja, einfach nur Danke, dass Ihr da seid und ich Eure 'kleine' Schwestern sein darf. Und mindestens genauso wichtig, danke Domenic für all deine Hilfe, Unterstützung, Geduld, Ruhe und dein Verständnis! Tausend dank, dass ich mich immer auf Dich verlassen kann!

I am heartily grateful to my supervisor Prof. Eric O'Neill for giving me the possibility to work on this interesting project and whose guidance and support always encourage me.

I would like to thank all my friends! Leanne, Maria and Dafni, thank you so much for all the good craic, support and help in the lab, but especially outside the lab. Oxford would have been a very different experience without you, with significantly less wine, tennis and chocolate. A big thank you to my housemates, Leanne (again), GG and Sibyl for always taking care of me and for always having the time for tea. And many thanks to all the rest of the EON members, present and past, Aswin, Nikola, Asmita, Michael, Ali, for the help and more importantly for all the fun in the lab.

Thank you all so much!

List of Tables

5.1 Chemicals 36
5.2 Cell culture reagents and supplements 38
5.3 Primary Antibodies for Western Blot 39
5.4 Primary Antibodies used for Immunofluorescence 40
5.5 Secondary Antibodies 41
5.6 Small interfering RNAs 42
5.7 Plasmids 43
5.8 TagMan probes 43

7.1 Relevant RASSF1A interactors and their functions 72

List of Figures

1.1 Stem Cell Niches in the Human Adult Brain 9

2.1 Models for Tumour Hierarchy 13
2.2 Genetic characterisation of primary and secondary glioblastoma . . 15
2.3 Classification of GBMs according to their Location 17

3.1 Waddington's Epigenetic Landscape 20
3.2 Mechanisms for Epigentic Gene Regulation 21

4.1 Mammalian Hippo Signalling Pathway 29
4.2 The structure of RASSF1A and RASSF1C 31

7.1 The human ES cell line H9 expresses pluripotency markers 60
7.2 RASSF1A expression is upregulated in spontaneously differentiated hESCs 62
7.3 Trilineage Differentiation of human ES cells 64
7.4 RASSF1A is activated by ATR but not ATM in hESCs 65
7.5 RASSF1A expression is upregulated with Differentiation into all three germ layers 66
7.6 Neuroectodermal Differentiation of human ES cells 68
7.7 RASSF1A during Neuroectodermal Differentiation of human ES cells 69
7.8 Hippo Pathway components during Neuroectodermal Differentiation 70
7.9 Knock-down of RASSF1A alters levels of SUV39H1 expression and its substrate H3K9me3 in hESCs 75

8.1 Schematic Overview of Human NSCs CX and VM as a model for Human Neural Differentiation 84
8.2 Undifferentiated CX and VM cells express NESTIN and RASSF1A but not OCT4 84
8.3 CX and VM cells express Neural Differentiation Markers 86
8.4 RASSF1A during Neural Differentiation of human NSCs 88

8.5 RASSF1A gene expression during Neural Differentiation of human NSCs . . . 88
8.6 Methylation of RASSF1A during Neural Differentiation of human NSCs . . . 89
8.7 Validation of RASSF1A siRNAs in CX cells . . . 90
8.8 RASSF1A Knock-down Control . . . 91
8.9 RASSF1A depletion impairs Neural Differentiation in human NSCs 92

9.1 SUV39H1 Antibody Validation . . . 97
9.2 SUV39H1 and Chromatin Marks during Neuroectodermal Differentiation of hESCs . . . 98
9.3 SUV39H1 antibody validation during Neural Differentiation . . . 99
9.4 SUV39H1 and Chromatin Marks during Neural Differentiation of hNSCs . . . 100
9.5 SUV39H1 gene expression during Neural Differentiation of human NSCs . . . 102
9.6 SUV39H1 is increased with Knock-down of RASSF1A in hNSCs . . 103
9.7 SUV39H1 localisation changes with RASSF1A Knock-down in CX cells . . . 104
9.8 SUV39H1 localisation changes with RASSF1A Knock-down in VM cells . . . 105
9.9 Quantification of SUV39H1 localisation with RASSF1A Knock-down in hNSCs . . . 106
9.10 H3K9me3 is reduced with RASSF1A knock-down . . . 107
9.11 Interaction of RASSF1A and SUV39H1 . . . 109
9.12 SUV39H1 knock-down in CX cells . . . 110
9.13 SUV39H1 knock-down in VM cells . . . 111

10.1 Model of RASSF1A-dependent SUV39H1 regulation . . . 120

11.1 Hypothesis of RASSF1A-dependent SUV39H1 regulation . . . 122

S1 Transfection of the neuroblastoma cell line SY5Y . . . 150
S2 CX and VM express pluripotency marker SOX2 . . . 151
S3 Transfection of CX cells using Lipofectamine 3000 . . . 152
S4 Optimisation of Nucleofection of CX and VM using Nucleofector 2b 153
S5 Optimisation of Nucleofection of CX and VM using Nucleofector 4D 154
S6 Knock-down of RASSF1A is lost at Day 7 of Differentiation . . . 155
S7 Regulation of SIRT1 with Differentiation and RASSF1A depletion . 156

S8 RASSF1A Knock-out CRISPR Clones 157

List of Abbreviations

2HG	2-hydroxyglutarate
AML	Acute myeloid leukaemia
APC	Astrocyte progenitor cell
ATRX	ATP-dependent helicas
BBB	Blood-brain barrier
BDNF	Brain-derived neutrophic factor
bFGF	Fibroblast growth factor
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
c-myc	Myelocytomatosis oncogene
C5	5th carbon atom
CH3	Methyl group
CHX	Cycloheximide
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
CPP	Cell-penetrating-protein
CSC	Cancer stem cell
CX	Cortex
DAXX	Death domain-associated protein 6
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E-cadherin	Epithelial cadherin
E3	Ubiquitin ligase

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGL	External granule layer
EMT	Epithelial-mesenchymal transition
EPI	Epiblast
ESC	Embryonic stem cell
EtOH	Ethanol
EZ	Enhancer of zeste
EZH2	Enhancer of zeste homolog 2
g	gravitational force
G9a	Protein G9a
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GLP	G9a-like protein 1
GPCR	G protein-coupled receptor
gRNA	guide RNA
H3F3A	H3 histone family member 3A
H3K27me	Trimethylation of histone H3 at lysine 27
H3K4me3	Trimethylation of histone H3 at lysine 4
H3K9ac	Acetylation of histone H3 at lysine 9
H3K9me3	Trimethylation of histone 3 lysine 9
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
hESC	Human embryonic stem cell
HIF1α	Hypoxia-inducible factor 1-alpha
HMG	High mobility group
HMT	Histone methyltransferase
hNSC	Human neural stem cell
HP1	Heterochromatin protein 1
ICM	Inner cell mass
IDH	Isocitrate dehydrogenase
IGL	Internal granule layer
iPS	Induced pluripotency stem
ITCH	Itchy homolog

JHDM	Jumonji-C domain-containing histone demethylase
KDM	Lysine-specific demethylase
KIBRA	Kidney and brain protein
Klf4	Krueppel-like factor 4
LATS1	Large tumour suppressor 1
LIF	Leukaemia inhibitory factor
LOH	Loss of heterozygosity
MBD1-3	Methyl-CpG-binding domain protein
MECP2	Methyl-CpG-binding protein 2
MeOH	Methanol
miRNA	Micro-ribonucleic acid
MOB1A/B	Mps one binder
mRNA	Messenger RNA
MSP	Methylation-specific PCR
MST1	Mammalian STE20-like protein kinase 1
NANOG	NANOG homeobox
NB	Neuroblastoma
NEB	New England Biolabs
NSC	Neural stem cell
OB	Olfactory bulb
OPC	Oligodendrocyte progenitor cell
PAX6	Paired box protein 6
PBS	Phosphate buffered saline
PE	Primitive endoderm
PFA	Formaldehyde
PHD	Plant homeodomain
Pit1	Pituitary specific positive transcription factor 1
PNS	Peripheral nervous system
PRC2	Polycomb repressive complex 2
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
RA	Ras-association
RASSF1	Ras association domain-containing protein 1
RISC	RNA-induced silencing complex
RMS	Rostral migratory stream
RNAi	RNA interference
RT	Room temperature

SAM	S-adenosyl-L-methionine
SARAH	SAv/RAssf/Hippo
Sav1	Salvador homologue 1
SCID	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
SET	Trithorax
SETDB1	SET domain bifurcated
SGZ	Subgranular zone
SHH	Sonic hedgehog protein
siNT	Non-targeting siRNA
SIRT1	Sirtuin 1
SUV39H1	Suppressor of variegation 3-9 homolog 1
SVZ	Subventricular zone
T	BRACHYURY
T site	Palindromic sequence
TBS	Tris buffered saline
TE	Throphoectoderm
TERT	Telomerase reverse transcriptase
TET	Ten-eleven translocation
TF	Transcription factor
TGFβ	Transforming growth factor beta
TP53	Tumour suppressor 53
TUBB3	βIII-tubulin
USP7	Ubiquitin-specific-processing protease 7
v-myc	Myelocytomatosis viral oncogene homolog
VM	Ventral mesencephalon
WHO	World Health Organization
YAP	Yes-associated protein

Part I

Introduction

Chapter 1

Embryonic Development

1.1 Human Embryogenesis

Human embryonic development starts with the fusion of the male (spermatozoon) and female (oocyte) gametes to form a diploid zygote. The diploid cell undergoes several mitotic divisions known as the process of cleavage. The cells, called blastomeres, formed during very early cell divisions (at least until the four cell state) are totipotent (Veiga et al., 1987). Totipotency is defined as the potential of a cell to be able to develop into an entire organism (Condic, 2014). With further cell divisions the embryo (called morula due to the 'mulberry'-like appearance) undergoes compaction, a process accompanied by the Epithelial cadherin (E-cadherin)-mediated formation of cell-cell adhesion. Subsequently on day 5 after fertilisation, cavitation leads to the formation of the blastocyst where the cells of the embryo segregate into embryonic (inner cell mass [ICM]) and extra-embryonic tissue (trophoectoderm [TE] - a cohesive one-layer epithelium). The embryonic stem cells have lost the potential to give rise to extra-embryonic membranes and trophoblast, but can still differentiate into cells of all three germ layers. This is defined as pluripotency. Loss of the zona pellucida, the membrane surrounding the ovum, leads to the implantation of the blastocyst into the endometrium. Following implantation at day 7 - 9 after fertilisation, the ICM segregates further into the primitive endoderm (PE)

or hypoblast and the primitive ectoderm (EPI) or epiblast, forming the bilaminar embryonic disc. The formation of the primitive streak by convergence of cells derived from the epiblast defines the anteroposterior and medial-lateral axis of the embryo (Carlson, 2015). With further gastrulation these cells migrate through the primitive streak to form new cell layers which later give rise to the three germ layers of a human embryo, ectoderm, endoderm and mesoderm (Carlson, 2015), whereas the hypoblast gives rise to extra-embryonic tissue, including the placenta and amniotic sac (Hill, 2017). The ectoderm generates the neural plate as a first step of neurulation, visible as a thickened cell layer. Lateral folding of the neural plate leads to the formation of the neural groove, which forms the neural tube upon closure, separating the neuroectoderm from the outer surface ectoderm. The neuroectoderm gives rise to the central nervous system (CNS) and brain . The surface ectoderm develops into epidermis, the peripheral nervous systems and adrenal glands develop from neural crest cells, a cell population arising from the neural plate (Schoenwolf G. C., 2015). The endoderm forms the inner organs such as gastrointestinal tract, liver, pancreas and the respiratory tract and the renal organs. Adipose and connective tissues, like cartilage, bone, fat tissue, the endothelium of blood vessels and blood cells, dermis and muscles (skeletal, smooth, cardiac) are derived from the mesoderm. The ability of embryonic stem cells (ESCs) to differentiate into all of these cell types of an organism is called pluripotency. Pluripotent human ESCs (hESCs) are cells derived form the ICM of the blastocyst. They have the potential to differentiate into the lineages of the three germ layers, but have lost the ability to form extraembryonic tissue.

1.2 Stem Cells and Differentiation

Stem cells are characterised and classified by their potential to undergo asymmetric cell division to self-renew and differentiate. Due to their high proliferative and differentiation potential, stem cells have been of enormous interest for therapy approaches in regenerative and cancer therapies. Their dual characteristics open a wide field for potential applications, however it implicates a need to understand how the maintenance of an ES cell state and how commitment to a more restricted fate or lineage is regulated.

1.2.1 Embryonic Stem Cells

Embryonic stem cells were first identified in animal models in the 1980s (Evans and Kaufman, 1981; Martin, 1981). In 1998, they were successfully isolated for the first time from human embryonic tissue by Thomson et al. (Thomson et al., 1998). Since then, hESCs have been intensively studied to elucidate regulatory mechanisms of human development and diseases. Today it is known that the core transcription factors such as Pit-1 (pituitary-specific positive transcription factor 1)/Octamer/Unc-86 (POU) class 5 homeobox 1 (POU5F1 or OCT3/4), NANOG homeobox (NANOG) or SRY (sex determining region Y)-box 2 (SOX2) are required for pluripotency maintenance in human and mouse ES cells. OCT4 and SOX2 form a heterodimer to regulate a large set of genes transcribed to maintain pluripotency. Additionally, Boyer *et al.* found that more than 90% percent of these genes are co-occupied by NANOG (Boyer et al., 2005) and their expression is either activated or repressed. In mice, it has been found that *Oct4*, *Sox2* or *Nanog* homozygous knock-outs result in embryonic lethality. *Oct4* homozygous knock-out mouse embryos do not develop past the blastocyst stage due to the failure of ICM formation (Nichols et al., 1998) and targeted disruption of *Sox2* leads to peri-implantation lethality in

homozygous mouse embryos due to impairment of the epiblast (Avilion et al., 2003). *Nanog*-deficient embryos lack the epiblast and collapse post-implantation (Mitsui et al., 2003). In 2006, Takahashi and Yamanaka succeeded in reprogramming mouse fibroblasts into an ES-cell like state by forced overexpression of the key transcription factors *Oct3/4*, *Sox2*, *Krueppel-like factor 4* (*Klf4*) and *myelocytomatosis oncogene* (*c-Myc*) (often abbreviated as OSKM), today known as induced pluripotency stem (iPS) cells (Takahashi and Yamanaka, 2006). Induced PS cells are accepted to have molecular, developmental and morphological similarities to ES cells (Amabile and Meissner, 2009). When injected into immunodeficient mice, both ES and iPS cells, form teratomas *in vivo*, a standard assay to confirm *in vitro* pluripotency (Takahashi and Yamanaka, 2006; Lensch et al., 2007). In 2007, human-derived fibroblasts were first reprogrammed by two independent laboratories using slightly different factors to induce pluripotency (Yu et al., 2007; Takahashi et al., 2007). An easy accessibility of human patient fibroblasts with skin biopsies in combination with the iPSC technology raises great hopes for regenerative medicine applications. However, their potential is also accompanied with a great risk of tumourigenesis due to the retroviral transfection method used for gene delivery of the pluripotency factors. In 2009, iPSCs were first generated by transferring reprogramming proteins into the nucleus. Kim *et al.* used cell-penetrating-proteins (CPPs)-anchored OSKM to generate iPSCs from human newborn fibroblasts, which were able to differentiate into all three germ layers (Kim et al., 2009a). When hESCs or iPSCs commit to ectoderm, endoderm and mesoderm, the cells undergo massive transcriptional changes. The pluripotency network is abolished and expression of genes that were suppressed is activated. In the developing embryo or in differentiating pluripotent stem cells, lineage specific genes are induced to ensure correct differentiation.

In the human neuroectoderm, Paired box protein 6 (PAX6) expression, a transcription factor suppressed by the pluripotency network, is induced. Interestingly, two of the PAX6 isoforms (PAX6a and PAX6b) have been found to suppress OCT4

and NANOG expression, but not SOX2. However, only PAX6a showed pro-neuronal properties (Zhang et al., 2010). PAX6 expression is acknowledged as a marker for neuroectoderm lineage specification. Similar to the neuroectoderm, the endoderm and mesoderm require tissue specific expression of determinants that regulate their specification and differentiation. SOX17, a high mobility group (HMG) box transcription factor, has been initially identified as a regulator of the definitive endoderm in mouse (Kanai-Azuma et al., 2002) and later in hESCs (Wang et al., 2011). BRACHYURY (T) is a transcription factor that binds to a palindromic sequence (T site) and activates expression of mesodermal specification genes. All three transcription factors (PAX6, SOX17 and BRACHYURY) are expressed during embryogenesis when the three germ layers are formed. They mark early lineage commitment from pluripotent to more committed cells and ensure the expression of genes of their respective lineage. Within the first seven weeks of human embryonic development, neural stem or progenitor cells (NSCs) divide symmetrically to increase the cell pool for further brain development (reviewed in Stiles and Jernigan, 2010). Following week 7, the NSCs divide asymmetrically, forming one self-renewing NSC and one specialised daughter cell, a neuron. This process is mainly controlled by the environment, the stem cell niche, where neurons migrate from to form the neocortex (Stiles and Jernigan, 2010). By the ninth week post-fertilisation, the foetal period of human development starts. During this period, the brain increases in size and surface area and becomes highly organised. Embryonic, including foetal neurogenesis, mainly contributes to the brain architecture formation. Neurogenesis was long believed to be a developmental process, only occurring pre- and perinatal. In 1998, Eriksson *et al.* showed for the first time that neurogenesis persists in the human adult brain by analysing bromodeoxyuridine (BrdU)-labelled human NSCs (hNSCs) in post-mortem human brain samples (Eriksson et al., 1998).

1.2.2 Adult Stem Cells

Adult stem cells are more fate restricted than pluripotent stem cells and can only give rise to cell types of their primed lineage (multipotency). In an adult, most cells of the body are post-mitotic and are terminally differentiated. However, for organ regeneration and tissue homoeostasis and repair, mammals harbour stem cells throughout the whole organism. They were first mentioned in 1968, when Becker *et al.* isolated cells with colony forming properties from mouse bone marrow (Becker et al., 1963). Today, bone marrow is a well described stem cell niche for haematopoietic and mesenchymal stem cells. Schofield *et al.* first described stem cell niches in 1978 as microenvironments that supports long-term cell activity and preserves stem cell abilities (Schofield, 1978). A stem cell niche is characterised by its structural, mechanical, and biochemical signals. Vasculature, supportive niche cells, extracellular matrix (ECM) and their secreted factors all have regulatory effects on the stem cell fate. In addition to bone marrow, other mammalian stem cell niches and their progenitors include skeletal muscles (satellite cells, reviewed in Yin et al., 2013), the intestinal crypt (crypt stem cells, Barker et al., 2007), hair follicles (hair follicle stem cells, Cotsarelis et al., 1990), the interfollicular epidermis (epidermal stem cells, Levy et al., 2005) and the CNS stem niches, the subventricular zone (SVZ) and subgranular zone (SGZ) with NSCs (Fig. 1.1).

1.2.3 Neural Stem Cells

NSCs are the multipotent cells of the nervous system. As previously mentioned, they reside in two stem cell niches of the brain: the SVZ lining the lateral ventricle and the SGZ of the hippocampal dentate gyrus (DG, Fig. 1.1). However, adult neurogenesis differs significantly among mammals. It has been shown in rodents (Lois et al., 1996) and primates (Kornack and Rakic, 2001) that SVZ stem cells

give rise to neuroblasts that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB). Neurogenesis in the DG of the hippocampus contributes essentially to the facilitation of memories, learning processes and mood regulation (Christian et al., 2014) and was confirmed in humans (Eriksson et al., 1998). In contrast to rodents and primates, neurogenesis in the adult human OB is limited or even non-existent (Roos et al., 2017). Additionally, studies by Ernst *et al.* concluded that adult neurogenesis leads to the addition of newborn neurons in the striatum, suggesting that hNSCs migrate from the SVZ to the striatum rather than the OB (Ernst et al., 2014). Noteworthy, adult neurogenesis can occur in noncanonical brain areas, e.g. in response to injuries, suggesting high plasticity of CNS cells (reviewed in Feliciano et al., 2015.)

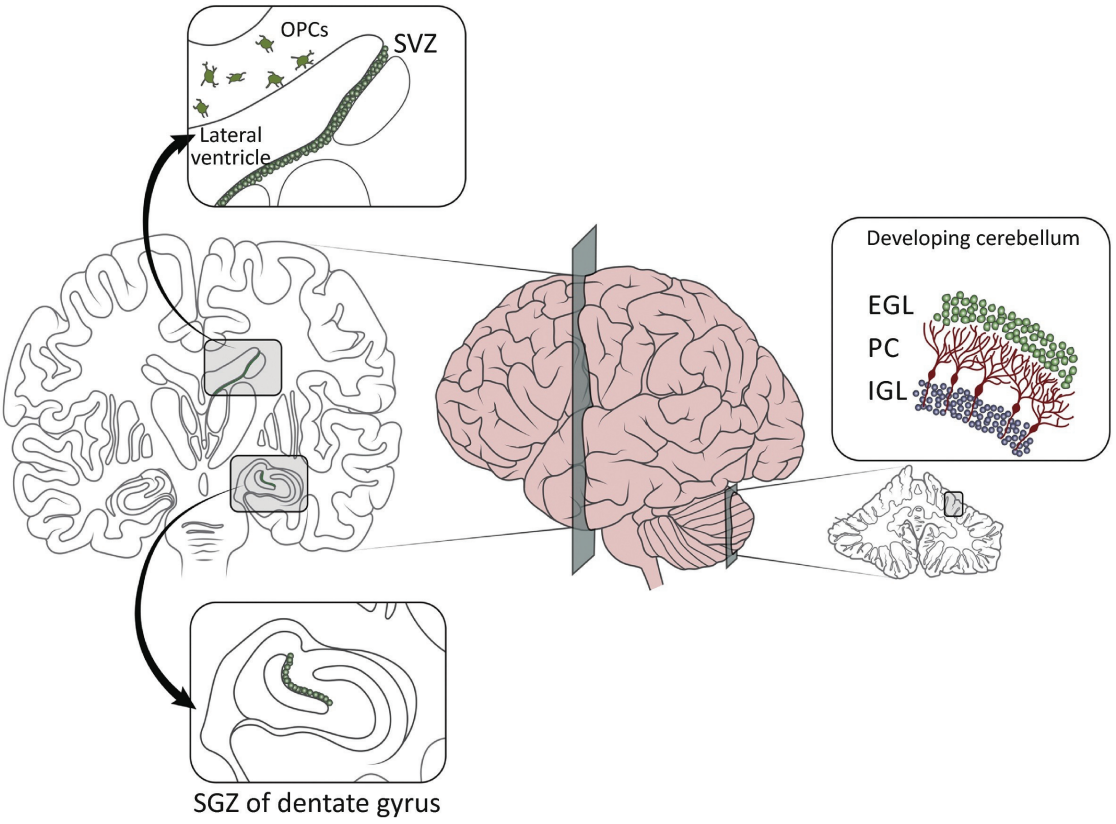


Figure 1.1: Stem Cell Niches in the Human Adult Brain: The human adult brain harbours two stem cell niches, the subventricular zone (SVZ) and the subgranule zone (SGZ) of the dentate gyrus (DG). Human NSCs (green) give rise to neuronal and glial precursors, particularly oligodendrocyte progenitor cells (OPCs). In the developing cerebellum NSCs are migrating from the external granule layer (EGL) through the Purkinje cell layer (neurons of the cerebellum) to the internal granule layer (IGL), the cerebellar cortex. Venkatesh and Monje, 2017, Reprinted with permission of Elsevier with licence number 4200220388272

Human NSCs are characterised by their potential to differentiate into neurons, astrocytes or oligodendrocytes, the three main cell types of the CNS. They have lost the expression of the pluripotency markers OCT4 and NANOG, but retain SOX2 expression. Additionally, the expression of lineage specific proteins including NESTIN, SOX1, and VIMENTIN is upregulated when pluripotent cells commit to the neural lineage. NESTIN and VIMENTIN are intermediate filament proteins important for proliferation and self-renewal of NSCs. SOX2 has been demonstrated to maintain NESTIN and Sonic hedgehog protein (SHH) expression (Favaro et al., 2009). SHH

is a ligand for the Hedgehog signalling pathway, which regulates pre- and postnatal neurogenesis. SOX1, like SOX2, is a transcription factor that prevents neural differentiation of NSCs. Interestingly, conditional *Sox2* knock-out in the mouse embryonic brain results in a complete loss of NESTIN/Glial fibrillary acidic protein (GFAP)-expressing NSCs and increased apoptosis (Favaro et al., 2009). GFAP is a class-III intermediate filament expressed in glial cells (precursors of astrocytes and oligodendocytes) and mature astrocytes. Astrocytes are 'star-like' cells with fine, long processes that form the supportive tissue of the brain and contribute to the blood-brain barrier (BBB) (reviewed in Alvarez et al., 2013). They are responsible for homeostasis of metabolites (glucose-lactate shuttle), ions (sodium/potassium-ATPase activity) and neurotransmitters (glutamate/glutamine shuttle). They are able to produce glycogen (Cataldo and Broadwell, 1986) and are connected to the neurovasculature to ensure nutrient supply for neurons (reviewed in Liu et al., 2017). Oligodendrocytes are mature glial cells of the CNS (known as Schwann cells in the peripheral nervous system [PNS]) mainly responsible for myelination of neuronal axons, a process to insulate neurons with a myelin sheath. Neurons are highly specialised cells of the nervous system that are responsible for transmission and integration of cellular communication signals (nerve impulses) from outside and inside an organism. A neuron consists of a soma (cell body), multiple dendrites and an axon. Dendrites are extensions of the soma, which are responsible for receiving signals from other axons (up to a 1000 per neuron) and converting them into electrical pulses. Multiple electrical impulses create an action potential, which is conducted by the axon to dendrites of other neurons (via a chemical synapse). Electrical insulation of axons assures a high action potential propagation velocity from one myelin sheath gap (Ranvier nodes) to another, known as saltatory conduction. When NSCs mature to become neurons, expression of βIII-tubulin (*TUBB3*, often referred to as TUJ1), a microtubule protein involved in axon guidance is induced (Katsetos et al., 1993). Axon guidance is the process of neurons extending their axons to form con-

nections that is tightly regulated by the actin cytoskleleton. Even though TUJ1 is accepted as a neuronal marker, fetal NSCs have been shown to co-express TUJ1 and GFAP in astrocyte progenitor cells (APCs), initiating a discussion about the use of TUJ1 as a neuronal marker (Draberova et al., 2008). In addition, a possible plasticity of glial cells to be able to transdifferentiate to neurons has been shown *in vivo* (Doetsch et al., 1999; Heinrich et al., 2010; Torper et al., 2013; Duan et al., 2015). In 1999, Doetsch *et al.* showed that astrocytes residing in the mouse SVZ or a subpopulation of these cells, are able to generate neurons (Doetsch et al., 1999). In 2010, Heinrich *et al.* confirmed that cortical astroglia in the adult mouse brain can be reprogrammed into neurons by induced expression of the pro-neuronal transcription factor neurogenin 2 (Neurog2, Heinrich et al., 2010). Furthermore, mouse astrocytes are able to transdifferentiate into neurons *in vivo* in response to ischemic brain injury (Duan et al., 2015). Human astrocytes, transplanted into rat brain, that stably express the pro-neuronal factors *Achaete-scute complex-like 1* (*Ascl1*), *Brain2* (*Brn2*) and *Myelin transcription factor-like protein 1* (*Myt1l*) have been shown to transdifferentiate into neurons (Torper et al., 2013) suggesting a potential plasticity of astroglia cells. The conversion of adult non-neuronal cells into neurons, particularly astrocytes due to their proximity, provides a promising therapeutic strategy for brain repair in the adult brain.

Adult neurogenesis has received significant attention not only for cell-based therapies in neurodegenerative diseases or injuries but also in the context of CNS cancer treatment. NSCs, loaded with oncolytic adenoviruses that preferentially replicate in cancer cells and subsequently destroy them, are currently used in a clinical trial (Clinical trial identifier NCT03072134). However, the concept of cancer initiating cells with stem cell properties, often referred to as cancer stem cells (CSCs), led to the identification of neurogenesis as a potential cause of CNS tumours.

Chapter 2

Cancer

2.1 Cancer Stem Cell Hypothesis

Stem cell proliferation, migration, differentiation and apoptosis is tightly controlled by intrinsic and extrinsic factors to maintain normal development, tissue homeostasis and regeneration. The dysregulation of these factors is associated with oncogenesis and is extensively studied in the context of treatment resistance and tumour reoccurrence. Stem and cancer cells share the ability to indefinitely self-renew and therefore it stands to reason that these cells have underlying similar regulatory processes. Tumour stem cells were first described in mouse myelomas by Park and colleagues (Park et al., 1971). In 1994, Lapidot *et al.* showed that a certain cell population of acute myeloid leukaemia (AML) cells can initiate leukaemia in severe combined immunodeficient (SCID) mice (Lapidot et al., 1994). More than ten years later, cells have been isolated from human brain glial tumours expressing stem cell markers (Ignatova et al., 2002), followed by the identification of tumour initiating CD133-positive cells from paediatric and adult brain tumours (Singh et al., 2004). It is nowadays known that tumours consist of different cell types with different genetic and epigenetic properties. This heterogeneity is one of the hallmarks of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011) and CSCs are considered to be the cell subpopulation that drive progression, reoccurrence and

metastasis. Initiation of mutagenic transformation is proposed by two theoretical mechanism (Fig. 2.1).

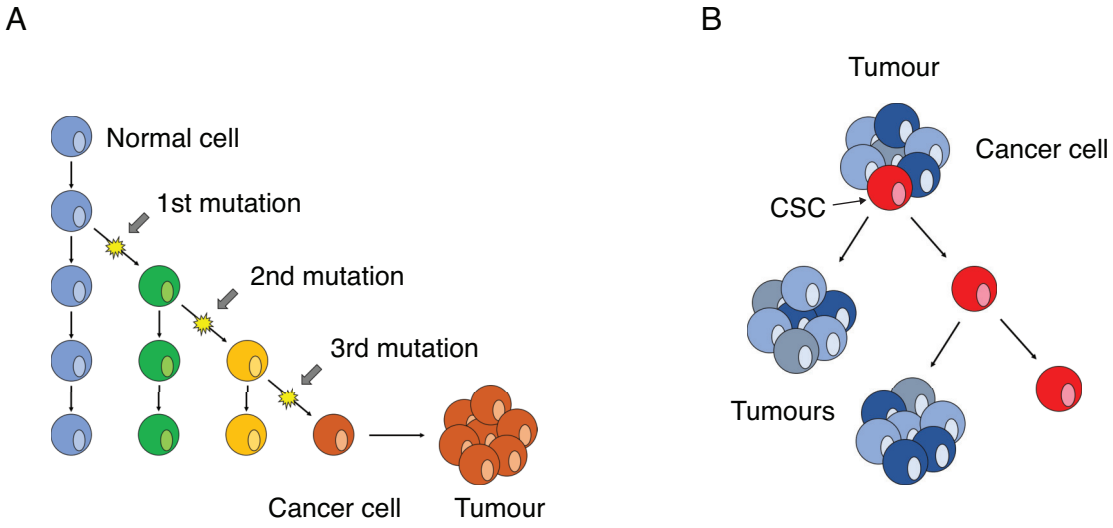


Figure 2.1: Models for Tumour Hierarchy: A) Tumourigenesis based on accumulation of mutations to form cancer cells (clonal model). B) Self-renewing cancer stem cell (CSC) as a origin of an tumour (CSC model). Adapted from Bradshaw et al., 2016; Adams and Strasser, 2008

These characteristics of cancer cell origin is reflected in the classification of CNS tumours.

2.2 Central Nervous System Tumours

In 2016, the World Health Organization (WHO) proposed a new classification system for CNS tumours based on the combination of geno- and phenotype information (Louis et al., 2016). CNS tumours are primarily diagnosed according to their cell/tissue of origin. Eighty percent of the CNS tumours are gliomas (arising from glial cells or their precursors of the brain). Thirty percent of all gliomas are malig-

nant. Glioblastoma multiforme (GBM), a WHO grade IV astrocytoma, is the most frequent and most malignant glioma. It is characterised by high heterogeneity (hence 'multiforme') due to the histopathological appearances of necrosis and microvascular hyperplasia. They are categorized into primary and secondary GBM. Primary GBM are *de novo* tumours affecting elderly patients, which show no histological precursor lesions. The diagnosis of secondary GBM requires evidence of progression from low-grade astrocytoma to a high-grade GBM and is primarily found in middle-aged adults (age between 30-45). Primary and secondary GBM are genetically different and can be distinguished by their *Epidermal growth factor receptor* (*EGFR*) and *isocitrate dehydrogenase* (*IDH*) status (Ohgaki and Kleihues, 2013). Primary GBM typically has high *EGFR* amplification in addition to *phosphatase and tensin homolog* (*PTEN*) mutations and loss of chromosome 10 ((Ohgaki and Kleihues, 2007; Fujisawa et al., 2000; Ohgaki et al., 2004)). *IDH* and *ATP-dependent helicase* (*ATRX*) mutations have been identified as marker for secondary GBM (Fig. 2.2). Other GBM mutations include *tumour suppressor 53* (*TP53*), *telomerase reverse transcriptase* (*TERT*) and *H3 histone family member 3A* (*H3F3A*, H3.3 K27M), the latter particularly in paediatric glioma.

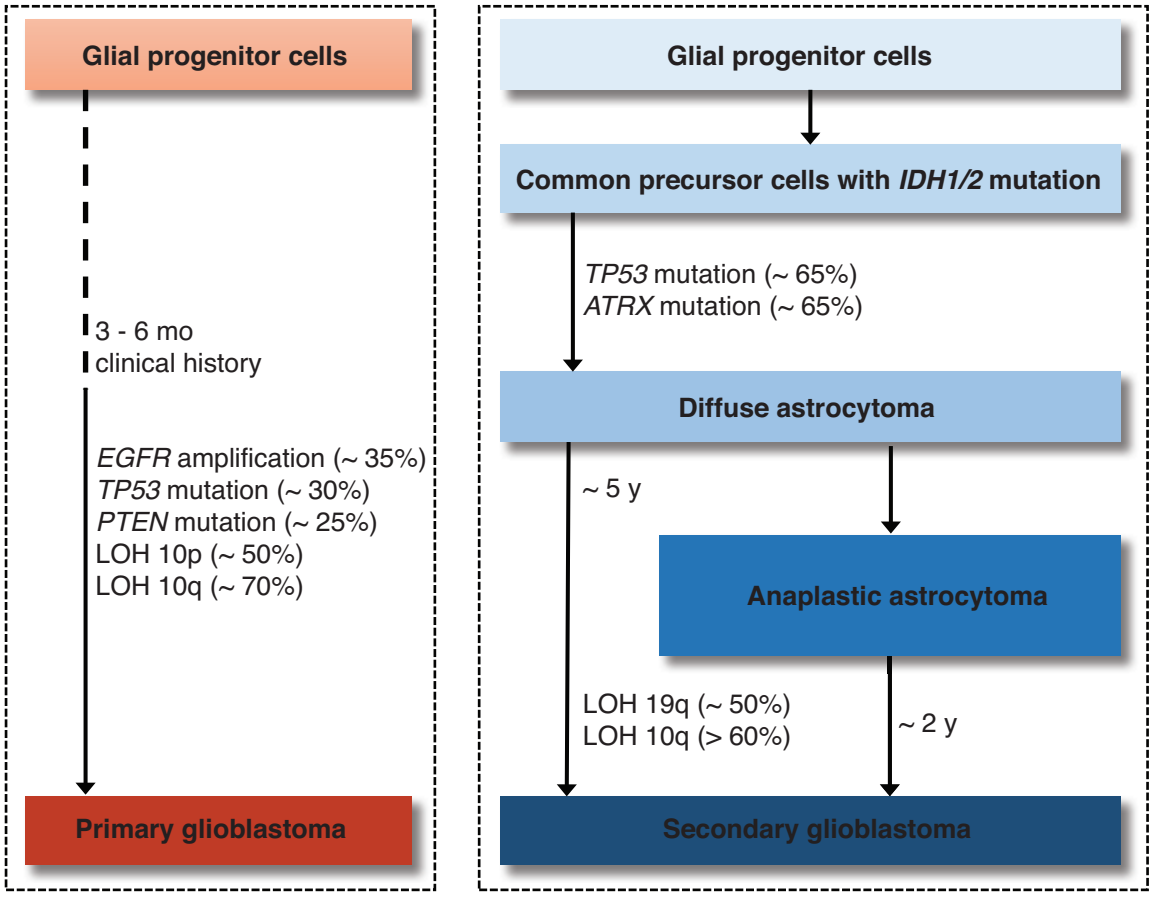


Figure 2.2: Genetic characterisation of primary and secondary glioblastoma: Primary glioblastomas are characterised by a short clinical history with mutations in *EGFR*, *TP53*, and *PTEN* and loss of heterozygosity (LOH) on chromosome 10. Secondary glioblastomas are marked by *IDH*, *TP53*, *ATRX* mutations and can arise from diffuse or anaplastic astrocytomas. Adapted from Ohgaki and Kleihues, 2013

IDH is an enzyme converting isocitrate to α-ketoglutarate. *IDH* mutations (IDH1: most frequent R132H, rare R132S, R132C, R132G and R132L; IDH2 R172K) are gain-of-function mutations leading to an abnormal production of 2-hydroxyglutarate (2HG) from α-ketoglutarate. Two-HG is considered to promote oncogenesis by stabilising hypoxia-inducible factor 1-alpha (HIF1α) and destabilising collagens (Sasaki et al., 2012). In addition, IDH mutant tumours display a hypermethylation phenotype due to the misregulation of α-ketoglutarate-dependent demethylating enzymes, which include ten-eleven translocation (TET) and the lysine-specific demethylases (KDMs, also known as jumonji-C domain-containing histone demethylases [JHDMs])

(Chowdhury et al., 2011; Xu et al., 2011). In 2012, Lu *et al.* conducted a study proving the inhibitory effect of 2HG on demethylation resulting in impaired differentiation (Lu et al., 2012). Indeed, dedifferentiation or inhibition of differentiation is considered as one of the hallmarks of cancer. Evaluation of high plasticity and therefore heterogeneity of GBM is surrogate for low treatment outcome and prognosis. GBM treatment is patient-centered, with radio- and/or chemotherapy (standard temozolomide, a DNA methylating agent) combined with surgery if the tumour is accessible. The median survival of GBM is less than two years.

2.3 Cancer Stem Cells in the Brain

The hierarchical tumour model (Fig. 2.1) is widely recognised over the clonal model, particularly in paediatric cancer. Brain and CNS tumours are the second most common cancers (after leukemia) in children and they are the leading cause for cancer-related death during childhood. The most prevalent paediatric CNS tumour is medulloblastoma. Medulloblastomas are embryonal tumours arising in the cerebellum or brain stem. Neuroblastoma (NB, embryonal) are the most common extracranial tumours in children. Both tumour types are acknowledged to arise from neural precursor cells (neuroblasts) or potentially dedifferentiated cells. In 2013, Jafri *et al.* undertook a retrospective study of 91 GBM patients by analysing their pre- and post-treatment magnetic resonance images (Jafri et al., 2013). They grouped the GBMs according to the involvement of the SVZ and cortex (Lim et al., 2007) and found that GBMs connected to both show worst overall and progression free survival (Fig. 2.3).

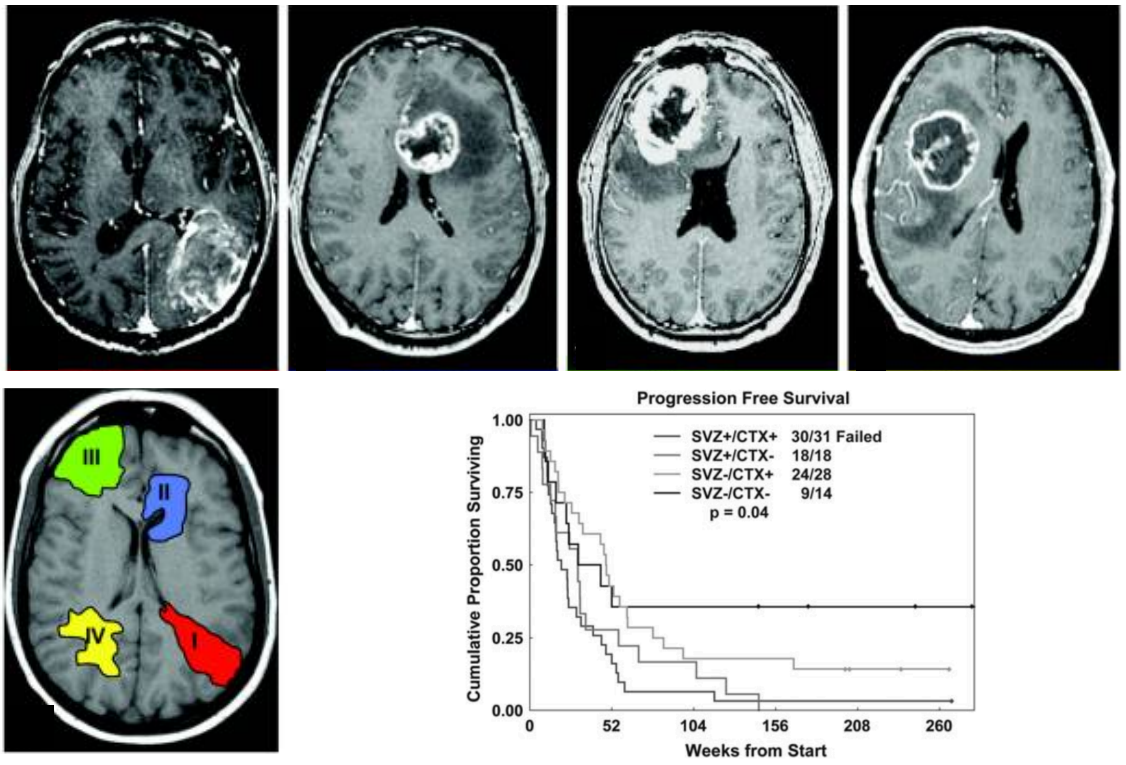


Figure 2.3: Classification of GBMs according to their Location: GBMs were categorised according to the involvement of the subventricular zone (SVZ) and/or the cortex (CTX), with I) tumours connected to SVZ and cortex, II) tumours connected to SVZ only, III) tumours connected to cortex only and IV) no involvement of SVZ or cortex. Adapted from Lim et al., 2007; Jafri et al., 2013

The fact that GBMs connected to the SVZ are more invasive and resistant to therapy is thought to be due to the potential of CSCs to repopulate the tumour. Events that are involved in the transformation of a normal to a cancer stem cell include mutations, dysregulation of the stem cell niche and impaired differentiation or dedifferentiation. Stem and cancer cells share the ability to indefinitely proliferate and differentiate into multiple lineages. These processes are regulated by intrinsic and extrinsic regulatory mechanisms, including the microenvironment, niche factors and the immune system as extrinsic and genetic, epigenetic and metabolic as intrinsic factors (reviewed in Lathia et al., 2015). In GBMs, signalling pathways known to

regulate brain development, are often 'hijacked' to sustain a pro-proliferative and invasive state (reviewed in Mehta and Lo Cascio, 2018). Notch signalling has been shown to regulate NSC self-renewal and is crucial to maintain the NSC pool *in vivo* (Alexson et al., 2006). However Jeon *et al.* showed that increased Notch signalling leads to malignant transformation of astrocytes (Jeon et al., 2008). The Transforming growth factor beta (TGFβ) pathway is known to regulate neural development (reviewed in Meyers and Kessler, 2017) and has also been shown to drive invasiveness of CSCs by enhancing cell migration (reviewed in Golestaneh and Mishra, 2005; Mehta and Lo Cascio, 2018). Additionally to cell signalling, stem and cancer stem cells often share the expression of certain markers including Cluster of differentiation (CD) 44, CD133, SOX2 and NESTIN (reviewed in Ortensi et al., 2013). The potential of NSC to migrate to sites of brain injury for repair (Imitola et al., 2004) has been associated with the invasiveness of CSCs (Ortensi et al., 2013) and the developmental process of epithelial-to-mesenchymal transition (EMT), associated with differentiation, is widely discussed as a major driver of GBM progression (Iwadate, 2016). A hallmark of CSCs that distinguishes them from normal stem cells is the impaired differentiation potential (Caren et al., 2015).

One of the major regulatory mechanisms of how differentiation is controlled, has been identified as epigenetics.

Chapter 3

Epigenetics

The term epigenetics was first introduced by Waddington in 1939 to differentiate between a certain geno- and phentotype. The Greek prefix 'επι' means 'over' or 'above' and can be understood as a term for genetics independent from deoxyribonucleic acid (DNA) sequence alterations. Today, epigenetics refers to heritable gene expression or the transcriptional memory of a cell.

Every somatic cell of an organism stores the information for the whole genome within their DNA sequence, however when cells differentiate and become more specialised, the gene expression pattern undergoes massive changes. In 1957, Waddington proposed a stochastic model of how cells differentiate to specific lineages (Fig. 3.1) After Takahashi and Yamanaka introduced iPSCs (Takahashi and Yamanaka, 2006), the landscape had to be redefined.

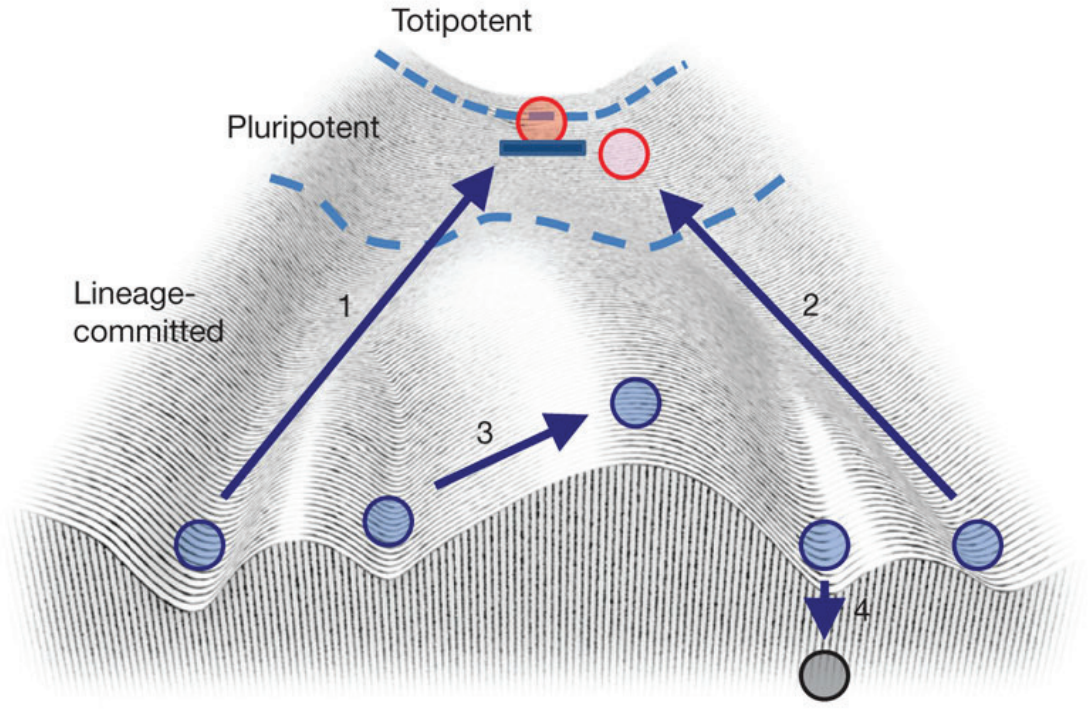


Figure 3.1: Waddington's Epigenetic Landscape: The most potent cell (totipotent) is on the top of an epigenetic landscape. When the 'cell rolls down the landscape', it commits to a certain cell lineage. Once differentiation is initiated, the cell cannot naturally stop or reverse its direction. After the invention of iPSCs, cells were able 'to roll up the landscape' by forced reprogramming (1). Additionally, cells have been shown to be able to 2) de- and 3) transdifferentiate or undergo 4) apoptosis or senescence. Waddington, 1957; Takahashi and Yamanaka, 2006; Yamanaka, 2009, Reprinted with permission of Nature Publishing Group, licence number 4195960940926

Over the last decade it has been controversially discussed to what extent iPSCs exhibit an epigenetic memory of their cell of origin. Differences in the establishment of the pluripotency ground state were reported depending on the tissue of origin, suggesting epigenetics as a barrier to reprogramming. Especially in the context of tumourgenesis it is important to understand the molecular and epigenetic mechanisms controlling cell differentiation and potential transition from normal to malignant cells to improve diagnostics and cancer therapy.

3.1 Epigenetic Mechanisms

Epigenetic mechanisms include the modification of histones, RNA interference and DNA methylation (Fig. 3.2)

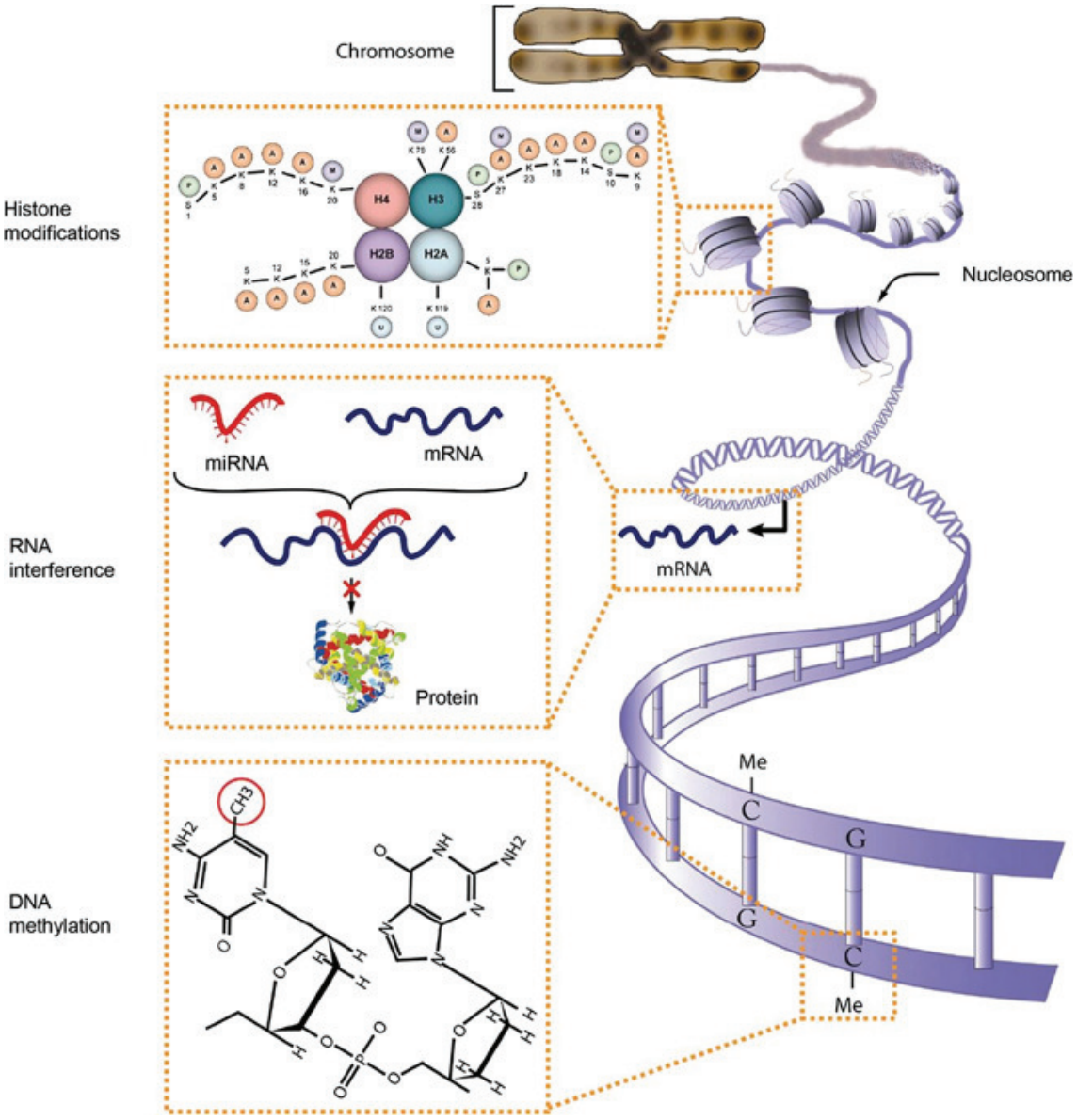


Figure 3.2: Mechanisms for Epigentic Gene Regulation Histone modifications, RNA interference (RNAi) and DNA methylation are three different mechanism to regulate gene expression. RNAi and DNA methylation are gene silencing mechanisms. Histone modification can be gene activating or repressing depending on the histone and the modification. Puumala and Hoyme, 2015, Reprinted with permission of American Academy of Pediatrics, licence number 4200441190221

Micro-ribonucleic acids (miRNAs) are post-transcriptional regulators that either prevent translation of messenger RNAs (mRNAs) by imperfect complementary binding (translation repression) and are therefore able to target up to several hundred genes simultaneously or they target mRNAs for degradation by a higher degree of complementarity (He and Hannon, 2004; Wahid et al., 2010). MiRNAs are small, non-coding RNAs (21-25 nucleotides) transcribed as long primary transcripts from either coding genes or intron regions of independent genes. After processing by RNA nucleases (Ribonuclease 3 DROSHA and Endoribonuclease DICER) (Denli et al., 2004) they bind to their target mRNA and recruit the RNA-induced silencing complex (RISC) which either directs the cleavage of the mRNA or facilitates the translational repression mechanism (Wahid et al., 2010). Another epigenetic mechanism for gene silencing is DNA methylation. The covalent binding of a methyl group (CH_3) to the 5th carbon atom (C5) of cytosine residues (5-methylcytosine, 5mC) is referred to as DNA methylation. DNA methylation is cumulated at, but not restricted to cytosine-phosphate-guanine (CpG) dinucleotide motifs known as CpG islands. Frequently these sites are regulatory gene elements, e.g. promoter or enhancer regions, and therefore represent a modification for gene repression. Methylation of DNA can either prevent transcription factors (TFs) from binding or can create new binding sites to allow repressor occupation. DNA methylation has been shown to be an initiating event for repressive histone modifications. Methylated DNA is recognised by methyl-CpG-binding domain proteins (MBD1-3) and methyl-CpG-binding protein 2 (MECP2) which recruit histone modifiers to establish repressive histone marks (Curradi et al., 2002). DNA methylation is catalysed by DNA methyl transferases (DNMT). Three DNMTs have been identified in humans: DNMT1 for DNA methylation maintenance during DNA replication and DNMt3a, DNMT3b for *de novo* DNA methylation. DNMTs received massive attention as cancer therapy targets due to their ability to establish a hypermethylated gene profile which can result in methylation and silencing of tumour suppressors (Serra et al.,

2014).

The third epigenetic mechanism that regulates gene expression is histone modifications. Histones are alkaline proteins that have an essential role in DNA packaging and chromatin condensation. The four core histones are H2A, H2B, H3 and H4. Two copies of each of the core histones form a octamer protein complex where 146 base pairs (bp) of DNA are wrapped around in superhelical turns. This basic unit of chromatin is called nucleosome (Luger et al., 1997). Nucleosomes are connected by linker DNA (up to 80 bp). The N-terminal protein tails of the histones are unstructured and project out of the histone core. These histone tails are targets of several post-translational modifications (PTMs) including phosphorylation, methylation, acetylation, ubiquitination and citrullination (reviewed in Bannister and Kouzarides, 2011). These histone PTMs contribute to either gene expression or repression depending on the amino acid targeted and the type of modification of the histone tail. Additionally, certain histone modifications influence the chromatin compaction state and are therefore needed to pack DNA in chromosomes. 'Open' chromatin structures consist of chains of nucleosomes reflecting decondensed, transcriptionally active DNA regions. This is referred to as euchromatin (Barski et al., 2007). Histone acetylation is a hallmark of euchromatin, even though this is not exclusive to this chromatin structure (Allis and Jenuwein, 2016). Acetylation of lysines weakens the interaction between negatively charged DNA and histones by neutralising their basic charge (Hong et al., 1993; Megee et al., 1995). In addition, acetylated lysine residues create binding motifs for bromodomain-containing proteins, first identified in 1999 (Dhalluin et al., 1999). Compacted, repressive chromatin, called heterochromatin, is formed by folding and looping nucleosomes and is generally characterised by histone methylation. Constitutive heterochromatin is found in genomic regions of permanently silenced genes whereas facultative heterochromatin characterises regions of differentially expressed genes (Bannister and Kouzarides, 2011).

3.2 The Histone Code

As previously mentioned, the N-terminal tails of the histones are unfolded and project out from the nucleosome plateau. The amino acids of these tails are subjects of several modifications established by enzymes known as histone 'writers'. Writers are histone methyltransferases (HMTs), histone acetyltransferases (HATs), kinases and ubiquitin ligases (E3). Different enzymes establish different marks regarding the chemical modification and the number. These histone modifications then create a binding site for histone 'readers'. Readers of the histone code are recruited to their modification site and subsequently regulate the transcription of the associated gene. Several protein domains that recognize specific histone modifications have been identified (reviewed in Yun et al., 2011). The most common domains recognising methylated lysines are chromatin organization modifier domain (chromo, Bannister et al., 2001), Tudor domain (Kim et al., 2006), malignant brain tumour domain (MBT, Kim et al., 2006) and plant homeodomain (PHD, Zeng et al., 2010). Tudor also recognizes arginine methylation. Bromo (Jeanmougin et al., 1997) and PHD domains recognise lysine acetylation. Histone modifications can be removed by 'erasers', i.e. histone demethylases (HDMs), deacetylases (HDACs), phosphatases and deubiquitinating enzymes (DUBs). The modification of histones is not only important for gene transcription regulation, but also contributes to the regulation of DNA damage repair (DDR, γH2AX), X-chromosome inactivation (by H3K27me3) and mitosis (H3S10 phosphorylation Bannister and Kouzarides, 2011).

3.3 Histone modifications during Differentiation

When cells undergo differentiation, the whole transcriptome changes to support cell type specific function of the mature cell. Stemness genes need to be permanently silenced, whereas the expression of differentiation genes needs to be induced and

constantly expressed. Chromatin remodelling allows or represses access to genomic regions coding for specific genes. From an epigenetic point of view, a certain gene can be in a repressed, poised or active state depending on the modifications of the histones near its promoter. A poised or bivalent chromatin state is characterised by opposing chromatin marks to ensure a rapid expression response in accordance to signals (Bernstein et al., 2006). A bivalent chromatin mark is the trimethylation of lysine 4 and 27 at histone H3 (H3K4me3 and H3K27me). H3K4me3 alone is an activation mark, whereas H3K27me marks gene repressive regions. Poletti *et al.* showed that during neural differentiation of hESCs, the repressive mark H3K27me3 is removed from the majority of the bivalent genes (Poletti et al., 2015). Genes that retain the activation mark were identified as lineage regulatory or differentiation genes (Bernstein et al., 2006) suggesting that these genes have a poised chromatin state to ensure an immediate gene expression following differentiation-inducing stimuli. Other activation marks were identified as H3K9 acetylation (H3K9ac) and H3K14ac. Their co-occurence is a hallmark for active gene promoters (Karmodiya et al., 2012). H3K27me3 is a critical chromatin mark for gene repression during cell differentiation and its unique methyltransferase enhancer of zeste homolog 2 (EZH2) (Kuzmichev et al., 2002) is the catalytic subunit of the polycomb repressive complex 2 (PRC2). PRC2 has been shown to suppress differentiation genes, potentially to maintain pluripotency (Boyer et al., 2006).

A repression mark that is established during differentiation is the trimethylation of H3K9 (Meshorer et al., 2006). H3K9me3 is a silencing histone mark responsible for formation of heterochromatin. It has been found to silence pluripotency genes in neurons (Golebiewska et al., 2009). In humans, eight HMTs exist, among which four are known to catalyse the trimethylation of H3K9. Suppressor of variegation 3-9 homolog 1 (SUV39H1), SUV39H2, SET domain bifurcated 1 (SETDB1) and SETDB2 all contain a SET domain, first identified in *Drosophila melanogaster* and named after Suppressor of variegation 3-9 Su(var)3-9, Enhancer of zeste and Trithorax (Rea

et al., 2000). The SET domain catalyses the addition of a methyl-group by targeting the ε-amino group of the lysine residue using the co-factor S-adenosyl-L-methionine (SAM) (Herz et al., 2013). Interestingly, SET domain-containing proteins have been shown to also methylate other non-histone proteins. Acetylated H3K9 marks active transcription sites. The NAD-dependent protein deacetylase sirtuin 1 (SIRT1) is responsible for removing the acetyl group. Protein G9a (G9a) and G9a-like protein 1 (GLP), two members of the SUV39 sub-family of HMTs, facilitate the mono- and dimethylation of H3K9 (Tachibana et al., 2005). Noteworthy, histone modifications not only influences gene expression by activating or repressing its transcription due to accessibility, but also due to post-transcriptional mechanisms, e.g. mRNA splicing (particularly H3K36me3, Kim et al., 2011).

3.4 SUV39H1 in Neural Differentiation

SUV39H1 was first identified in screens as a suppressor of position-effect variegation, a tool to study chromatin regulation in *Drosophila melanogaster* (Tschiersch et al., 1994). It trimethylates H3K9 using the monomethylated lysine residue (an active histone mark) as a substrate and mediating its di- and trimethylation (Rice et al., 2003). The N-terminal chromo domain of SUV39H1 recognises the H3K9me1 mark and its C-terminal SET domain catalyses additional methylation. The H3K9me3 mark creates a binding site for heterochromatin protein 1 (HP1) (Aagaard et al., 1999; Bannister et al., 2001), which facilitates heterochromatin compaction. *Suv39h1/Suv39h2* double knock-out mice develop normally until embryonic day E12.5 but show impaired growth and genome stability due to pericentric heterochromatin defects leading to pre-natal lethality. Additionally, the knock-out mice were predisposed to B-cell lymphomas (human Non-Hodgkin lymphomas) (Peters et al., 2001). Overexpression of *Suv39h1* in mice led to growth retardation and

impaired erythroid differentiation (Czvitkovich et al., 2001). SUV39H1 has been shown to be upregulated in glioma cell lines and glioma tissue compared to normal brain tissue and its knock-down resulted in a decreased proliferation, migration and clonogenic ability (Spyropoulou et al., 2014). Additionally, elevated levels of SUV39H1 in glioma has been shown to have anti-apoptotic effects (Lai et al., 2017). In cerebral ischemia models, a brain condition characterised by hypoxic metabolic stress due to shortage of blood supply in the brain, SUV39H1 inhibition exhibited neuroprotective functions (Schweizer et al., 2015). Inhibition of SUV39H1 in the hippocampus improved age-dependent deficiencies in mice (Snigdha et al., 2016) and Brain-derived neurotrophic factor (BDNF)-mediated degradation of SUV39H1 stimulated neurite outgrowth in mouse cortical neurons (Sen and Snyder, 2011). Interestingly, SUV39H1 has been shown to directly silence OCT4 during mESC differentiation (Scarola et al., 2015) and it is crucial in zebrafish development for terminal differentiation (Rai et al., 2006). As SUV39H1 is crucial for differentiation during development, high levels in glioma suggests pro-cancerous effects, suggesting SUV39H1 has various roles during embryonic development and tissue homoeostasis in adulthood. SUV39H1 protein stability and activity has been shown to be regulated by several mechanisms. SIRT1 regulates SUV39H1 activity by deacetylating lysine 266 within the SET domain and enhancing SUV39H1 activity towards H3K9me3 (Vaquero et al., 2007). Interestingly, SIRT1 has been shown to be required for the oncogenic transformation of human neural stem cells (Lee et al., 2015). Furthermore, SUV39H1 protein degradation is mediated by E3 ubiquitin ligase Double minute 2 protein (MDM2) and Ubiquitin-specific-processing protease 7 (USP7, also know as HAUSP) (Mungamuri et al., 2016). Interestingly, MDM2 self-ubiquitination is suppressed by complex formation of MDM2 with USP7 and Death domain-associated protein 6 (DAXX). This complex is interrupted by Ras association domain-containing protein 1 isoform A (RASSF1A), an upstream regulator of the Hippo signalling pathway, which destabilises MDM2 (Song et al., 2008b).

Chapter 4

The Hippo Signalling Pathway

The Hippo pathway, first discovered in *Drosophila* screens, is a fundamental and evolutionarily highly conserved signalling pathway that regulates organ growth, stem cell function and tissue regeneration (Fig. 4.1, reviewed in Zhao et al., 2011). The mammalian core players of the Hippo pathway are the related serine/threonine kinases, mammalian STE20-like protein kinase 1 (MST1) and MST2 and the large tumour suppressor 1 (LATS1) and LATS2. MST1/2 in complex with the non-catalytic protein Salvador homologue 1 (Sav1) phosphorylate LATS1/2 (Chan et al., 2005) and the regulatory proteins Mps one binder (MOB1A/B), which in turn enhances complex formation (Praskova et al., 2008) and activates LATS1/2. Activated LATS1/2 phosphorylates the transcriptional co-activator Yes-associated protein (YAP) on five different consensus HXRXXS motifs (Zhao et al., 2007), as well as its vertebrate-specific paralogue Transcriptional co-activator with PDZ-binding motif (TAZ). Phosphorylation at serine 127 of YAP (Ser89 for TAZ) creates a binding consensus to the adaptor protein 14-3-3. As part of the canonical pathway binding to 14-3-3 leads to cytoplasmic retention and consequent inactivation of YAP/TAZ (Kanai et al., 2000). Accumulation in the cytoplasm stimulates their ubiquitin-mediated proteolysis (Zhao et al., 2010). However, Matallanas *et al.* showed that phosphorylation of YAP in response to proapoptotic signals promotes the complex formation with p73 in the nucleus, which induces transcription of apoptotic genes

(Matallanas et al., 2007).

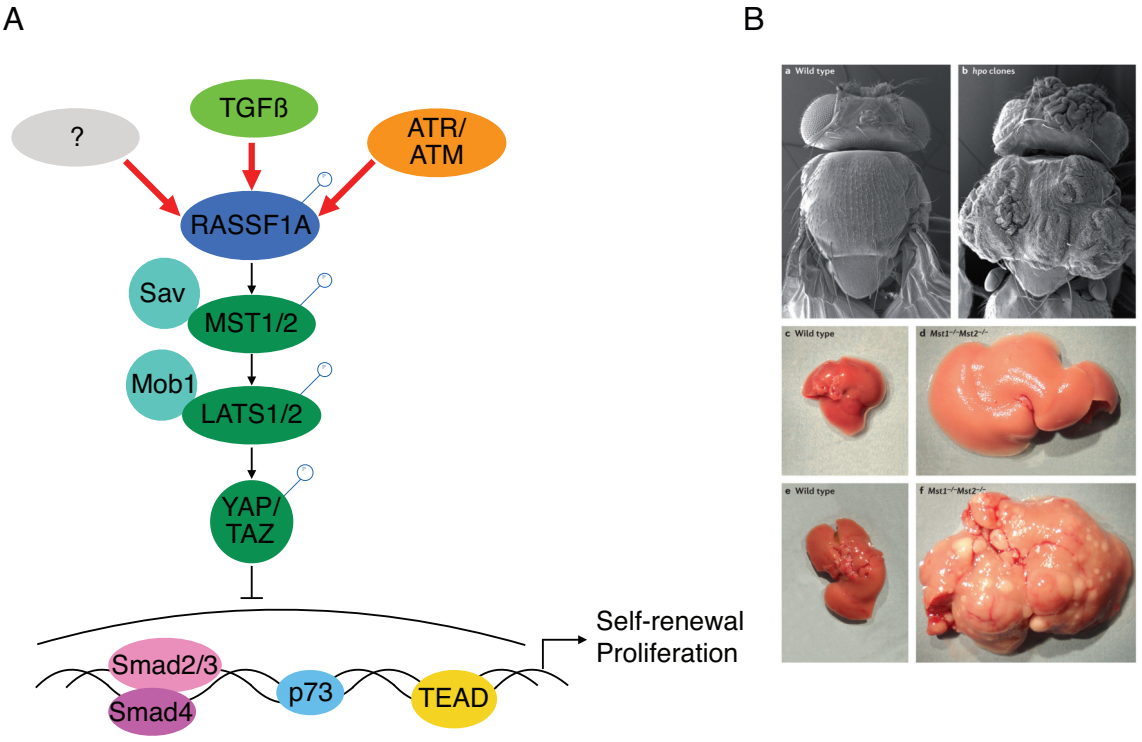


Figure 4.1: Mammalian Hippo Signalling Pathway: A) The mammalian Hippo Pathway core components (MST1/2, LATS1/2, YAP/TAZ) including RASSF1A, an upstream regulator of the pathway. RASSF1A is regulated by ATR/ATM and Transforming growth factor beta (TGFβ). Smads, p73 and TEAD are transcription factors co-regulated by YAP/TAZ. B) Electron microraphs of wildtype and Hippo (Hpo) mutant *Drosophila* and mouse MST1/2 double mutant liver. Johnson and Halder, 2014 Reprinted with permission of Nature Publishing Group, licence number 4200221269691

When the Hippo pathway is switched off, YAP/TAZ remain in the nucleus and form complexes with transcription factors, e.g TEADs and SMADs, to drive or suppress their target gene expression. These genes are known to regulate cell fate, regeneration, stemness, apoptosis, EMT/metastasis and mechanotransduction (reviewed in Varelas, 2014).

4.1 Hippo Signalling in Cancer Stem Cells

Hippo pathway dysregulation is described in many various cancers (Harvey et al., 2013) and components of the pathway are discussed as prognostic markers (Misawa et al., 2009; Xu et al., 2009) and therapeutic targets (Park and Guan, 2013). The Hippo pathway has a role in regulation of proliferation, self-renewal, differentiation and apoptosis. In hESCs, TAZ maintains pluripotency by controlling nuclear shuttling of Smads and therefore mediating TGFβ signalling (Varelas et al., 2008). However, Zhou *et al.* showed that TAZ has different roles in regulation of differentiation dependent on the localisation. TAZ nuclear translocation induced differentiation in mouse epiblast stem cells (Zhou et al., 2017). Hippo pathway has been shown to maintain quiescence of NSCs in *Drosophila* (Ding et al., 2016) and elevated levels of YAP is associated with medulloblastoma (Fernandez et al., 2009) and GBM growth (Orr et al., 2011). Additionally to the co-effectors YAP/TAZ, different components of the Hippo pathway have been found to play a role in stem cells and cancer. In *Mst1/2* double knock-out mESCs, differentiation into the three germ layers was distorted (Li et al., 2013). Neurofibromin (NF2 or Merlin) is an upstream regulator of the Hippo Pathway and has been found to be decreased in GBM (Lau et al., 2008). Other upstream modulators of the Hippo pathway that have been identified include Cluster of differentiation 44 (CD44, Xu et al., 2010), Kidney and brain protein (KIBRA, Genevet et al., 2010; Moleirinho et al., 2013) and G protein-coupled receptors (GPCRs, Yu et al., 2012) (revieved in Yu and Guan, 2013).

In 2000, Dammann *et al.* first identified a new gene, RASSF1 on the common homozygous deletion area 3p21.3 (Dammann et al., 2000), which later was described to regulate MST1/2 (Avruch et al., 2006) and was ultimately identified as part of the *Drosophila* Hippo/Salvador/Lats tumour suppressor pathway (Guo et al., 2007).

4.2 Ras association domain-containing protein 1

The RASSF1 gene consists of eight exons, which are either alternatively spliced (isoforms A, D, E, F, G) or transcribed from a different promoter region (isoforms B, C, H). The two predominant isoforms, RASSF1A and RASSF1C are ubiquitously expressed in normal tissues (Fig. 4.2), RASSF1B is expressed predominantly in cells of haematopoietic origin and isoforms D and E are specifically expressed in cardiac and pancreatic cells, respectively (Agathanggelou et al., 2005). *RASSF1A* and *RASSF1C* share exon 3-6, which encode an ataxia telagectasia mutated (ATM) kinase phosphorylation consensus sequence (Hamilton et al., 2009), a RalGDS/AF6 or Ras-association (RA) domain and a SAv/RAssf/Hippo (SARAH) domain associating with MST1/2 (Praskova et al., 2004; Matallanas et al., 2007; Weyden and Adams, 2007).

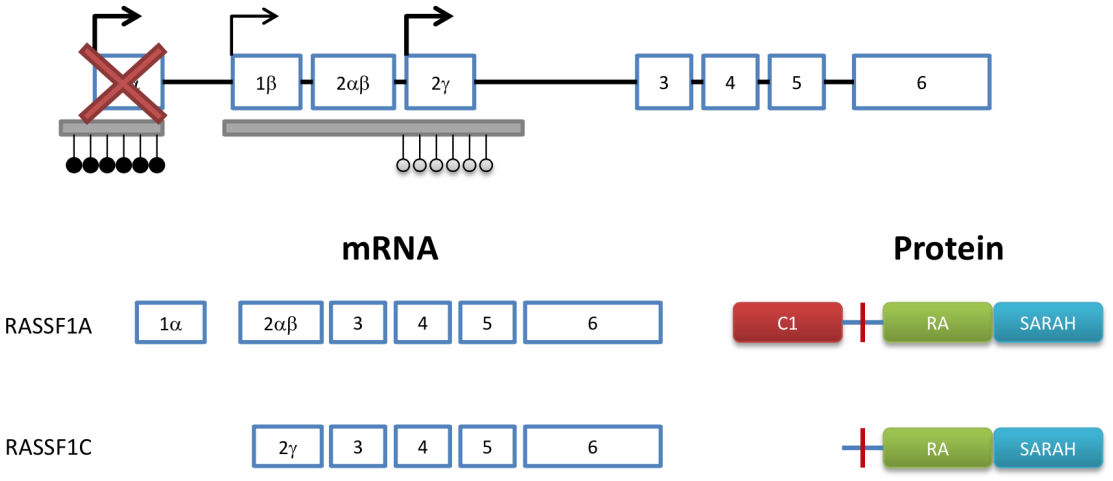


Figure 4.2: The structure of RASSF1A and RASSF1C *RASSF1A* consists of exons 1α, 2α/β, 3, 4, 5, and 6, while *RASSF1C* is a product of exons 2γ, 3, 4, 5, and 6. Both isoforms contain a SARAH and a C-terminal RA domain. RASSF1A has an additional C1 domain that is not present in RASSF1C. Adapted from Weyden and Adams, 2007

RASSF1A is a well established tumour suppressor and methylation or loss of the

gene is associated with various cancers (Kunstman et al., 2013; Visnovsky et al., 2013; Daniunaite et al., 2014; Drilon et al., 2014; Hagrass et al., 2014). It is known to inhibit the accumulation of cyclin D1 and hence block cell cycle progression (Shivakumar et al., 2002). In addition, activation of RASSF1A by ATM (Hamilton et al., 2009), the death receptor Tumor necrosis factor receptor superfamily member 6 (Fas, Vichalkovski et al., 2008) or Tumor necrosis factor alpha (TNFα, Foley et al., 2008) promotes pro-apoptotic signals whilst depletion of RASSF1A in retinal pigment epithelial cells results in disrupted cell migration and polarity (Arnette et al., 2014). RASSF1A is phosphorylated and activated by ATM/ATR in response to DNA damage (Hamilton et al., 2009; Pefani et al., 2014). Upon phosphorylation, RASSF1A dimerises, which enhances the association to MST2 and stimulates its kinase activity, hence activating the Hippo pathway. On the contrary, RASSF1C is often expressed in cancer and is described to promote growth and reduce sensitivity to chemotherapeutics (Reeves et al., 2013). Guo *et al.* reported an opposed expression of RASSF1A and RASSF1C in oesophageal squamous cell carcinoma (Guo et al., 2014) and the expression ratio of RASSF1A to RASSF1C is suggested to be a critical prognostic factor (Reeves et al., 2013).

Epigenetic changes like DNA methylation and histone acetylation play a crucial role in cancerogenesis. However, the inheritance of gene expression patterns to maintain a distinct transcriptional state are evolutionarily important for gene silencing, position-effect variegation, X-chromosome inactivation, transcriptional memory and cellular differentiation (Foster et al., 2007; Saxena and Carninci, 2011; Elgin and Reuter, 2013, reviewed in Bheda and Schneider, 2014). *RASSF1A* is often methylated in brain tumours (reviewed in Dammann et al., 2005), including glioma (Gao et al., 2004), glioblastomas (Piperi et al., 2010), medulloblastomas (Lusher et al., 2002) and neuroectodermal tumours (Inda and Castresana, 2007). In neuroblastomas, *RASSF1A* methylation negatively correlates with overall and relapse-free survival (Misawa et al., 2009). Additionally, it has been reported that tumours con-

nected with the subventricular zone (SVZ) are associated with tumour progression and worse survival (Jafri et al., 2013; Carlsson et al., 2014). However, the biological relevance and functions of RASSF1A in stem and cancer cells remains elusive.

4.3 Aim of Study

To understand how tumourigenesis arises it is important to understand normal cell physiology. Our lab has recently been shown that RASSF1A contributes to the differentiation potential of mouse embryonic stem cells by modulating YAP binding partners to mediate differentiation (Papaspyropoulos et al., 2018). Loss of RASSF1A expression by epigenetic silencing due to promoter methylation has been described in various brain tumours (Dammann et al., 2005; Munoz et al., 2012). Additionally, LOH at chromosome 3p21, the RASSF1A locus, is associated with neural crest-derived tumours including phaeochromocytomas and neuroblastomas (Astuti et al., 2001; Ejeskar et al., 1998). Furthermore, RASSF1A is a key regulator of apoptosis (Matallanas et al., 2007; Levesley et al., 2011), restricts cell cycling (Donninger et al., 2014) and contributes to genomic stability (Pefani et al., 2014; Pefani and O'Neill, 2016) which classifies RASSF1A as a tumour suppressor. RASSF1A methylation is linked to paediatric and secondary glioblastoma (Munoz et al., 2012) and has been shown to be of prognostic value in neuroblastomas (Misawa et al., 2009), a tumour affecting primarily children. Childhood cancers are often referred to as developmental malignancies (Federico et al., 2011) as exposure to mutagens is limited. To date, nothing is known about the role of RASSF1A in the developing nervous system. In this project, we ask whether RASSF1A is an essential driver of differentiation in human embryonic and fetal stem cells focussing on neuroectodermal and neural differentiation. We are particularly interested in the loss of RASSF1A and its potential contribution to the transformation of non-malignant cells. Furthermore,

we ask if the loss of RASSF1A impairs the differentiation capacity of normal cells and if potential effects are Hippo pathway dependent. RASSF1A has been shown to interact with the methyltransferase SUV39H1 (Weimann et al., 2013). Additionally, the Hippo kinase MST1 was reported to phosphorylate H2BS14, a chromatin mark associated with apoptosis (Cheung et al., 2003). YAP/TAZ, the Hippo pathway effectors, recruit the nucleosome remodelling and histone deacetylase (NuRD) complex to restrict chromatin accessibility by H3 deacetylation leading to transcriptional repression of target genes (Kim et al., 2015). Therefore, we ask if loss of RASSF1A/Hippo signalling due to epigenetic silencing contributes to glioblastoma onset by disrupting the crosstalk between RASSF1A/Hippo signalling from the epigenetic machinery regulating differentiation.

Part II

Materials and Methods

Chapter 5

Materials

5.1 General Laboratory Reagents

Tissue culture plastic was purchased from Sarstedt and Corning and glassware was purchased from Fisher and VWR, unless otherwise stated.

Table 5.1: Chemicals

Name	Provider
2-Propanol	Fisher Scientific
Agarose	Sigma-Aldrich UK
ATM inhibitor (KU55933)	Merck
ATR inhibitor (VE821)	Merck
Bis-Tris 20x buffer	Thermo Fisher Scientific
Bovine serum albumin (BSA)	Thermo Fisher Scientific
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich UK
Dithiothreitol (DTT)	Sigma-Aldrich UK
Dual Colour Brighter	Biorad
Ethanol (EtOH)	Fisher Scientific

Continued on next page

Table 5.1 – *Continued from previous page*

Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich UK
Formaldehyde (PFA)	Thermo Fisher Scientific
Glycine	Fisher Scientific
HiMark Pre-Stained Protein Standard	Life technologies
Hydrochloric acid	Fisher Scientific
LB broth	Sigma-Aldrich UK
Lipofectamine 3000	Thermo Fisher Scientific
Methanol (MeOH)	Fisher Scientific
Milk powder	Marvel
NEM	Sigma-Aldrich UK
NuPAGE MOPS SDS Running Buffer (20X)	Thermo Fisher Scientific
Phosphatase inhibitors	Roche
Phosphate buffered saline (PBS) tablets	Oxoid
Protease inhibitors	Roche
ROCK inhibitor Y	Tocris
Sodium chloride	Fisher Scientific
Sodium deoxycholate	Sigma-Aldrich UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich UK
Tris Acetate 20x buffer	Thermo Fisher Scientific
Tris base	Fisher Scientific
Triton X 100	Sigma-Aldrich UK
Tween 20	Sigma-Aldrich UK

5.2 Cell Culture Reagents

Table 5.2: Cell culture reagents and supplements

Name	Cat. no	Provider
Accutase	SCR005	Merck Millipore
Matrigel® hESC-Qualified	354277	Corning
DMEM/F-12 w/o Hepes	DF-042-B	Merck Millipore
DMEM/F-12, Hepes	11330032	Gibco™
Epidermal growth factor (EGF)	GF144	Merck Millipore
Fibroblast growth factor (bFGF)	GF003	Merck Millipore
Laminin	L2020	Sigma-Aldrich UK
mFreSR™	5855	STEMCELL™ technologies
mTeSR™1	05850	STEMCELL™ technologies
ReNcell NSC freezing media	SCM007	Merck Millipore
ReNcell NSC maintenance media	SCM005	Merck Millipore
ROCK inhibitor Y-27632	1254	Tocris Bioscience
STEMdiff™ Neural Induction Media	05835	STEMCELL™ technologies
STEMdiff™ Neural Progenitor Media	05833	STEMCELL™ technologies
STEMdiff™ Trilineage Differentiation Kit	05230	STEMCELL™ technologies
Versene®	15040066	Thermo Fisher Scientific

5.3 Antibodies

Table 5.3: Primary Antibodies for Western Blot

Antibodies	Dilution	Cat. no.	Provider
BRACHYURY	1:1000	sc-20109	Santa Cruz
FLAG (M2)	1:1000	F1804	Sigma-Aldrich
GAPDH	1:2000	97166	Cell Signaling
GFAP	1:1000	130300	Invitrogen
H3 (96C10)	1:1000	3638	Cell Signaling
H3K27me3	1:1000	9733	Cell Signaling
H3K4me3	1:1000	ab8580	Abcam
H3K9ac	1:1000	9649	Cell Signaling
H3K9me3	1:1000	6F12H4	Millipore
MDM2	1:1000	sc-813	Santa Cruz
MST1	1:1000	ab124787	Abcam
MST1/2	1:1000	sc-62211	Santa Cruz
MST2	1:1000	ab52641	Abcam
NANOG	1:1000	4903	Cell Signaling
NESTIN	1:1000	sc-21248	Santa Cruz
OCT4	1:1000	2840	Cell Signaling
PAX6	1:1000	901301	Biolegend
pMST1/2	1:1000	3681	Cell Signaling
pS127-YAP	1:1000	4911	Cell Signaling
pS131-RASSF1A	1:100	custom-made, Hamilton et al., 2009	Eurogentec
RASSF1	1:1000	14-6888082	Ebioscience
RASSF1A	1:1000	HPA040735	Atlas Antibodies

Continued on next page

Table 5.3 – *Continued from previous page*

SIRT1	1:1000	8469	Cell Signaling
SOX17	1:1000	81778	Cell Signaling
SOX2	1:1000	3579	Cell Signaling
SUV39H1	1:1000	8729	Cell Signaling
SUV39H1	1:1000	39785	Active motif
SUV39H2	1:1000	ab5264	Cell Signaling
TAZ	1:1000	4883	Cell Signaling
TUJ1	1:1000	801201	Biolegend
YAP	1:1000	sc-101199	Santa Cruz

Table 5.4: Primary Antibodies used for Immunofluorescence

Antibodies	**Dilution**	**Cat. no.**	**Provider**
BRACHYURY	1:200	sc20109	Santa Cruz
GFAP	1:400	130300	Invitrogen
H3K9me3	1:400	6F12H4	Millipore
NANOG	1:300	4903	Cell Signaling
NESTIN	1:100	MAB353	Millipore
OCT4	1:400	2840	Cell Signaling
PAX6	1:400	901301	Biolegend
RASSF1	1:100	HPA040735	Atlas Antibodies
RASSF1	1:100	146888082	Ebioscience
SIRT1	1:200	8469	Cell Signaling
SOX17	1:200	81778	Cell Signaling
SOX2	1:400	3579	Cell Signaling

Continued on next page

Table 5.4 – *Continued from previous page*

SUV39H1	1:100	8729	Cell Signaling
TUJ1	1:400	801201	Biolegend

Table 5.5: Secondary Antibodies

Antibodies	**Dilution**	**Cat. no.**	**Provider**
Alexa fluor® goat anti-mouse 488nm	1:500	A-11001	Thermo Fischer Scientific
Alexa fluor® goat anti-rabbit 488nm	1:500	A-11008	Thermo Fischer Scientific
Alexa fluor® donkey anti-goat 488nm	1:500	A-11055	Thermo Fischer Scientific
Alexa fluor® goat anti-rat 488nm	1:500	A-11006	Thermo Fischer Scientific
Alexa fluor® 594 goat anti-mouse IgG (H+L)	1:500	A-11005	Thermo Fischer Scientific
Alexa fluor® 594 goat anti-rat IgG (H+L)	1:500	A-11007	Thermo Fischer Scientific
Alexa fluor® 594 donkey anti-goat IgG (H+L)	1:500	A-11058	Thermo Fischer Scientific
Alexa fluor® 594 goat anti-rabbit IgG (H+L)	1:500	A-11037	Thermo Fischer Scientific

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Table 5.5 – *Continued from previous page*

Anti-rabbit IgG, HRP-conjugated antibody	1:5000	7074	Cell Signalling
Anti-goat IgG, HRP-conjugated antibody	1:5000	705-035-003	Jackson Immuno Research
Anti-mouse IgG, HRP-conjugated antibody	1:5000	115-035-003	Jackson Immuno Research
Anti-rat IgG, HRP-conjugated antibody	1:5000	112-035-003	Jackson Immuno Research

5.4 RNAi

All siRNAs were purchased from Eurofins MWG.

Table 5.6: Small interfering RNAs

Gene	Sequence
siLuciferase	GCCAUUCUAUCCUCUAGAGGAUG
siNT	UAAGGUAUGAAGAGAUAC
siRASSF1	ACGCACAAGGGCACGUGAA
siRASSF1A #1	GACCUCUGUGGCGACUU
siRASSF1A #2	UCUUCUGCUCAAUCUCAGC
siSUV39H1	CUAAGAAGCGGGUCCGUAU

5.5 Plasmids

Table 5.7: Plasmids

Construct name	Backbone	Provider/Reference
pcDNA3	pcDNA3	Addgene
Flag-RASSF1A	pcDNA3	Hamilton et al., 2009
Flag-RASSF1A-S131A	pcDNA3	Hamilton et al., 2009
zsGreen	pQXCIP	Pefani et al., 2016
zsGreen-RASSF1A	pQXCIP	Pefani et al., 2016

5.6 TaqMan assays

All TaqMan assays were purchased from Thermo Fisher Scientific.

Table 5.8: TagMan probes

Gene	Detection	Assay ID
18S	18S rRNA	Hs99999901_s1
ACTB	Beta-Actin	1355178 C8
GAPDH	GAPDH	Hs99999905_m1
RASSF1	total RASSF1	Hs00200394_m1
RASSF1	RASSF1A	Hs00945257_m1
RASSF1	RASSF1C	Hs0094679_m1
RASSF1	RASSF1A Unmethylated	1355178 C7
RASSF1	RASSF1A Methylated	1355178 C6
SUV39H1	SUV39H1 # 1	Hs00957889_m1
SUV39H1	SUV39H1 # 2	Hs00957892_m1

Chapter 6

Cell biology

6.1 Cell Lines and Culture

The human embryonic stem cell line H9 (WA009) was purchased from WiCell. The cells were thawed according to the manufacturer's protocol. Briefly, cell culture plates (Corning) were coated with Matrigel™ (Corning) in a final concentration of 83.33 µg/ml in DMEM/F-12 (Gibco). The Matrigel™ was allowed to polymerise for 1 h at room temperature (RT) before being aspirated. The ROCK inhibitor Y-27632 (Tocris) was added to mTeSR™1 media to a final concentration of 10 µM before being added to the plate. The cells were thawed at 37°C in a water bath, transferred to 10 ml mTeSR™1 and spun down at 200 g for 5 min. Using a 5 ml serological pipette (Costar) the cells were gently resuspended and added to the plate before being placed in an incubator at 37°C, 5% CO2. Medium was changed every day. When the colonies became too dense or spontaneous differentiation occurred, the cells were split using Versene® (Thermo Fisher Scientific). Briefly, cells were rinsed with Versene® and treated in Versene® for 5-9 min until the first cells became detached. The Versene® was aspirated and cells were resuspended in mTeSR™1. The cells were plated with a split ratio between 1:5 and 1:20.

For Cryopreservation, the cells were split, spun down and gently resuspended in mFreSR™ media. The cryogenic vials were placed in a Mr. Frosty (Sigma-Aldrich)

at -80℃. The cells were transferred to liquid nitrogen for long term storage.

The human fetal neural stem cell lines ReNcell VM and CX were purchased from Merck Millipore. The cells were thawed according the manufacturer's protocol. Briefly, tissue culture flasks (Corning) were coated with 20 µg/ml laminin (Sigma-Aldrich) for at least 4 h at 37℃, 5% CO2. The laminin solution was aspirated and the coated flasks were rinsed once with 1 x PBS. Complete ReNcell NSC Medium was prepared by adding FGF2 and EGF (both Merck Millipore) to a final concentration of 20 ng/ml. Ten ml of complete ReNcell NSC Medium was added to the coated flasks and the flasks were incubated at 37℃, 5% CO2 until usage. The cells were thawed in the water bath and transferred to 15 ml conical tube. Carefully, 9 ml of pre-warmed ReNcell NSC Maintenance Medium was added. The cells were centrifuged for 5 min at 300 x g, resuspended in 5 ml of pre-warmed media, plated on the laminin-coated flasks and incubated at 37℃, 5% CO2. Medium was changed the following day. At a confluency of approximately 80%, the cells were passaged according to the manufacturer's protocol. The cells were washed once with PBS and incubated with Accutase™ for 3 min at 37℃. After the cells detached, 5 ml of pre-warmed ReNcell NSC Maintenance Medium was added. The cells were transferred to a 15 ml conical tube and centrifuged at 300 x g for 5 min. The supernatant was aspirated and the cells were resuspended in 2 ml of media supplemented with EGF and FGF. Appropriate numbers of cells were plated on fresh laminin-coated flasks with complete ReNcell NSC Medium.

For Cryopreservation, the cells were dissociated, resuspended in ReNcell NSC Maintenance Medium and centrifuged at 300 g for 5 min. The cells were resuspended in ReNcell freezing medium (Merck Millipore) to a concentration of 2 x 10 cells/ml and 1 ml of cell per cryogenic vial were placed in a Mr. Frosty (Sigma-Aldrich) at -80℃. For long term storage the cells were transferred to liquid nitrogen.

SH-SY5Y cells were kindly provided Prof. Ye, IBME, Oxford, UK. SH-SY5Y cell were grown in Roswell Park Memorial Institute (RPMI) media supplemented with 10 % (v/v) fetal bovine serum (FBS) and non-essential amino acids (NEAA) and 100 U/ml Penicillin/Streptomycin (all purchased from Gibco). SH-SY5Y cells were nucleofected according to the protocol for hNSCs.

6.2 Differentiation Assays

We used STEMdiff™ Trilineage Differentiation Kit (STEMCELL technologies) to differentiate H9 cells into the three germ layers ectoderm, endoderm and mesoderm. Therefore, H9 cells were detached as previously described. For ectodermal differentiation, the cells were resuspended in ectodermal differentiation media containing 10 µM Y-27632 (Tocris). H9 cells for mesodermal and endodermal differentiation were resuspended in mTeSR™1 containing 10 µM Y-27632. After 24h, the media were change to prewarmed ectodermal, endodermal or mesodermal differentiation media without addition of supplements. Cells were cultured for seven days, changing media daily. To differentiate H9 cells to the neuroectoderm, cells were split as described above and resuspended in STEMdiff™ neural induction media supplemented with 10 µM Y-27632. Cells were cultured in STEMdiff™ neural induction media for seven days and then transferred to neural progenitor medium. Medium was changed daily without addition of supplements.

To induce neural differentiation, ReNcells were cultured as described previously in ReNcell NSC Maintenance Medium but without addition of FGF and EGF. Cells were allowed to attach for at least 24h before induction of differentiation.

6.3 Transient Transfection

To achieve transient transfection in hNSCs the cells were nucleofected using the Nucleofector 2b device. Therefore, 2-4 x 10^6 cells were spun down at 300 g for 5 min. The cell pellet was resuspended in 100 µl nucleofection solution L (Cell Line Nucleofector® Kit L, VCA-1005, Lonza) and 100 - 300 nM siRNA or 2 µg plasmid were added to the cell mix. For nucleofection optimisations 1 µg pmaxGFP® vector (provided by the nucleofection kit) was added and different programmes were used for validation. The cell solution was transferred to a cuvette provided by the same kit. The cuvette was placed in the Nucleofector 2b and the programme G-013 was applied. Immediately after the nucleofection, 500 µl prewarmed medium was added to the cuvette and the cells were transferred to laminin-coated plates in pre-warmed maintenance media using the provided pasteur pipettes. Cells were allowed to attach for 24 h before induction of differentiation.

The human ESC line H9 was reverse transfected using Lipofectamine 3000 (Invitrogen) according to the manufacture's protocol. Briefly, 5 µl Lipofectamine 3000 was diluted in 145 µl OptiMEM™ (Cat. no. 11058021, Gibco) and 100 nM or 2 µg plasmid was diluted in OptiMEM™ to a total volume of 150 µl. The lipofectamine mix was added to the siRNA/plasmid mix and incubation was allowed for 15 min at RT. The Lipofectamine/RNA/plasmid mix was transferred to a Matrigel™-precoated 6 cm plate, H9 cells resuspended in mTeSR™1 were added to the mix. The cells were incubated for at least 48 h before collection.

6.4 Drug Treatments

H9 cells were treated with 10 µM TGFβ, 10 µM ATR (VE821) and 1 µM ATM (KU55933) inhibitors, which were added to the maintenance media (mTeSR™1). DMSO was used as a negative control. Cells were collected and lysed according to

the protocol for western blot analysis.

6.5 Molecular Biology

6.5.1 Transformation of Bacteria

Prior to transformation, LB agar plates and LB broth (both Sigma) were prepared. Therefore, 20 g LB agar was dissolved in 500 ml Milli Q water and 31 g 2YT broth was dissolved in 1 l Milli Q water. Both were sterilised by autoclavation at 121℃, 15 psi for 30 min. The LB agar was allowed to cool down to approximately 50℃ before addition of appropriate antibiotics to a final concentration of 100 µg/mL. The agar was poured to 10 cm dishes (Thermo Fisher Scientific) and was allowed to solidify before being stored at 4℃.

To transform the plasmids into bacteria, One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific) were thawed on ice. Fifty µl bacteria were incubated with 2 µl ligation mix (plasmid or control) on ice for 15 min. For transformation the bacteria were placed in a heat block for 30 s at 42℃ following an incubation on ice for 5 min. One ml LB broth was added to the bacteria and incubation of bacteria at 37℃ was allowed for 1 h while shaking at 750 rpm (Eppendorf heat block). The bacteria were spun down at 4500 rpm (rotor 5454R, Eppendorf) and resuspended in 100 µl LB broth. Transformation reactions were spread onto LB agar plates supplemented with appropriated antibiotics and were incubated o/n at 37℃.

6.5.2 Isolation of Plasmid DNA

Colonies were picked with a sterile pipette tip and transferred to 5 ml LB broth containing the appropriate antibiotic in a round-bottom 14 ml tube (Nunc™, Thermo Fisher Scientific). Starter cultures were incubated for 6-10 h at 37℃ while shaking at 225 rpm (Innova 44 Incubator). Overnight culture of 200 ml LB broth containing appropriate antibiotics were inoculated with 500 µl bacterial starter culture. Bacteria were allowed to grow o/n at 37℃ while shaking at 225 rpm and they were harvested by centrifugation at 6000 g for 15 min at 4℃. Isolation of the plasmid DNA was performed according to the manufacturer's instructions (QIAGEN Plasmid Maxi Kit). Briefly, bacterial pellets were resuspended, lysed (buffer P1 and P2) and precipitated (buffer P3). The supernatant was applied to Qiagen-tip 500 to bind plasmid DNA to the column. After washing with buffer QC, DNA was eluted with buffer QF and precipitated with isopropanol. After centrifugation and washing with 70 % (w/v) ethanol, the DNA was air-dried and resuspended in 200 µl nuclease-free water.

6.6 Molecular Biochemistry

6.6.1 Western Blotting

The cells were lysed in SDS lysis buffer (50 mM Tris-HCl pH = 6.8, 2% SDS) and sonicated for 15 cycles, 15 s on, 15 s off (Bioruptor). The protein concentration was determined using NanoDrop 1000 by measuring the absorbance of the samples at a wavelength of 280 nm, with an optical density (OD) OD=1 equals 50 µg/ml. The samples were normalised using NuPAGE® LDS sample buffer (4x) and 1x Laemmli loading buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 100 mM DTT, 0.01% bromophenol blue). The samples were boiled for 10

min at 98℃ and stored at -20℃. For SDS-PAGE, the samples were boiled again for 10 min at 98℃ to denature the proteins. The samples were loaded to NuPAGE® Novex 10% Bis-Tris Gel 1.5 mm. Proteins were allowed to stack for 20 min at 100 V followed by approximately 2 h of 120 V for separation. SDS-PAGE was performed in NuPAGE® MOPS SDS running buffer. Blotting of the proteins to PVDF membranes was carried out at 100 V for 80 min in Transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% (v/v) methanol). Subsequently, membranes were blocked according to the manufacturer's protocol in either 5% (w/v) non-fat milk (Marvel) in 0.1% PBST or 5% (w/v) BSA (Sigma-Aldrich) in 0.1% TBST. Antibodies were applied as given in table 5.3. Detection was carried out using Pierce™ ECL Western Blotting Substrate or Millipore Immobilon Western Chemiluminescent HRP Substrate. Western blots were quantified using Fiji software.

6.6.2 Immunoprecipitation

All steps of the IP were strictly performed on ice or at 4℃. Cells of a 1 x 10 cm dish per condition were washed twice in PBS, lysed in 250 µl IP buffer (137 mM NaCl, 2 mM EDTA, 20 mM Tris pH 8.0 and 1% Triton X-100) and scraped into a 1.5 ml eppendorf tube. The cell lysates were rotated for 15 min and spun down for 30 min at 14,000 rpm (20,800 g) at 4℃ (5417R, Eppendorf). The supernatant was transferred to a new eppendorf tube and the protein concentration was determined using BCA kit (Thermo Fisher Scientific). Therefore, a BSA concentration series was prepared (BSA, Thermo Fisher Scientific) the cell lysates were diluted 1:10 in water and standards + samples were measured in duplicate. The samples were normalised and pre-cleared with 10 µl Protein G Magnetic beads (PureProteome Protein G Magnetic Bead System, LSKMAGG02, Millipore) for 30 min at 4℃. The samples were placed on a magnet (DynaMag-2 Magnet, 12321D, Thermo Fisher Scientific) for approximately 30 s and the supernatant was carefully transferred to

a new eppendorf tube. Fifteen percent of the lysates were transferred to a new eppendorf tube as input control. Input controls were resuspended in LDS sample buffer (4x) and stored at -20. The rest of the lysates were incubated with 2 µg antibody per sample. Incubation was allowed for 2h at 4℃ before 20 µl beads were added to each sample. After incubation of the beads for one additional hour, the beads were washed 3 times in 1 ml IP buffer and the proteins were eluted from the beads with 60µl LDS buffer (1X). Input and IP samples were boiled for 10 min at 98℃ to fully elute and denature the proteins. All samples were stored at -20℃.

6.6.3 RNA Isolation and Reverse Transcriptase PCR

RNA was isolated using the RNeasy Kit (Invitrogen) according to the manufacturer's protocol. Briefly, cell pellets were lysed in the 350 µl lysis buffer (RLT) provided by the kit. The same volume 70% (v/v) ethanol was added and the mix was transferred to a column. Following a wash step, DNase (RNase-Free DNase Set, Qiagen) was incubated on the column, followed by additional wash steps. RNA was eluted in 15-30 µl nuclease-free water and RNA was quantified using NanoDrop 1000 by measuring the absorbance at a wavelength of 260 nm with an OD=1 equals 40 ng/µl RNA.

6.6.4 Quantitative Real Time Polymerase Chain Reaction

For qRT-PCR, total RNA was isolated using the Qiagen RNeasy Kit according to manufacturer's protocol including DNA digestion. The concentration of RNA was measured by NanoDrop 1000 (Thermo Fisher Scientific) and 750 ng to 1 µg of RNA was reversed transcribed using the Superscript III Reverse Transcriptase Kit (Life Technologies). Therefore, 1 µl deoxynucleotide triphosphates (dNTPs, U1511, Promega) 1 µl Oligo(dT)18 Primer (BIO-38029, Bioline) and 1 µl random

hexamer primers (BIO-38028, Bioline) were added to 750 ng to 1 µg of RNA in a total volume of 13 µl. The mix was denatured for 5 min at 65℃ (MyCycler Version 1.065, Biorad) followed by 1 min chill on ice. For the cDNA synthesis, 4 µl first strand buffer, 1 µl 0.1 M DTT, 1 µl nuclease-free water and 1 µl SuperScript™ III Reverse Transcriptase was added to the mix. The mix was incubated at 50℃ for 60 min followed by an inactivation of 15 min at 70℃. The cDNA was stored at -20℃. For qRT-PCR, the cDNA was pre-diluted 1:5 in nuclease-free water. Eight µl diluted cDNA, 10 µl TaqMan™ Gene Expression Master Mix (Cat. no. 4369016, Thermo Fisher Scientific) and 1 µl TaqMan™ probe for the specific gene and for the control gene were added to each well of a MicroAmp™ Optical 96-Well Reaction Plate (N8010560, Thermo Fisher Scientific). Quantitative RT-PCR was carried out in a StepOnePlus™ Real-Time PCR machine (Thermo Fisher Scientific) with the following steps:

Hold	50℃	2 min
	95℃	10 min
40 cycles	95℃	15 s
	60℃	1 min
Hold	4℃	∞

The differential mRNA expression of genes was calculated with the comparative Ct method as followed: ΔCt = Ct (Target gene) - Ct (Housekeeping gene); ΔΔCt = ΔCt (treated) - ΔCt (untreated); the relative expression difference (rel. Exp) is calculated by rel. Exp = $2^{-\Delta\Delta Ct}$. TaqMan® assays were performed in multiplex format.

6.6.5 Methylation-specific Polymerase Chain Reaction

For Methylation-specific PCR (MSP), genomic DNA was isolated from undifferentiated and differentiated cells using the DNeasy Blood and Tissue Kit (Quiagen) according to the manufacturer's protocol for cultured cells. Briefly, cells were centrifuged at 300 g for 5 min, resuspended in 200 µl PBS and treated with 20 µl proteinase K. Cells were lysed in 200 µl lysis buffer (buffer AL) and 200 µl ethanol (96-100%) was added before vortexing for 15 s. A DNeasy Mini spin column was placed in a 2 ml collection tube and the mix was added to the column. The tubes were centrifuged for 1 min at 6000 g, the flow-through was discarded and the tube was placed in a new collection tube. The column was washed once with 500 µl wash buffer 1 (buffer AW1) followed by a 1 min centrifugation at 6000 g before it was placed in a new collection tube. A second wash step was carried out with 500 µl wash buffer 2 (buffer AW2) followed by a centrifugation of 3 min at 20,000 g. The column was placed in a 1.5 ml eppendorf tube and DNA was eluted with in 100 µl nuclease-free water by 1 min centrifugation at 6000 g. The DNA concentration was measured at the NanoDrop 1000 by measuring the absorbance of the samples at a wavelength of 260 nm, with an OD=1 equals 50 ng/µl DNA.

For the bisulfite conversion, the MethylEdge™ Bisulfite Conversion kit (Cat. no. N1301, Promega) was used. Therefore, 500 ng DNA in 20 µl nuclease-free water of samples and controls were placed in a 200 µl PCR tube and DNA was converted according to the manufacturer's protocol. Briefly, 130 µl of bisulfite ME conversion reagent was added to the DNA and the mix was incubated in a thermocycler as follows:

Incubation	
98°C	8 min
54°C	60 min
4°C	hold

Converted DNA was stored or processed for desulphonation and cleanup on a ME Spin Column provided by the MethylEdge™ Bisulfite Conversion kit. Therefore, 600 µl ME binding buffer and the bisulfite-treated sample were added to the spin column and mixed by inverting the tube. Columns were spun at 10,000 g for 30 s. Flow-through was discarded and 100 µl 1 x ME wash buffer (including ethanol) were added to the column, followed by a 30 s centrifugation at 10,000 g. Desulphonation was carried out for 15 min at RT in 200 µl desulphonation buffer. Samples were spun (10,000 g, 30 s) and washed twice with 200 µl 1 x ME wash buffer before converted DNA was eluted in a fresh 1.5 ml eppendorf tube using 20 µl nuclease-free water followed by centrifugation for 30 s at 10,000 g.

Methylation of DNA was assessed with TaqMan® probes specific to unmethylated and methylated RASSF1A of samples and human methylated and converted methylated controls (N1231 and N1221, Promega).

6.6.6 Immunofluorescence

For immunofluorescence, cover slips (Cat. no. 631-0149, VWR) were either coated with 20 ng/ml laminin (L2020, Sigma) for hNSCS or 83.33 µg/ml Matrigel™ for hESCs according to the protocol for coating plates, as previously described. The cells were allowed to attach to the coated cover slips for at least 48h before being washed in PBS, fixed in 5% PFA/PBS (Invitrogen) and permeabilised in 0.3% v/v Triton X-100 (Thermo Fisher Scientific) in PBS for 5 min. The cover slips were washed again in 1 ml PBS and blocked in 3% BSA/PBS (Invitrogen) for 1-2 h at RT. Primary antibodies were applied according to table 5.4 (diluted in 3% BSA/PBS) and incubated o/n at 4℃. The cells were washed 3 times before and after the secondary antibody was incubated for 1-2 h at RT. The cover slips were mounted with DAPI mounting media, dried o/n and sealed. Images were taken with a Zeiss LSM780 microscope and processed with ZEN software. The fluorescence sig-

nal of single cells was measured and quantified by calculating the corrected total cell fluorescence (CTCF) with CTCF = Integrated density - (Area of selected cell x Mean fluorescence of background) using the ImageJ software. Total fluorescence was quantified by the Microscopy core facility, CRUK/MRC Oxford Institute for Radiation Oncology, Oxford, UK. Therefore, the maximum intensity projection was taken of each image and the total fluorescence intensity was measured by Fiji software. Each total fluorescence value was corrected by subtraction of the background signal. Additionally, the nuclear percentage of the fluorescence intensity signal was determined by measuring the fluorescence intensity signal that overlaps with DAPI. Cell number was determined in each image by counting DAPI events with the Fiji software.

6.6.7 CRISPR/Cas9

For RASSF1A specific CRISPR knock-out, gRNAs have been designed by the Genome Engineering Service of the Weatherall Institute of Molecular Medicine (WIMM), Oxford, UK. They were designed to target the 5' and 3' end of exon 1 of the RASSF1 gene to ensure isoform specific knock-out. The 3' gRNA was cloned into a the pSpCas9(BB)-2A-GFP (PX458) vector (Addgene, #48138), encoding GFP and Cas9. The 5'gRNA was cloned into the same backbone encoding Ruby (RFP) instead of GFP. CX and VM cells were nucleofected with 3 µg of each gRNA according to the protocol described before. Expression was allowed for 48h before 3-4 x 10^6 cells/ml were collected and resuspended in PBS. The cells were sorted for expression of both (GFP and RFP) by fluorescence-activated cell sorting (FACS, BD FACS Aria III) as single cells into 96-well plates containing hNSC maintenance media. Cell sorting was performed by the flow-cytometry facility (Department of Oncology, Oxford). The clones were cultured at 37℃, 5% CO2. Sequences of the gRNAs are:

5’ gRNA: CACCGGTCGTTGTGGCCGTCCGGGG

3’ gRNA: AAACGATACGAGTGGAGTGCGACC

Part III

Results

Chapter 7

Human Embryonic Stem Cells

7.1 Introduction

Human ESCs are cells derived from the ICM of the blastocyst. They have the potential to give rise to the three germ layers of a human embryo; ectoderm, endoderm and mesoderm. The ectoderm develops into the neural crest and neural tube that later give rise to the nervous system and brain. The endoderm forms the inner organs such as liver, pancreas, intestine and lungs. Muscles, bone, cartilage, adipose and connective tissues are derived from the mesoderm. The ability of embryonic stem cells to differentiate into all cell types of an organism is called pluripotency. The pluripotency factors OCT4, NANOG and SOX2 are core transcription factors that govern the transcriptional pluripotency network by maintaining self-renewal and restricting differentiation. When embryonic stem cells differentiate, pluripotency factors are lost and cell-type specific transcriptional and proteome signatures are established. Differentiation is an important characteristic of stem cells and the inability to differentiate is often associated with cancer.

The Hippo pathway has been extensively studied in context of development and cancer. However, the role of RASSF1A in development, as an upstream regulator of the Hippo pathway, is still widely under-explored. Our lab has previously shown that RASSF1A has a role in mouse ESC differentiation and reprogramming (data not

shown). In the first chapter, we asked whether RASSF1A exhibits similar functions in human ESCs and if RASSF1A specifically contributes to lineage specification of differentiating hESCs. Here, we were particularly interested in early human lineage commitment events from pluripotent stem cells to multipotent stem cells. Since RASSF1A is often methylated in CNS tumours and its methylation is of prognostic value (Grawenda and O'Neill, 2015), we then focussed on neuroectodermal differentiation to elucidate the function of RASSF1A in neural stem cells with regards to a potential dysregulation in brain tumours.

7.2 RASSF1A in Human Embryonic Stem Cells

To address the question of the role of RASSF1A in early human development we used the hES cell line H9 as a model system. H9 are adjusted to feeder-free conditions. They are routinely grown on a matrix (Matrigel®) in hESC maintenance media mTeSR1™. To validate the cell line, H9 cells were cultured for several rounds before they were assessed for pluripotency characteristics. Subsequently, H9 were stained for the pluripotency markers OCT4, SOX2 and NANOG by immunofluorescence (Fig. 7.1).

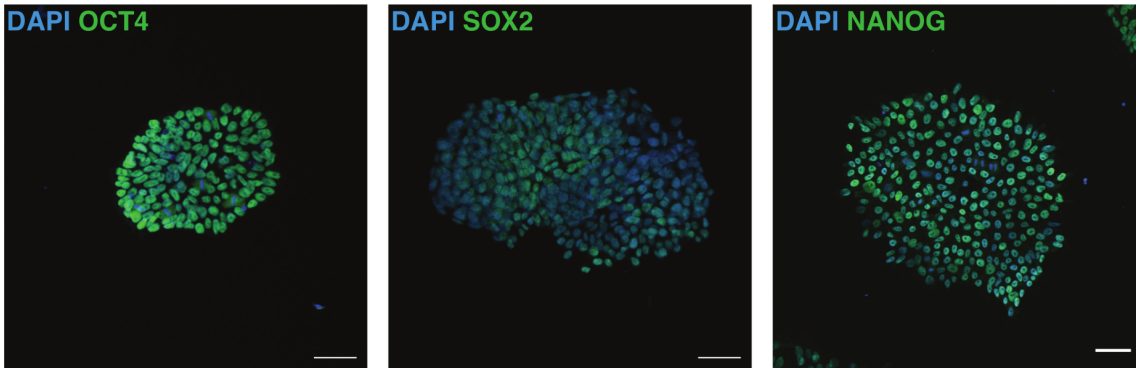


Figure 7.1: The human ES cell line H9 expresses pluripotency markers: The hESC line H9 was grown feeder-layer free on Matrigel™ coated plates in maintenance media mTESR1™. Expression of pluripotency markers was assessed by immunofluorescence with the indicated antibodies. Scale bar represents 50 µm.

We confirmed that H9 cells characteristicly grow in round colonies and express the pluripotency markers OCT4, SOX2 and NANOG. They are able to differentiate into the germ layers ectoderm, endoderm and mesoderm which is accompanied by loss of pluripotency, shown by SOX2 loss, and gain of lineage specific markers (Fig. 7.3).

7.2.1 RASSF1A is upregulated in differentiated hESCs

As in human embryos, human embryonic stem cells can, under chemically defined conditions, differentiate *in vitro* into all cell types of the three germ layers. However, despite culturing H9 cells in the maintenance media mTESR1™ that supports the hESCs self-renewal by FGF and TGFβ signalling, the cells can undergo spontaneous differentiation. This phenomenon has been widely reported (Sathananthan and Trounson, 2005; Manuilova et al., 2001). Park *et al.* was able to show that spontaneous differentiation is not directed towards a specific lineage and that progenitors from all three germ layers can arise by spontaneous differentiation (Park et al., 2004). These spontaneously differentiated cells occur mainly at the edge of the cell colony and they have lost the expression of NANOG (Fig. 7.2A). Differentiated cells lose colony morphology, which is a strong indication of differentiation. Some of these cells showed neuronal-like morphology. Therefore, spontaneously differentiated cells were stained for PAX6 expression to determine if the RASSF1A-positive cells were of neuroectodermal origin (Fig. 7.2B and C).

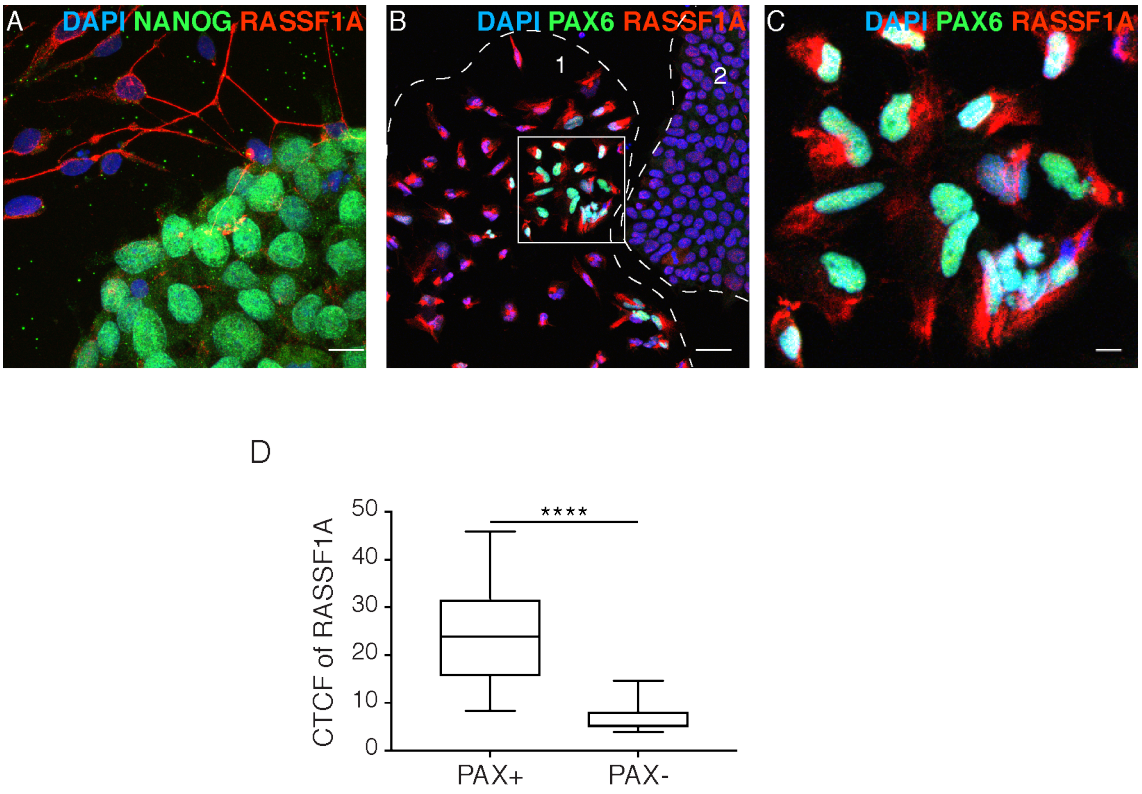


Figure 7.2: RASSF1A expression is upregulated in spontaneously differentiated hESCs: H9 cultured in mTeSR1™ media undergo spontaneous differentiation. A-C) RASSF1A and pluripotency and lineage marker expression (NANOG, PAX6, respectively) was assessed by immunofluorescence. D) RASSF1A expression was quantified by calculating the corrected total cell fluorescence (CTCF) of PAX+ cells within the phenotypical differentiated region 1 and PAX- cells (within the undifferentiated colony region 2). Statistical significance was determined by a two-tailed t-test, **** $P < 0.0001$ Scale bar represents 10 µm for A) and C) and 50 µm for B).

We found that RASSF1A expression was increased in spontaneously differentiated cells that lose NANOG expression and is mainly localised to the cytoplasm (Fig. 7.2A). Furthermore, PAX6-positive cells that are committed to the neuroectodermal lineage express significantly higher levels of RASSF1A (Fig. 7.2B-D). These data suggest that expression of RASSF1A is induced in differentiation, particularly in cells committing to the neuroectodermal lineage.

Our group has previously shown that RASSF1A is upregulated with Leukaemia inhibitory factor (LIF) withdrawal-induced differentiation of mESCs (Papaspyropoulos

et al., 2018). Upon depletion of LIF in the media of mESCs, mTOR signalling is activated and cells commit to differentiation (Cherepkova et al., 2016). To investigate if RASSF1A is also upregulated with differentiation of hESCs and to determine if the increase in expression is lineage specific, we performed a trilineage differentiation assay (Fig. 7.3).

Therefore, cells were cultured in chemically defined media to allow ectoderm, endoderm and mesoderm differentiation. Differentiation was allowed for one week before cells were assessed for the pluripotency marker SOX2 and the differentiation marker PAX6 (ectoderm), SOX17 (endoderm) and BRACHYURY (mesoderm).

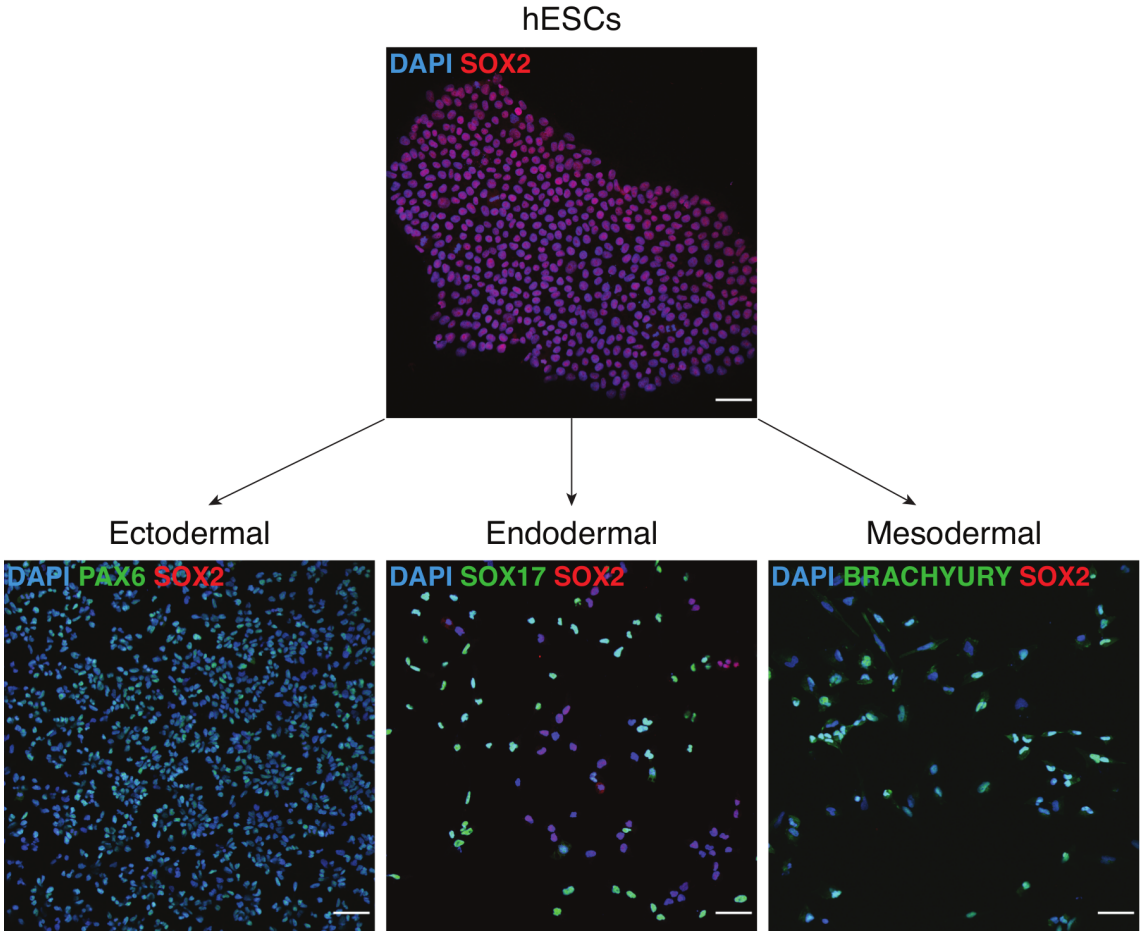


Figure 7.3: Trilineage Differentiation of human ES cells: H9 were differentiated for seven days to the three embryonic germ layers; ectoderm, endoderm and mesoderm using chemically defined media. Individual lineage marker expression was analysed by immunofluorescence. Scale bar represents 50 µm.

Figure 7.3 confirms that H9 cells are able to differentiate into the three different germ layers (pluripotency hallmark). The ectodermal cells form rosette-like structures and gain PAX6 expression (as shown previously). The endodermal differentiated cells gain SOX17 expression and the mesodermal differentiated cells start expressing BRACHYURY. The decreased expression of SOX2 and the upregulation of respective markers reflect the loss of pluripotency and confirms the functionality of the differentiation assay.

Previously, colleagues have shown that RASSF1A gets phosphorylated at serine 131

and is activated by the DNA damage kinases ATR (Pefani et al., 2014) and ATM (Hamilton et al., 2009). Upon activation, RASSF1A dimerises, which enhances binding to the core kinases of the Hippo pathway MST1 and MST2. To validate the antibody for pS131-RASSF1A specificity in H9, cells were treated with ATR (VE821) and ATM (KU55933) inhibitors, additional to the combination of both. DMSO was used as negative control for the inhibitor solvent. TGFβ signalling, known to target RASSF1A for protein degradation in cancer cells (Pefani et al., 2016) and siRNA-mediated transient knock-down of RASSF1A were used as additional antibody specificity controls (Fig. 7.4).

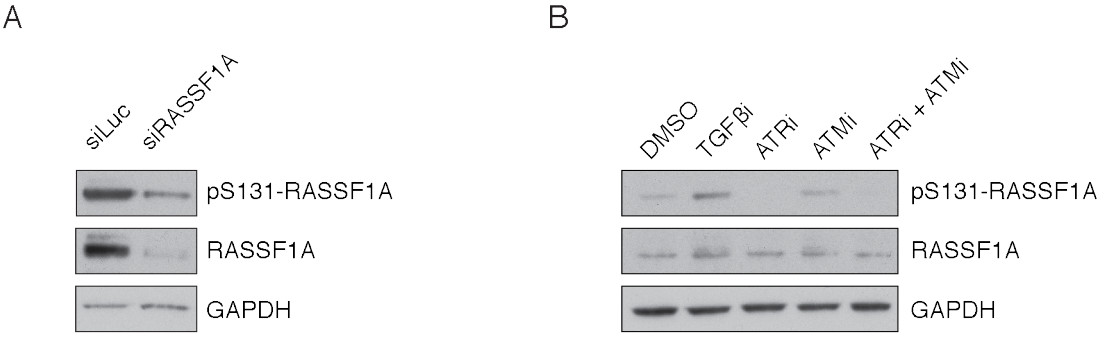


Figure 7.4: RASSF1A is activated by ATR but not ATM in hESCs: A) RASSF1A transient knock-down was used as a control for antibody specificity B) H9 cells were treated with TGFβ, ATR (VE821) and ATM (KU55933) inhibitors for 6 h. TGFβ inhibition was used as control for RASSF1A stabilisation. Phosphorylation and total RASSF1A levels were assessed by western blotting. GAPDH was used as a loading control.

In H9 cells, RASSF1A is phosphorylated at serine 131 and activated by ATR shown by a decrease of pS131-RASSF1A with ATR inhibition. However, ATM inhibition had no effect on RASSF1A phosphorylation compared to the DMSO control. Inhibition of both, ATR and ATM activity, showed a decrease in pS131-RASSF1A to similar levels as ATR inhibition alone. As expected, upon TGFβ treatment, RASSF1A was increased. Knock-down of RASSF1A confirmed the specificity of the antibodies against phosphorylated and total RASSF1A .

To investigate if RASSF1A contributes to lineage specification of differentiating human embryonic stem cells and if this potential contribution is activation specific, Western Blot analysis was performed with the antibodies validated in Figure 7.4.

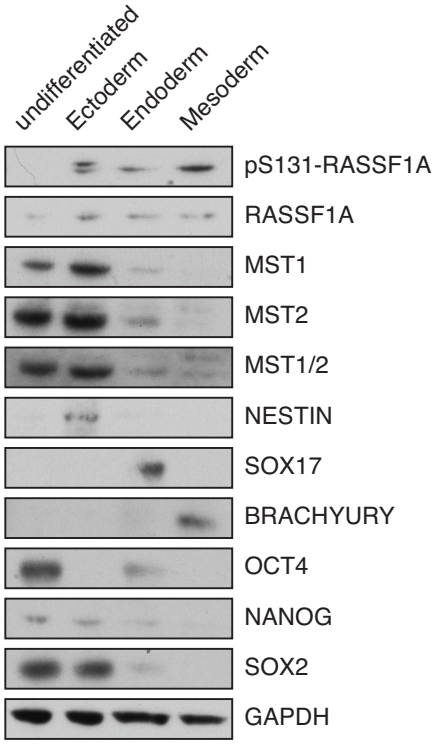


Figure 7.5: RASSF1A expression is upregulated with Differentiation into all three germ layers: Human ESCs were differentiated to the ectodermal, endodermal and mesodermal lineage. Differentiation was allowed for seven days and was confirmed by western blot analysis against appropriate pluripotency (OCT4, NANOG, SOX2) and differentiation (NESTIN, SOX17, BRACHYURY) markers.

Total RASSF1A expression and its phosphorylation was increased with ectodermal, endodermal and mesodermal differentiation compared to undifferentiated hESCs (Fig. 7.5). Induction of differentiation to the three germ layers was confirmed by expression of specific markers. NESTIN was used as a marker for ectodermal lineage commitment and SOX17 and BRACHYURY confirmed endodermal and mesodermal differentiation, respectively. The pluripotency markers OCT4, NANOG and SOX2

were decreased with differentiation. As expected, SOX2 was still expressed in the ectodermal lineage after seven days of differentiation. SOX2 has been described as a persistent marker for NSCs (Ellis et al., 2004).

Interestingly, MST1/2, the core kinases of the Hippo pathway, were expressed the highest in undifferentiated and ectodermal cells. Hindley *et al.* showed that MST1 is activated in neuroepithelial stem cells derived from hESCs (Hindley et al., 2016). Together, these data encouraged us to further investigate the role of RASSF1A in neural lineage commitment.

7.2.2 RASSF1A during Neuroectodermal Differentiation

To elucidate a role of RASSF1A in human neural development we focussed on neuroectodermal differentiation of hESCS. Neuroectodermal differentiation was induced using chemically defined media that inhibits TGFβ/BMP signalling (often referred to as dual-SMAD inhibition). The cells were allowed to differentiate for three days before being reseeded in differentiation media for another four days.

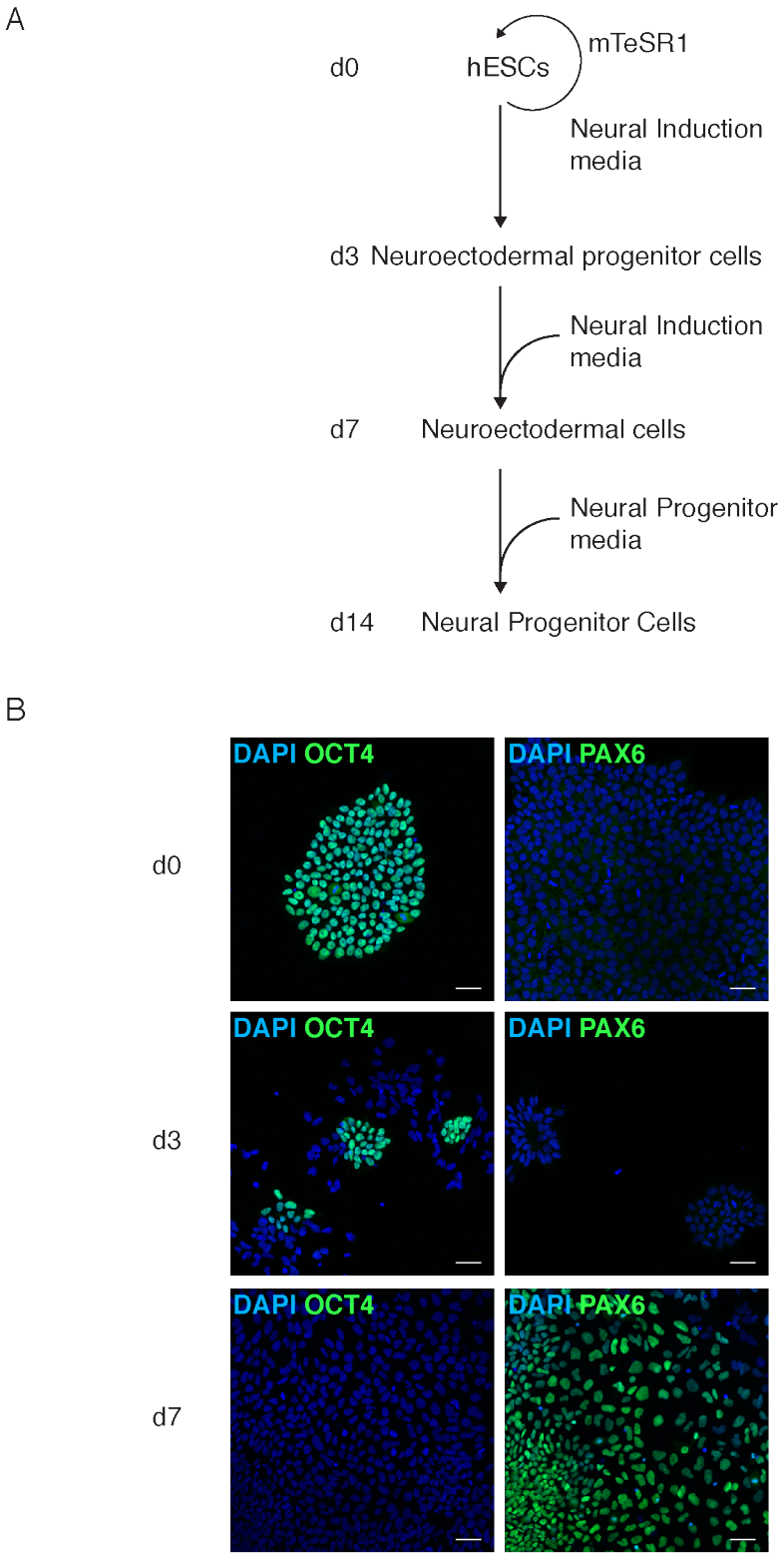


Figure 7.6: Neuroectodermal Differentiation of human ES cells: A) Schematic overview for neuroectodermal differentiation of H9 cells. B) Immunofluorescent staining of differentiating hESCs confirming differentiation-associated changes of morphology and pluripotency/differentiation marker (loss of pluripotency marker OCT4 and gain of differentiation marker PAX6 . Scale bar represents 50 µm.

With induction of differentiation into the neuroectodermal lineage, hESCs cells lose the expression of the pluripotency marker OCT4 and induce expression of neuroectodermal differentiation markers including PAX6 (Fig. 7.6B) and NESTIN (Fig. 7.7).

An increased RASSF1A protein expression during neuroectodermal lineage commitment was confirmed by immunofluorescence (Fig. 7.7) and Western Blot (Fig. 7.8).

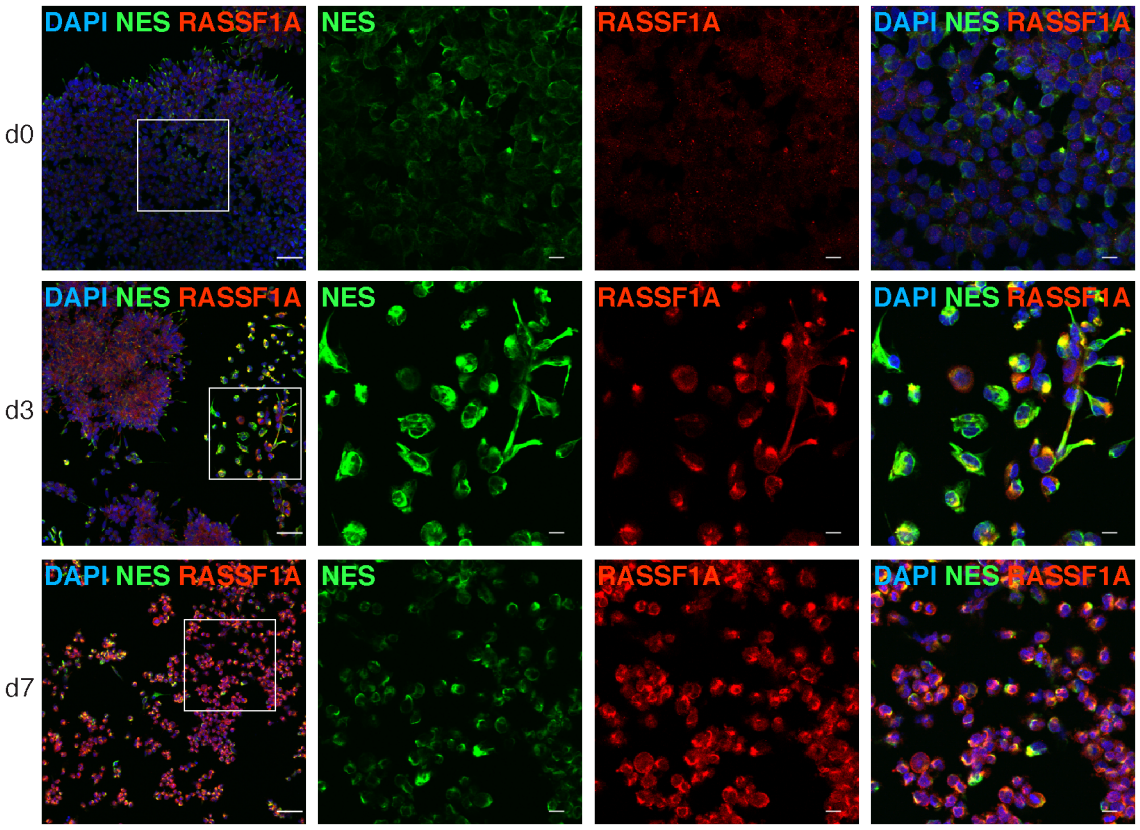


Figure 7.7: RASSF1A during Neuroectodermal Differentiation of human ES cells: Immunofluorescent staining of RASSF1A and NESTIN (NES) during neuroectodermal differentiation of hESCs. Scale bar represents 50 µm and 20 µm for zoomed images (boxed region).

Noteworthy, differentiated hESCs alter their *in vitro* growth behaviour from colony

shaped to monolayer growth, a characteristic *in vitro* differentiation hallmark.

RASSF1A is an upstream regulator of the Hippo Pathway. Hence, we hypothesised that the downstream core components of the Hippo pathway may be affected by the increase of overall and activated RASSF1A.

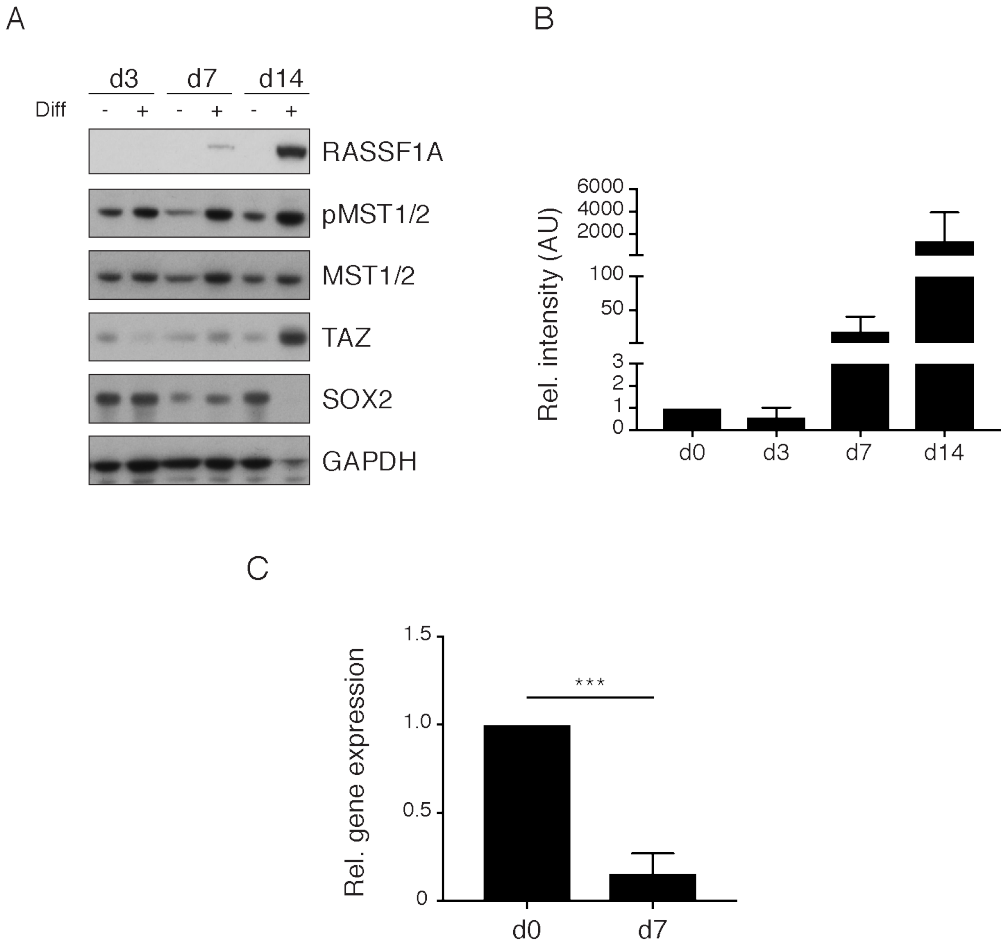


Figure 7.8: Hippo Pathway components during Neuroectodermal Differentiation: Human ESCs were differentiated and A) western blot analysis showed that upon neuroectodermal differentiation RASSF1A, MST1/2 and TAZ are upregulated. B) Western blot quantification of RASSF1A expression represented by relative arbitrary unit (AU). C) RASSF1A mRNA levels were significantly decreased with differentiation (p=0.0002). Error bars represent standard deviation of n=3 three biological replicates. Statistical significance was determined by a two-tailed t-test, *** $P < 0.001$

RASSF1A protein levels are upregulated with neuroectodermal differentiation (Fig. 7.8). Additionally, the expression of other core components of the Hippo Pathway, MST1/2 and TAZ, were induced upon neuroectodermal differentiation. An increased phosphorylation of the core kinases MST1/2 was observed with differentiation, suggesting the underlying mechanism of how RASSF1A influences differentiation might involve the core kinases of the Hippo Pathway. RASSF1A mRNA levels were significantly down-regulated with neuroectodermal differentiation (Fig. 7.8C). A discrepancy between gene transcription and translation has been reported for other genes, but is widely under-explored (Lundberg et al., 2010). SOX2 expression is lost after 14 days of differentiation, suggesting a certain maturation of the cells. Whether the cells are post-mitotic or terminally differentiated would be of interest.

7.2.3 Epigenetic changes during Differentiation

Differentiating cells undergo massive transcriptional, and consequently, translational changes. Differential gene expression of cells committing to different cell types is often conducted by epigenetic changes. We therefore asked if the induction of RASSF1A expression in differentiating cells might influence epigenetic regulators and their targets, particularly in cells committing to the neural lineage.

To select potential candidates that might influence or might be influenced by increased RASSF1A expression during differentiation, we analysed known RASSF1 interactors and their role in development. (Table 7.1). Data were collected from BioGRID (Stark et al., 2006), UniProt (Apweiler et al., 2004; Consortium, 2017) and IntAct (Orchard et al., 2014) and represent only selected RASSF1A interactors.

Table 7.1: Relevant RASSF1A interactors and their functions

Interactor	Relevant Role	Assay	Confirmed	Publication
ATM	DNA Damage Response	Protein-Peptide	Co-IP	Kim et al., 1999, Hamilton et al., 2009
ATR	DNA Damage Response	Protein-Peptide	Co-IP	Kim et al., 1999, Pefani et al., 2014
H2A type 2-B	DNA compaction	Affinity Capture-MS		Huttlin et al., 2015
KDM1A	Demethylation of H3K4 and H3K9	Affinity Capture-Luminescence, Y2H		Weimann et al., 2013
LATS1	Hippo Pathway		Co-IP	Guo et al., 2007
LATS2	Hippo Pathway	FRET, Reconstituted Complex		Mo et al., 2016
MDM2	E3 ubiquitin-protein ligase		Co-IP	Song et al., 2008b, Zhang et al., 2013
SAV1	Hippo Pathway	Reconstituted Complex	Co-IP	Guo et al., 2007, Donninger et al., 2011

Continued on next page

Table 7.1 – *Continued from previous page*

STK3 (MST2)	Hippo Pathway	Affinity Capture-MS, Proximity Label-MS, Y2H	Co-IP	Varjosalo et al., 2013, Huttlin et al., 2015, Hauri et al., 2013, Huttlin et al., 2017, Couzens et al., 2013, Fenton et al., 2004, Liu et al., 2007, Guo et al., 2007, Matallanas et al., 2007, Romano et al., 2010
STK4 (MST1)	Hippo Pathway	Affinity Capture-MS, FRET, Proximity Label-MS, Y2H		Behrends et al., 2010, Varjosalo et al., 2013, Huttlin et al., 2015, Hauri et al., 2013, Huttlin et al., 2017, Mo et al., 2016, Couzens et al., 2013, Liu et al., 2007
SUV39H1	Trimethylation H3K9	Affinity Capture-Luminescence, Y2H		Weimann et al., 2013
SUV39H2	Trimethylation H3K9	Y2H		Vinayagam et al., 2011, Weimann et al., 2013

Continued on next page

Table 7.1 – *Continued from previous page*

TGFβR1	TGFβ signalling	Co-IP	Pefani et al., 2016
TGFβR2	TGFβ signalling	Co-IP	Pefani et al., 2016

Interestingly, SUV39H1, a histone methyltransferase that trimethylates histone H3 and lysine 9 (H3K9me3), has been shown to interact with RASSF1 by Yeast-Two-Hybrid and Affinity-Capture-Luminescence experiments (Weimann et al., 2013). The H3K9me3 mark is a histone modification for gene repression and chromatin compaction and is established during differentiation to permanently silence transcription of specific genes, including pluripotency genes. Additionally, RASSF1A interacts with MDM2, an ubiquitin E3 ligase that, among other functions, has been shown to promote proteosomal degradation of SUV39H1 through its ubiquitination at lysine 87. SUV39H1 activity is also regulated by SIRT1-mediated deacetylation. RASSF1A has been shown to regulate SIRT1-mediated deacetylation of XPA in response to ultra violet light-induced DNA damage (Donninger et al., 2015). Since RASSF1A is able to modulate SIRT1 and MDM2 activity towards other proteins and both proteins have been shown to regulate SUV39H1, we asked if SUV39H1 might be affected by loss of RASSF1A in tumours with *de novo* RASSF1A promoter methylation. We performed western blotting on transient RASSF1A knock-down lysates (Fig. 7.9). Knock-down of SUV39H1 showed antibody specificity for SUV39H1.

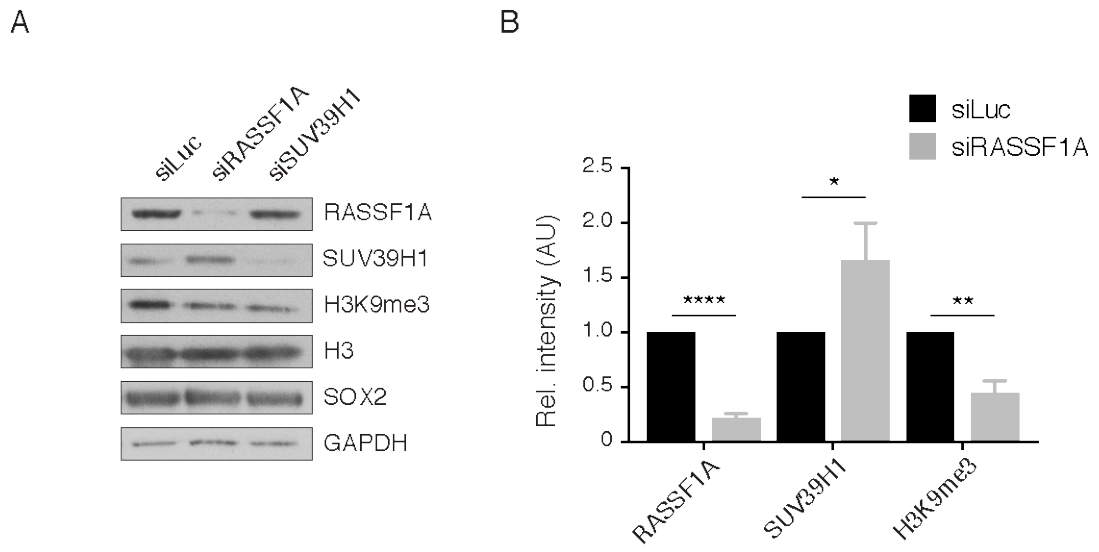


Figure 7.9: Knock-down of RASSF1A alters levels of SUV39H1 expression and its substrate H3K9me3 in hESCs: Human ESCs were transfected with siRNA targeting either RASSF1A or SUV39H1. A) Levels of protein expressed were analysed by western blotting 48h post-transfection. GAPDH is used as a loading control. B) Western blot quantification of RASSF1A, SUV39H1 and H3K9me3 with RASSF1A knock-down. Error bars represent n=3 biological replicates. Statistical significance was determined by a two-tailed t-test, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$

Interestingly, as a consequence of siRNA-mediated knock-down of RASSF1A, SUV39H1 protein levels are increased in hESCs. Intriguingly, the SUV39H1 substrate H3K9me3 is decreased to a similar extend as observed with SUV39H1 knock-down. Total histone levels and SOX2 are not affected by either RASSF1A knock-down or SUV39H1 knock-down. This suggests that RASSF1A regulates SUV39H1 protein levels and its activity, therefore affecting SUV39H1 substrate methylation of H3K9.

7.3 Discussion

In this chapter, we investigated the role of RASSF1A during differentiation of human pluripotent stem cells. Firstly, we set up the human embryonic stem cell line H9 and verified their pluripotency by immunofluorescence (Fig. 7.1). We observed that hESCs spontaneously differentiate and that these differentiated cells have an increased RASSF1A expression. Interestingly, spontaneous differentiation occurred exclusively at the edges of hESC colonies. Rosowski *et al.* made similar observations in the hES cell line H1 after induction of ectoderm differentiation with BMP4. They discussed the different mechanical forces and paracrine signalling as well as growth factor gradients at the edges of hESC colonies as potential causes of differentiation (Rosowski et al., 2015). Interestingly, the Hippo Pathway has been shown to conduct cell response to mechanical stimuli (Dupont et al., 2011).

To identify if RASSF1A expression is increased in all the lineages of the primary germ layers we performed ectoderm, endoderm and mesoderm differentiation assays (trilineage differentiation). At day 7 of differentiation, the ectodermal cells lose the expression of OCT4 (Fig. 7.5) but maintain expression of NANOG and SOX2. Interestingly, OCT4 is considered as the major pluripotency factors as other factors can compensate for loss of SOX2 or NANOG but not for loss of OCT4 (reviewed in Radzisheuskaya and Silva, 2014). Additionally, Kim et. al. had been able to generate human iPS cells from human NSCs by overexpression of OCT4 alone (Kim et al., 2009b), indicating that altered OCT4 expression is sufficient for ectodermal differentiation and dedifferentiation. The endodermal cells have decreased expression of the pluripotency markers and increased expression of SOX17, a transcription factor specifically expressed in the endodermal lineage. Mesodermal differentiation was verified by loss of all pluripotency transcription factors compared to undifferentiated, ecto- and endodermal cells. Expression of BRACHYURY confirmed mesodermal differentiation. Remarkably, all the lineages show a different expression pattern for

the pluripotency factors. In recent years, much effort has been undertaken to understand the transcriptional regulatory circuitry maintained by OCT4, SOX2 and NANOG to affect pluripotency and self-renewal of hESCs.

Human ESCs, which are pluripotent and have the potential to differentiated into all three germ layers, express low levels of RASSF1A. Upon differentiation, RASSF1A expression is increased with ectodermal, endodermal and mesodermal lineage commitment. Respective differentiation was validated by expression of lineage specific markers, shown either by Western Blot or immunofluorescent staining (Fig. 7.3 and 7.5) additionally to loss of pluripotency markers. We did not observe any lineage preferences of RASSF1A expression. Additionally, RASSF1A is phosphorylated at serine 131 and activated with differentiation into all three germ layers. However, MST1/2, the core kinases of the Hippo Pathway, were expressed the highest in differentiated ectodermal cells, suggesting an upregulation of Hippo signalling specifically in early ectoderm lineage specification. Indeed, an increased phosphorylation of MST1/2 was observed with neuroectodermal differentiation compared to undifferentiated hESCs (Fig. 7.8A). MST1/2 are important for embryonic development, as the double knock-out mouse is embryonic lethal at E9.5 (Oh et al., 2009; Song et al., 2010). In response to oxidative stress, MST1/2 induces apoptosis of neurons (Yuan et al., 2009). Apoptosis is considered as a quantity and quality control mechanism in the developing brain. It controls the stem cell population and ensures correct integration of mature neurons into the neural circuits (Ryu et al., 2016; Jaeger et al., 2015). Thus, apoptosis is essential for correct differentiation.

The expression of the Hippo pathway effector TAZ was also upregulated with neuroectodermal differentiation, particularly at day 14 of differentiation when SOX2 expression was lost. The same was observed for YAP (Fig. 9.2). YAP/TAZ have been shown to maintain the neural stem cell pool in mouse brains (Cao et al., 2008) and to enhance neural stem cell characteristics (Han et al., 2015). A further characterisation of the hESC-derived, SOX2 negative progenitor cells is necessary to

indicate their developmental stage. The expression of specific neural stem (SOX2, SOX1, NESTIN) and differentiation marker (GFAP, TUJ1, Microtubule-associated protein 2 [MAP2]) needs to be tested to elucidate the grade of lineage commitment and maturation including post-mitotic terminal differentiation (Proliferation marker protein Ki67). Controversially, in opposition to protein levels we found RASSF1A transcription to be significantly downregulated with ectodermal differentiation (Fig. 7.8). As it is usually assumed that mRNA and protein expression levels correlate, this remains only a presumption. Several papers have discussed poor correlation of gene expression and protein levels (Greenbaum et al., 2003; Vogel et al., 2010; Lundberg et al., 2010; Schwanhausser et al., 2011) due to differences in half-lives of mRNA and proteins, post-transcriptional and post-translational modifications and/or transcription and translation rates. Irrespective of the apparent variation in transcript levels detected by qRT-PCR, protein levels were clearly regulated and associated with differentiation. We intend to further explore this relationship by looking at the array of RASSF1 transcripts and the effects of translation control to better understand the discrepancy. It would be interesting to test whether RASSF1A protein is stabilised in neuroectodermal cells. Cycloheximide (CHX) is a drug that blocks *de novo* protein synthesis by inhibiting translational elongation (Schneider-Poetsch et al., 2010). A CHX chase experiment could elucidate if increased RASSF1A levels are due to post-translational mechanisms. Additionally, parallel metabolic pulse labelling and its downstream applications Stable isotope labelling by amino acids in cell culture (SILAC) and mass spectrometry for protein and next generation sequencing for transcripts (Schwanhausser et al., 2011) would be highly informative about copy numbers, half-lives and the correlation of RASSF1A mRNA and protein. E3 ligases that have been identified to mediate ubiquitin-dependent degradation of RASSF1A are Itchy homolog (ITCH, Pefani et al., 2016), Cullin 4A (CUL4A, Jiang et al., 2011) and S-phase kinase-associated protein 2 (SKP2, Song et al., 2008a). Until today, deubiquitinases targeting RASSF1A are not known. In the context

of stem cells and differentiation, the identification of RASSF1A ubiquitinating and deubiquitinating enzymes would be helpful to further understand RASSF1A stabilisation. RASSF1A has been shown to disrupt the complex formation of E3 ligase MDM2, deubiquitinase USP7 and DAXX, promoting self-ubiquitination of MDM2 and the stability of p53. (Song et al., 2008b). Furthermore, MDM2 mediates p73 ubiquitination but does not promote its degradation (Zeng et al., 1999; Balint et al., 1999), suggesting a proteasome-independent role of MDM2 mediated ubiquitination. Wu et al. showed that MDM2 inhibits p73-dependent apoptosis and cell cycle arrest but not its transcriptional activity (Wu and Leng, 2015). In fact, p73 is stabilised upon MDM2 binding (Ongkeko et al., 1999). Although p73 has been linked to neural stem cell self-renewal and maintenance (Gonzalez-Cano et al., 2010; Talos et al., 2010), it has also been shown to promote neuronal differentiation (De Laurenzi et al., 2000; Velletri et al., 2013). Interestingly, p73 binds to YAP and its interaction promotes p73-dependent transcription (Strano et al., 2001), apoptosis (Matallanas et al., 2007) or differentiation (Papaspyropoulos et al., 2018).

To explore possible functions of RASSF1A in differentiated cells, we summarised RASSF1A interactors according to their relevance in differentiation. SUV39H1/RASSSF1A interaction has been found in two independent screens (Weimann et al., 2013). Interestingly, SUV39H1 has been shown to be upregulated in gliomas (Spyropoulou et al., 2014). As SUV39H1 catalyses the trimethylation of H3K9, which is a heterochromatin mark characteristic for transcriptional shutdown, a hyperactive SUV39H1 could lead to transcriptional silencing of tumour suppressors. However, overexpression of SUV39H1 in transgenic mice resulted in impaired growth and erythroid differentiation (Czvitkovich et al., 2001). In summary, these data suggest that SUV39H1 has different biological roles during development and adulthood. As RASSF1A is often epigenetically silenced in tumours of the central nervous system, we asked whether a similar relationship could exist in pluripotent cells. We found that SUV39H1 is significantly upregulated with siRNA-

mediated depletion of RASSF1A (Fig. 7.9). RASSF1A levels were not affected by SUV39H1 knock-down. However, the histone mark H3K9me3 is significantly reduced with RASSF1A knock-down compared to the control. An upregulation of SUV39H1 and a downregulation of H3K9me3 suggest that SUV39H1 is not functional regarding its methyltransferase activity. Possible explanations could be a mislocation of SUV39H1, including blocked translocation to the nucleus, inhibition of chromatin recognition/binding, reduced SUV39H1 activity and increased demethylation of H3K9me3. Additionally, it would be of interest if the effect is Hippo pathway dependent. The SUV39H1 NLS mediating SUV39H1 nuclear import could be phosphorylated by MST1/2 and/or LATS1/2 and SUV39H1 transcription might be directly affected by YAP/TAZ transcriptional activity. This data encouraged us to further investigate the role of RASSF1A and SUV39H1 in human neural stem cells.

Chapter 8

Human Neural Progenitor Cells

8.1 Introduction

Neurogenesis and gliogenesis are defined as the developmental processes that generate neurons and glial cells, respectively. It had been a long standing dogma that neurogenesis only occurs during embryonic development. However, in 1998 Eriksson *et al.* published the first evidence for neurogenesis in the human hippocampus (Eriksson et al., 1998). Today, it is known that new neurons are formed throughout the adult life in a process referred to as adult neurogenesis. The SVZ and SGZ were identified as neural stem cell niches of the adult brain, which harbour NSCs that are capable of differentiation. As described earlier, these stem cell niches are often believed to contribute to glioblastoma resistance and recurrence as they can repopulate the tumour (Lim et al., 2007). To investigate the role of RASSF1A during differentiation of human neural stem/ progenitor cells (hNSCs) we purchased the two cell lines ReNcell CX and VM from Merck/ Millipore. CX cells are derived from the cortical region and VM cells are derived from the ventral mesencephalon (midbrain) region of an 14 week and 10 week old human fetus, respectively (Donato et al., 2007). Both cell lines are immortalised by retroviral transduction with c-myc (in CX) or v-myc (in VM). Expression of the oncogene is regulated under the cytomegalovirus (CMV) promoter and addition of the growth factors EGF and FGF

to the media ensures self-renewal of CX and VM cells. Upon withdrawal of EGF and FGF, expression of myc is decreased (Villa et al., 2004) and the cells are able to differentiate. Differentiation was assessed by expression of lineage specific markers. GFAP is an intermediate filament protein characteristicly expressed in glial cells and mature astrocytes, however GFAP-positive cells have been shown to give rise to neurons and oligodendrocytes *in vivo* (Casper and McCarthy, 2006). TUJ1 is a βIII-tubulin antibody clone specific to neurons. It does not detect glial βtubulins. Here, we use the CX and VM cells to model human neural differentiation at a later stage of neurogenesis than compared to hESCs. Whether RASSF1A contributes to neural differentiation of multipotent NSCs towards more mature neural cells is unknown.

8.2 Characterisation of Human Neural Stem Cells

Glioblastomas are the most common lethal cancer in children. Paediatric tumours are, in contrast to adult tumours, thought to occur due to developmental defects rather than accumulation of mutations. Indeed, progenitor and cancer cells share certain characteristics and therefore a dysregulation of the extrinsic and intrinsic factors of progenitor cells is heavily discussed as a cause of cancer at an early age. We therefore decided to investigate to role of RASSF1A in the two human neural stem cell lines CX and VM.

The CX and VM cells were cultured according to the manufacturer's protocol. They are monolayer cells that grow on laminin-coated plates, an extracellular matrix (ECM) protein to mimic the stem cell niche (Flanagan et al., 2006). To maintain their self-renewal, the cells are cultured with addition of EGF and FGF. The cells have a population doubling time of approximately 30 hours (data not shown). Undifferentiated cells express the NSC marker NESTIN (Fig. 8.1) and SOX2 (Fig. S2). To differentiate the cells, EGF and FGF were withdrawn from the media. When CX and VM cells differentiate, they express the neuronal cell marker TUJ1 (βIII-tubulin) and the glial cell marker GFAP confirming differentiation (Fig. 8.1).

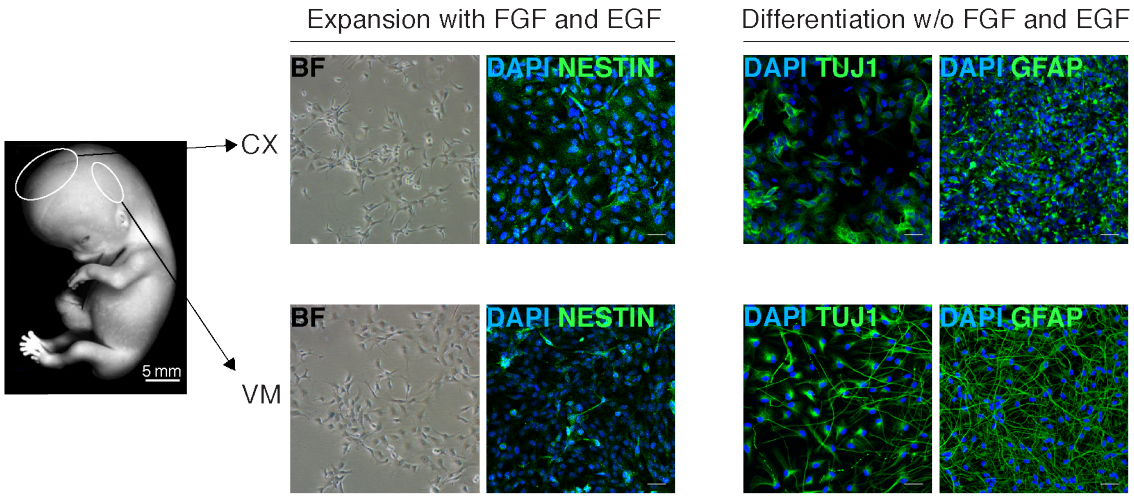


Figure 8.1: Schematic Overview of Human NSCs CX and VM as a model for Human Neural Differentiation: CX and VM cells are derived from the cortical (CX) and midbrain (VM) region of a human fetus. To maintain CX and VM cultures, FGF and EGF were added to the cells. With withdrawal of FGF and EGF, CX and VM differentiate and commit to more mature cell types of the CNS. Marker expression was assessed after 14 days of differentiation with the indicated antibodies by immunofluorescence. Scale bar represents 50 µm.

Next, we analysed if undifferentiated CX and VM cells express RASSF1A (Fig. 8.2). To assess their developmental stage, we asked whether they still express the major pluripotency marker OCT4.

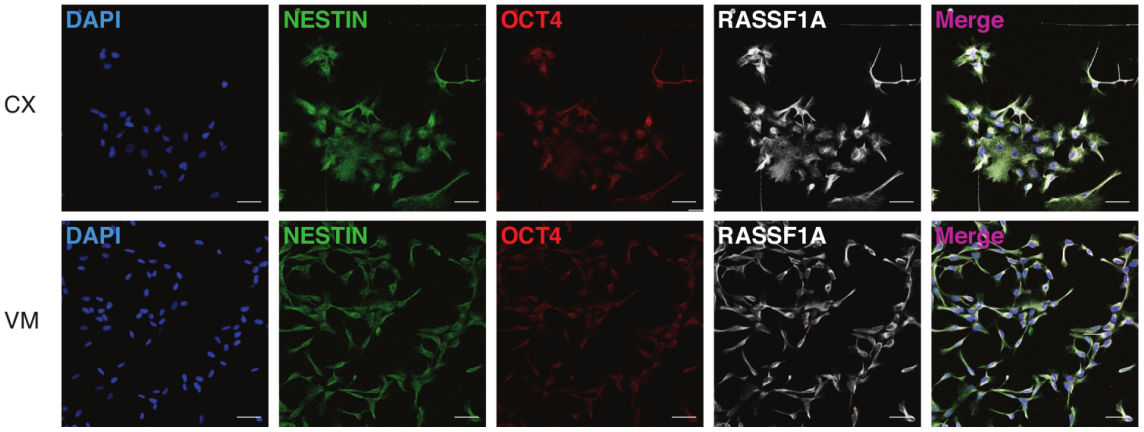


Figure 8.2: Undifferentiated CX and VM cells express NESTIN and RASSF1A but not OCT4: Undifferentiated CX and VM cells were assessed for marker and RASSF1A expression by immunofluorescence with indicated antibodies. Scale bar represents 50 µm.

RASSF1A is expressed in undifferentiated CX and VM cells (Fig. 8.2). Neural stem cell properties were confirmed by expression of NSC marker NESTIN and the pluripotency marker SOX2 (Supplementary Fig. S2). As expected hNSCs do not express OCT4 (Fig. 8.2).

To validate the GFAP and TUJ1 antibody specificity and to further explore the characteristics of hNSCs, we differentiated the cells and used western blot and immunofluorescent staining to assess their differentiation capacities.

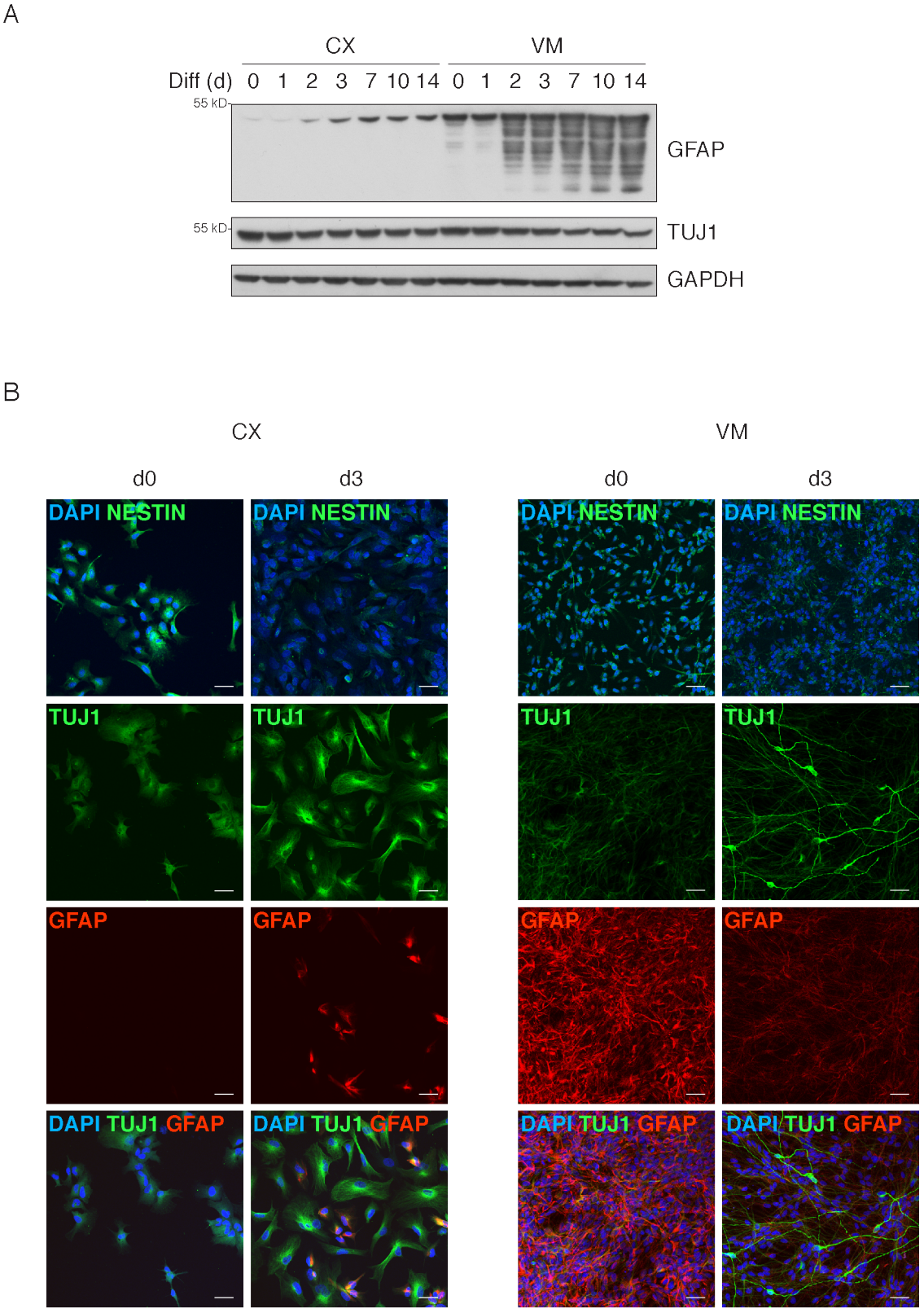


Figure 8.3: CX and VM cells express Neural Differentiation Markers: A) The differentiation potential of CX and VM cells was was assessed over 14 days by GFAP and TUJ1 expression using western blot analysis. B) Differentiation was confirmed by immunofluorescence after three days with the indicated antibodies. Scale bars represent 50 µm.

Western blot analysis shows that GFAP expression is increased in both cell lines (Fig. 8.3). Western blot and IF confirm that CX cells undergo gliogenesis. GFAP has a molecular weight of approximately 50 kDa. However, the GFAP antibody recognises multiple sub-bands in differentiating VM cells. Multiple GFAP bands have been reported as different isoforms, which were shown to be expressed in different areas of the mouse brain (Kamphuis et al., 2012). Possibly, the expression of different isoforms is induced with differentiation of VM cells, but not with CX. TUJ1 expression does not change when differentiation was assessed by western blot, however immunofluorescence staining shows an increase in both cell lines. Differentiated VM cells show typical morphology of glial and neuronal cells. Differentiated CX cells express the neuronal marker TUJ1, however the morphology of the TUJ1-positive cells deviates from the typical neuronal cell shape. Notable, undifferentiated VM cells show high expression of GFAP, which is decreased with differentiation. In mice, GFAP-positive cells have been shown to differentiate to neurons, astrocytes and oligodendrocytes (Casper and McCarthy, 2006; Ahmed et al., 2012), suggesting a certain plasticity of these cells. Additionally, GFAP is a marker for reactive astrocytes (Hol and Pekny, 2015) Here, we confirm that CX and VM express the differentiation marker after 3 days of differentiation.

8.3 RASSF1A during Neural Differentiation of hNSCs

To address if an increased RASSF1A expression is specific to early lineage commitment of embryonic stem cells or if with hNSCs differentiation RASSF1A levels change, we determined RASSF1A expression levels by western blot analysis.

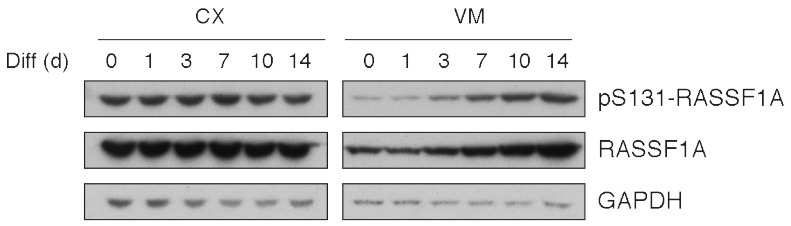


Figure 8.4: RASSF1A during Neural Differentiation of human NSCs: CX and VM cells were differentiated for 14 days. Western blot analysis was used to determine RASSF1A activation and total RASSF1A expression with differentiation.

Interestingly, RASSF1A is gradually increased over time with differentiation in VM but not CX cells. Additionally, phosphorylation of RASSF1A is increased similar to total expression levels in VM cells. Expression levels of RASSF1A are higher in CX than in VM cells.

We then asked if RASSF1A is transcriptionally upregulated with differentiation of hNSCS.

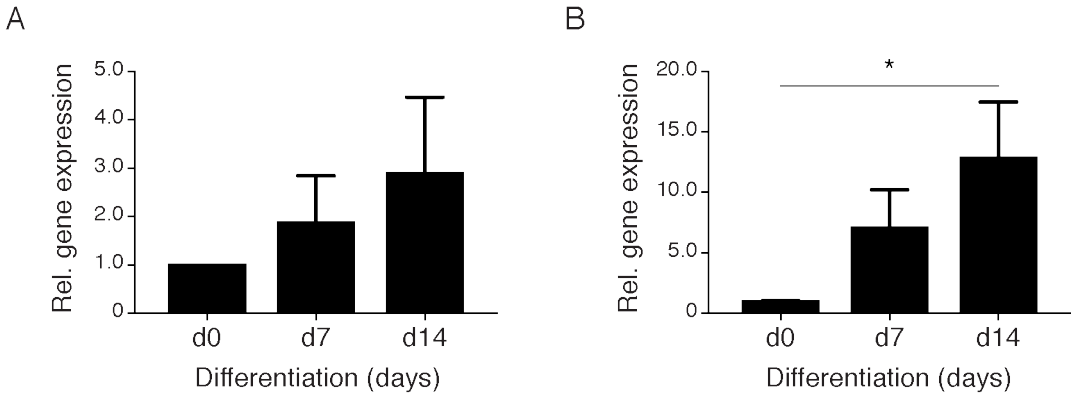


Figure 8.5: RASSF1A gene expression during Neural Differentiation of human NSCs: A) CX and B) VM cells were differentiated for 14 days. Samples were taken at indicated time points. QRT-PCR was performed to determine relative RASSF1A gene expression during differentiation. Presented is the relative gene expression to undifferentiated cells. GAPDH was used as a input control. Error bars represent n=3 biological replicates. Statistical significance was determined by a two-tailed t-test, * $P < 0.05$.

RASSF1A expression is transcriptionally upregulated with neural differentiation in VM cells (7.1-fold at day 7 and 12.9 fold, p=0.022 at day 14, Fig. 8.5B). In CX cells, RASSF1A mRNA expression increases 1.9 fold with seven days of differentiation and 2.9 fold (not significant), with 14 days of differentiation (Fig. 8.5A).

Since RASSF1A is frequently epigenetically silenced by promoter methylation in cancer, we asked if the activation of gene expression with differentiation is due to gene demethylation. Therefore, we differentiated CX and VM cells and performed methylation specific PCR to identify methylation levels on RASSF1A (Fig. 8.6).

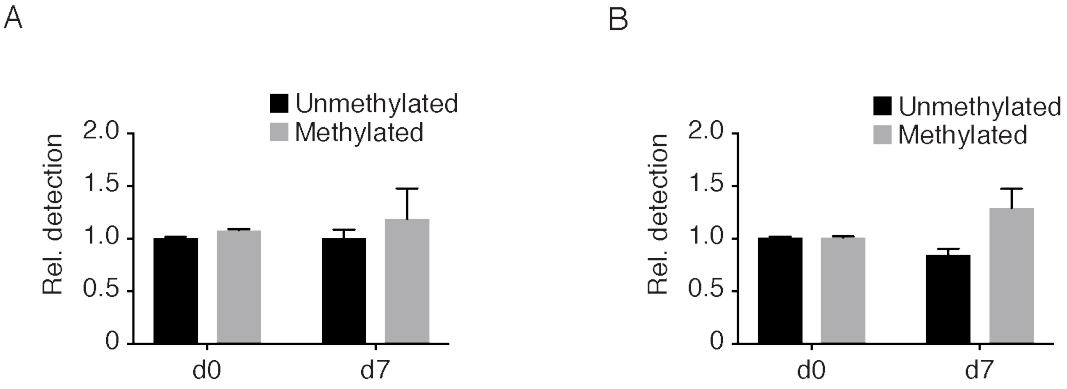


Figure 8.6: Methylation of RASSF1A during Neural Differentiation of human NSCs: A) CX and B) VM cells were differentiated for 7 days . Methylation specific PCR was performed to detect levels of changes in unmethylated and methylated RASSF1A with differentiation relative to methylation-independent Beta-actin. Error bars represent n=3 biological replicates.

Activation of RASSF1A expression in VM cells is not due to demethylation of the promoter. As expected, no difference in promoter methylation was observed in CX cells. Beta-actin was used as a DNA input control and the TaqMan® assay was chosen to be methylation-independent.

To modulate RASSF1A protein levels in hNSCs we performed Lipofectamine 3000 transfection, a method based on cationic lipid transfection (Fig. S3). Since no over-expression of RASSF1A was achieved, we validated Nucleofection™, a transfection method based on electroporation. Transfection of CX and VM was optimised using the the Cell Line Optimization Nucleofector® Kit with the Nucleofector™ 2b device (Supplementary Fig. S4). CX and VM cells were transfected using solution L, programme G-013 in further experiments.

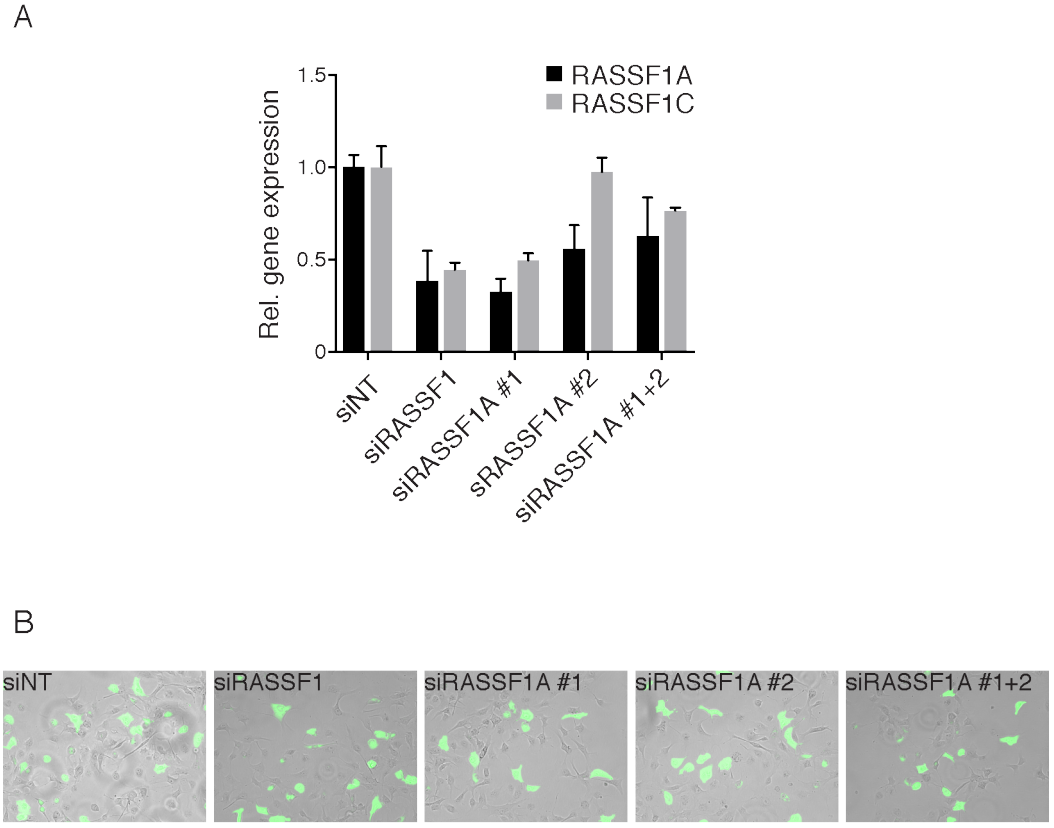


Figure 8.7: Validation of RASSF1A siRNAs in CX cells: CX cells were co-transfected with GFP and siRNA against either RASSF1A using Nucleofection™. A non-targeting siRNA (siNT) was used as a negative control and a siRNA against RASSF1 as a positive control. A) Knock-down efficiency of the two isoforms, RASSF1A and RASSF1C, was determined by qRT-PCR. B) GFP was visualised with fluorescence microscope. Error bars represent n=3 technical replicates.

To ensure that the siRNAs are specific for the RASSF1A isoform, qRT-PCR was performed to determine levels of RASSF1A and RASSF1C mRNA expression. Transfection with siRASSF1 resulted in a 50% knock-down of both isoforms compared to the non-targeting control. RASSF1A and RASSF1C expression were also decreased by 50% when transfected with siRASSF1A #1. However, siRASSF1A #2 was specific for RASSF1A as no decrease in RASSF1C mRNA levels was detected. We therefore used siRASSF1A #2 in further experiments.

Since RASSF1A is increased with neural differentiation in VM cells, we asked whether depletion of RASSF1A affects the differentiation potential of hNSCs. Therefore, CX and VM cells were transfected with siRASSF1A and differentiation was induced 24 h post-transfection.

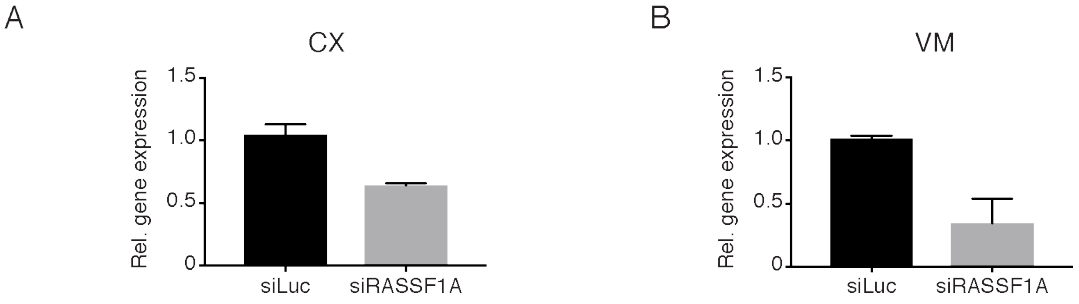


Figure 8.8: RASSF1A Knock-down Control: A) CX and B) VM cells were transfected with siRASSF1A and cells were collected 24 h post-transfection. A siRNA against Luciferase was used as a negative control. Initial knock-down of RASSF1A was confirmed at d0 by qRT-PCR. Error bars represent n=3 biological replicates.

We confirmed that RASSF1A expression is reduced due to transient knock-down when differentiated was induced (Fig. 8.8).

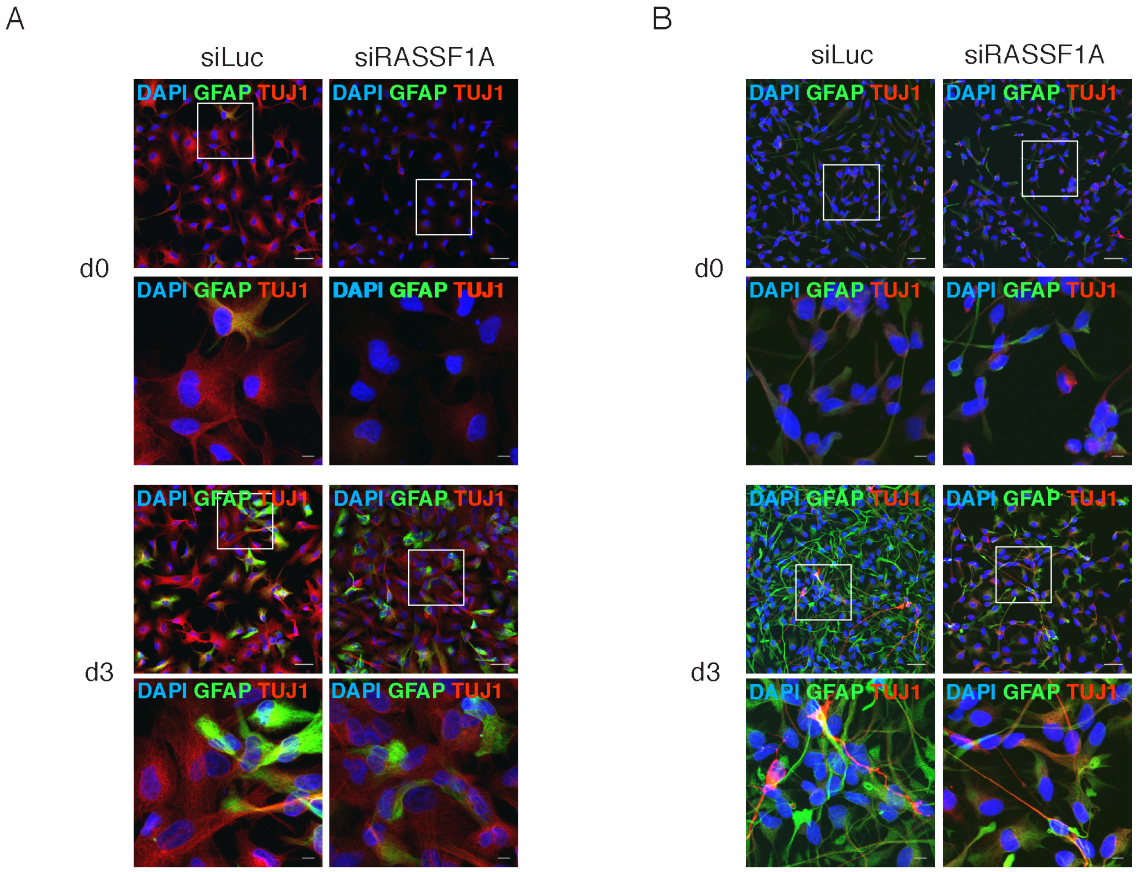


Figure 8.9: RASSF1A depletion impairs Neural Differentiation in human NSCs: A) CX and B) VM cells were transfected with siRASSF1A and differentiation was induced 24 h post-transfection. The cells were allowed to differentiate for three days and expression levels of the differentiation marker GFAP and TUJ1 were determined by immunofluorescence. Scale bar represents 50 µm and 10 µm for zoomed images.

Upon differentiation of RASSF1A-depleted cells, CX and VM cells show less expression of GFAP and TUJ1 than compared to the control in immunofluorescence staining (Fig. 8.9). VM cells fail to differentiate into neuronal cells and show less neurite outgrowth. Consequently, we conclude that the absence of RASSF1A impairs differentiation.

8.4 Discussion

In this chapter, we studied the role of RASSF1A in human neural stem cells. Interestingly, the two cell lines, which are derived from different regions of the human fetal brain show different properties regarding their RASSF1A expression. The cell line derived from the cortical region (CX) has high RASSF1A expression, which is not changing with differentiation. However, the midbrain cell line (VM) expresses RASSF1A at lower levels but its expression is increased with differentiation. Additionally, differentiation markers reveal differences of the two cell lines. VM cells have high GFAP expression when undifferentiated and the morphology of these cells suggest a strong glial phenotype. However, they are able to differentiate into neurons, which show high TUJ1 expression and have a typical spindle-like neuronal morphology. On the contrary, CX cells express TUJ1 in an undifferentiated state. This expression increases with differentiation, but the morphology of theses cells do not demonstrate a typical neuronal morphology. Since both cell lines express the neural stem cell marker NESTIN and SOX2, the question arises what impact the source of origin has on these stem cells. As expected, NESTIN is lost with differentiation, suggesting a maturation of these cells. However, the TUJ1-positive CX cells do not exhibit dendritic or axonal outgrowth and are therefore not considered as functional neurons. They induce expression of GFAP upon differentiation, suggesting that the CX cells are more primed towards the glial lineage. The fact that the two cell lines show different characteristics in regards to RASSF1A expression was very intriguing. Thus, all experiments were conducted in both cell lines.

In cancer, RASSF1A promoter methylation is frequently observed. Since methylation of the RASSF1A promoter has been intensively studied in this context, we wondered if methylation and demethylation of the promoter is involved in the regulation of RASSF1A expression in normal cells. We did not find any evidence that the increase in RASSF1A expression in VM cells is due to demethylation of the

promoter.

Interestingly, with RASSF1A depletion VM cells failed to differentiated to neurons (Fig. 8.9). Moreover, differentiation marker expression is decreased in CX cells. Additionally, GFAP expression was decreased with knock-down and differentiation of VM cells compared to the controls. We therefore speculate that the underlying mechanism of RASSF1A loss in cancer might be due to impaired differentiation capacities. To clarify if CX and VM cells are impaired in their differentiation capacity upon knock-down of RASSF1A, the expression of stem cells marker including NESTIN and SOX2 need to be assessed. Additionally, since CX and VM cells express a basal level of GFAP/TUJ1 the quantification of the fluorescence intensity per cell is needed to confirm a significant impact of loss of RASSF1A on their differentiation. The histone mark H3K9me3 has been shown to silence genes when cells undergo neural differentiation (Golebiewska et al., 2009; Parsons, 2012). We therefore felt encouraged to further explore the role of the RASSF1A-SUV39H1 axis in neuroectodermal and differentiation.

Chapter 9

Epigenetics

9.1 Introduction

Differentiation is accompanied by massive epigenetic changes. As a cell differentiates and commits to a more specialised cell type, many genes need to be permanently repressed. SUV39H1 uses H3K9me1 as a substrates and catalyses its di- and trimethylation, which is recognised by HP1. Therefore, SUV39H1 is essential for the establishment of heterochromatin and gene repression. In the previous chapters we verified that loss of RASSF1A contributes to impaired neural differentiation. In hESCs, transient knock-down of RASSF1A resulted in increased SUV39H1 levels, but decreased H3K9me3. Since SUV39H1 promotes long-term gene silencing by heterochromatin formation, we asked if the RASSF1A-dependent regulation of SUV39H1 also persists in hNSCs and if this regulation could contribute to carcinogenesis.

This led to the hypothesis that RASSF1A may have a role in SUV39H1 localisation. Therefore we continued to study the impact of loss of RASSF1A on SUV39H1 with regards to protein stabilisation, activity and localisation.

9.2 Epigenetic Regulation of Neural Differentiation

To validate the SUV39H1 antibody (Cell signaling Technologies), we performed a siRNA mediated transient knock-down of SUV39H1, which we confirmed by qRT-PCR with two different TaqMan® assays (Fig. 9.1).

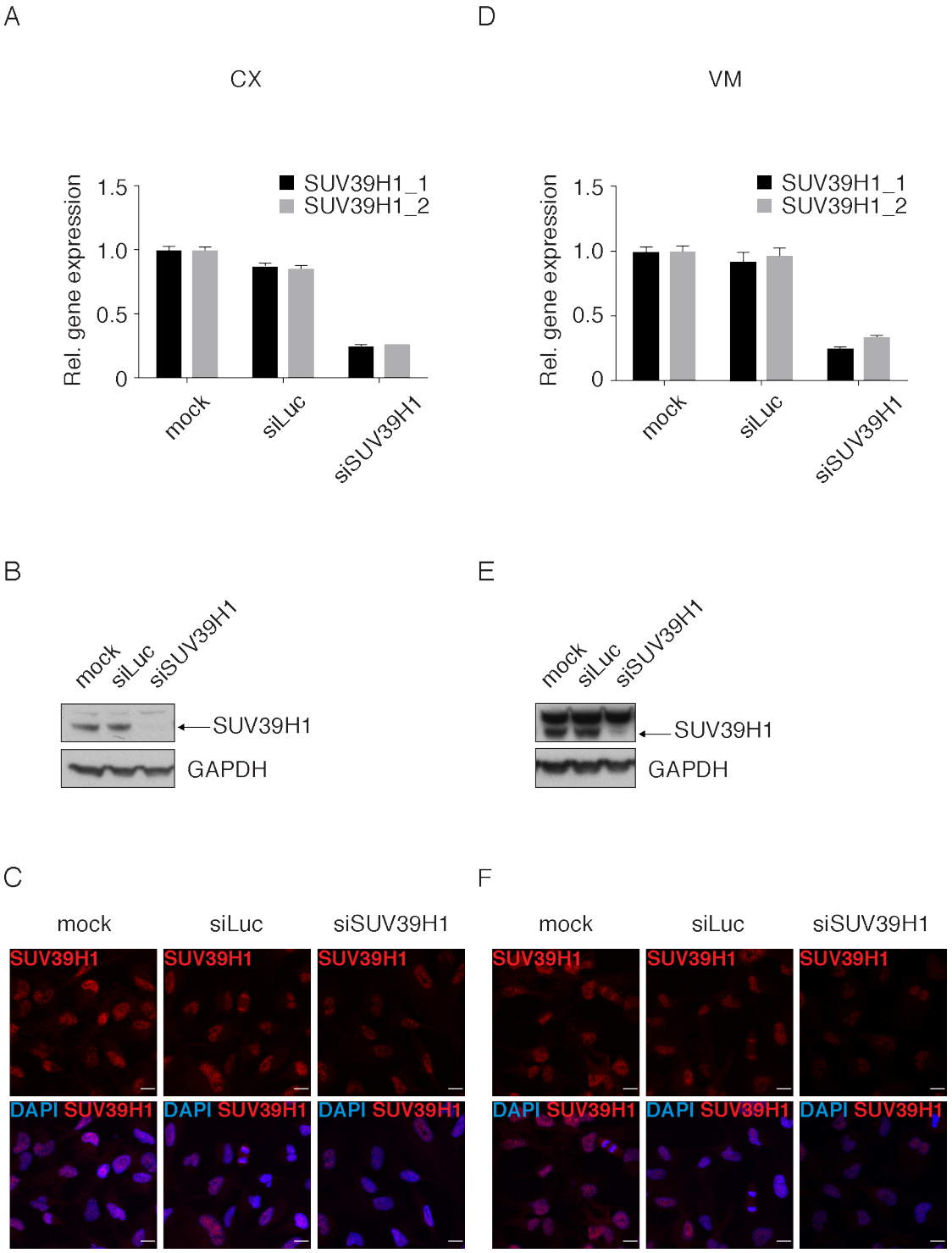


Figure 9.1: SUV39H1 Antibody Validation: Transient knock-down of SUV39H1 was performed in CX (A-C) and VM (D-F) cells. Quantitative RT-PCR (A,D) confirmed Knock-down. Western Blot analysis (B, E) was performed to identify the specific SUV39H1 band. In immunofluorescence (C, F) the antibody was specific. Error bars represent three technical replicates of n=3. Scale bar represents 10 µm.

The SUV39H1 (Cell Signaling Technologies®) antibody is specific in immunofluorescence. However, in western blot analysis the antibody detected an unspecific band above the specific SUV39H1 band. Noteworthy, other SUV39H1 antibodies (Thermo Scientific and Active motif) did not detect the unspecific band but the overall SUV39H1 signal was much weaker (data not shown). For future experiments the Cell Signaling Technologies antibody was used to detect SUV39H1 unless otherwise indicated.

9.3 RASSF1A and Chromatin marks

Since SUV39H1 is increased with RASSF1A knock-down in hESCs, we asked whether this is a consequence of impaired neuroectodermal differentiation. Therefore, we examined SUV39H1 levels during neuroectodermal differentiation of hESCs (Fig. 9.2).

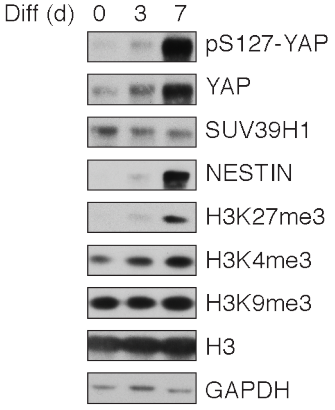


Figure 9.2: SUV39H1 and Chromatin Marks during Neuroectodermal Differentiation of hESCs: Human ESCs were differentiated for seven days. Cell samples were taken at day 0 (undifferentiated), day 3 and day 7. Western blot analysis was performed for SUV39H1, total and phosphorylated YAP and histone marks. NESTIN was used as a differentiation marker and GAPDH served as a loading control.

When hESCs differentiate into the neuroectodermal lineage, NESTIN expression is

massively increased, particularly after seven days of differentiation. Interestingly, SUV39H1 and H3K9me3 levels do not change with differentiation. As previously observed, NESTIN expression is increased during neuroectodermal lineage specification, particularly after seven days of differentiation. Interestingly, total expression and phosphorylation at serine 127 of YAP were increased with differentiating hESCs. Additionally to the gene repression mark H3K9me3, we monitored changes of H3K4me3 (gene activation) and H3K27me3 (gene repression). Both marks play an important role in regulating developmental genes, thus an increase in the context of early differentiation is expected.

We then wanted to test how SUV39H1 expression is affected during neural differentiation, when hNSCs commit to more mature neural cells. Firstly, to confirm specificity of the SUV39H1 antibody during neural differentiation, transient knock-down of SUV39H1 was performed and cells were differentiated for seven days. Samples were collected at day 0 (24 h post transfection) and day 1. SUV39H1 expression was assessed by western blot.

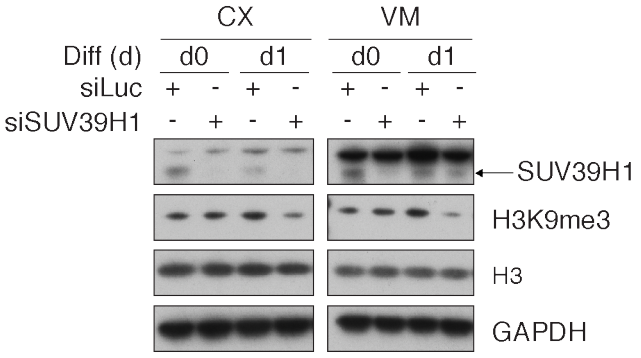


Figure 9.3: SUV39H1 antibody validation during Neural Differentiation: Knock-down of SUV39H1 was performed and cells were collected at day 0 (24 h post-transfection) and day 1. Expression of SUV39H1 and its histone mark H3K9me3 were determined by western blot analysis.

Depletion of SUV39H1 was sufficient at day 0 and day 1. The histone mark H3K9me3 is increased with differentiation in the knock-down controls. Interestingly, a reduction of H3K9me3 is not noticeable at day 0, but a reduction in the chromatin mark at d1 was detected in both cell lines. This observation indicates either a delay in demethylation (catalysed by the four lysine demethylases JMJD2A-D) or the reduced mark is differentiation-specific.

Next, we differentiated CX and VM cells for 14 days and analysed SUV39H1 expression by western blot.

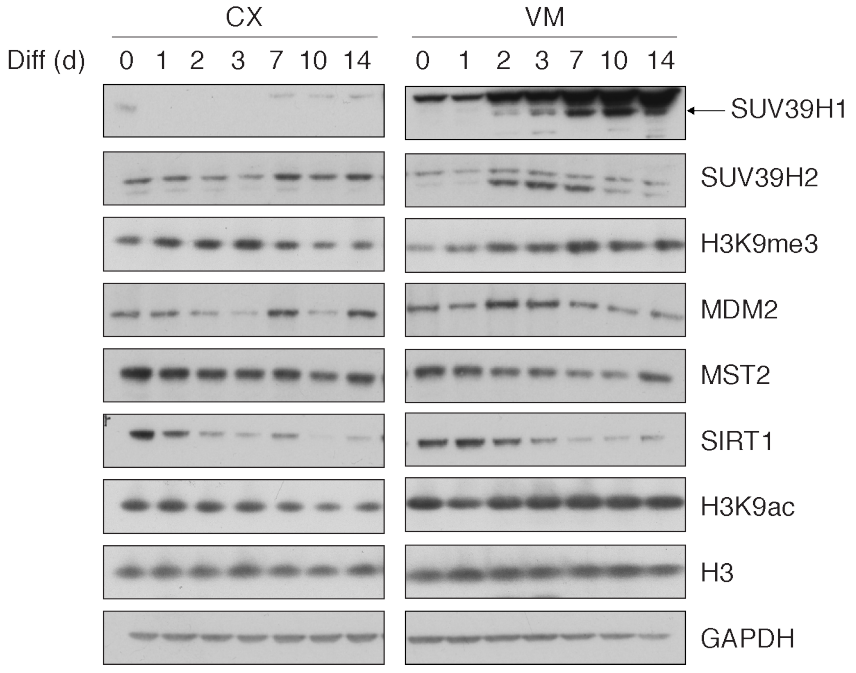


Figure 9.4: SUV39H1 and Chromatin Marks during Neural Differentiation of hNSCs: CX and VM cells were differentiated in total for 14 days. Cells were collected at seven time points during this time. Expression levels of chromatin marks and their modifiers were assessed by western blot analysis.

SUV39H1 expression was increased with neural differentiation in VM cells (Fig 9.4). However, undifferentiated CX cells express SUV39H1 at low levels but lose expres-

sion with differentiation. Noticeably, SUV39H1 expression is significantly lower in CX cells than in VM cells. The band detected with the SUV39H1 antibody at 7, 10 and 14 days post initiation of differentiation in CX cells was previously identified as unspecific (Fig. 9.3). Accordingly, the H3K9me3 mark is reduced with differentiation in CX cells. In VM cells, SUV39H1 expression and H3K9me3 are increased with differentiation (Fig 9.4). No significant changes of SUV39H2 and MST2 were observed with differentiation of hNSCs. SIRT1, a H3K9 deacetylase, which has been shown to enhance SUV39H1 activity by deacetylation, was decreased with differentiation in both cell lines. The H3K9ac mark did not change during differentiation of VM cells, but is decreased in CX cells. No differentiation-specific changes of MDM2 were observed in CX cells, but MDM2 was decreased after 3 days of differentiation in VM cells. Since SUV39H1 and H3K9me3 are increased with differentiation indicating that the cells undergo increased transcriptional shutdown, a typical hallmark for differentiation. Thus, the VM cells seem to have a greater differentiation potential,

Next, we tested if the increase in SUV39H1 protein expression is a consequence of the upregulation of SUV39H1 transcription. Therefore, qRT-PCR analysis was performed in undifferentiated (d0) and differentiated (d7 and d14) CX and VM cells.

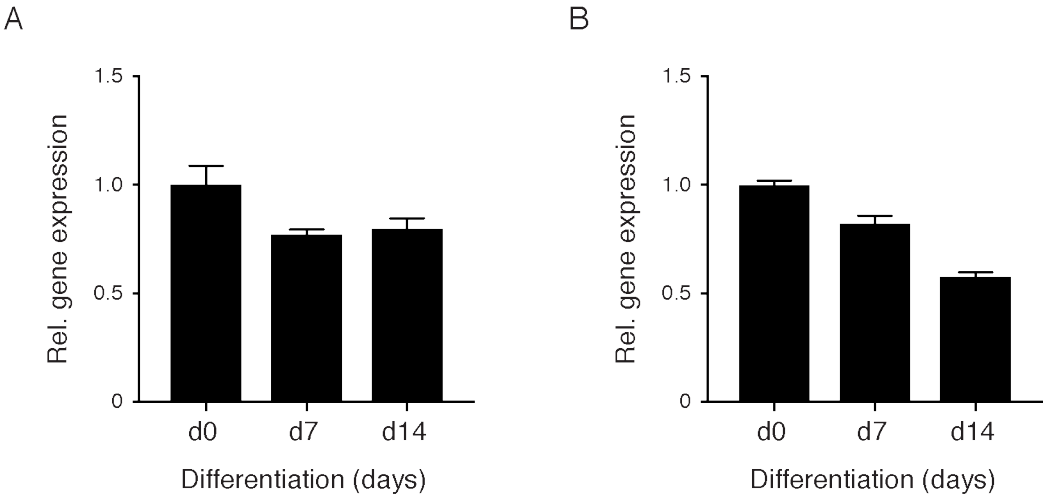


Figure 9.5: SUV39H1 gene expression during Neural Differentiation of human NSCs: A) CX and B) VM cells were differentiated for 14 days. Samples were taken at indicated time points. QRT-PCR was performed to determine SUV39H1 gene expression during differentiation. Presented is the relative gene expression to undifferentiated cells. GAPDH was used as a input control. Error bars represents n=3 three technical replicates.

SUV39H1 transcript levels are slightly decreased with differentiation in CX cells (Fig. 9.5). In VM cells, the decrease is more pronounced , however protein levels are significantly increased. Reasons for this discrepancy could be similar to those discussed earlier for RASSF1A transcript levels in neuroectodermal differentiation (Chapter 8).

To investigate the hypothesis that RASSF1A regulates SUV39H1 protein levels in hNSCs (as previously observed in hESCs), a transient knock-down of RASSF1A was performed in CX and VM cells.

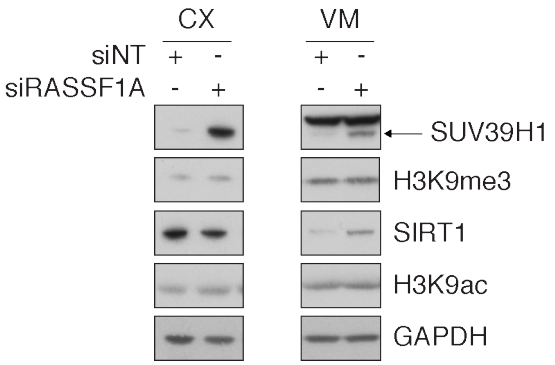


Figure 9.6: SUV39H1 is increased with knock-down of RASSF1A in hNSCs: CX and VM cells were nucleofected with siNT and siRASSF1A. SUV39H1 and SIRT1, as well as their corresponding chromatin marks were analysed 24 h post-transfection by western blot with the antibodies indicated.

SUV39H1 was increased with RASSF1A knock-down in CX and VM cells (Fig. 9.6). In western blot no changes in H3K9me3 with RASSF1A depletion was observed. Interestingly, SIRT1 was increased with RASSF1A knock-down in VM cells, but not in CX cells. The H3K9ac mark was not affected.

Consequently, to investigate whether RASSF1A depletion affects the cellular localisation of SUV39H1, CX (Fig. 9.7) and VM (Fig. 9.8) were stained for SUV39H1 by immunofluorescence.

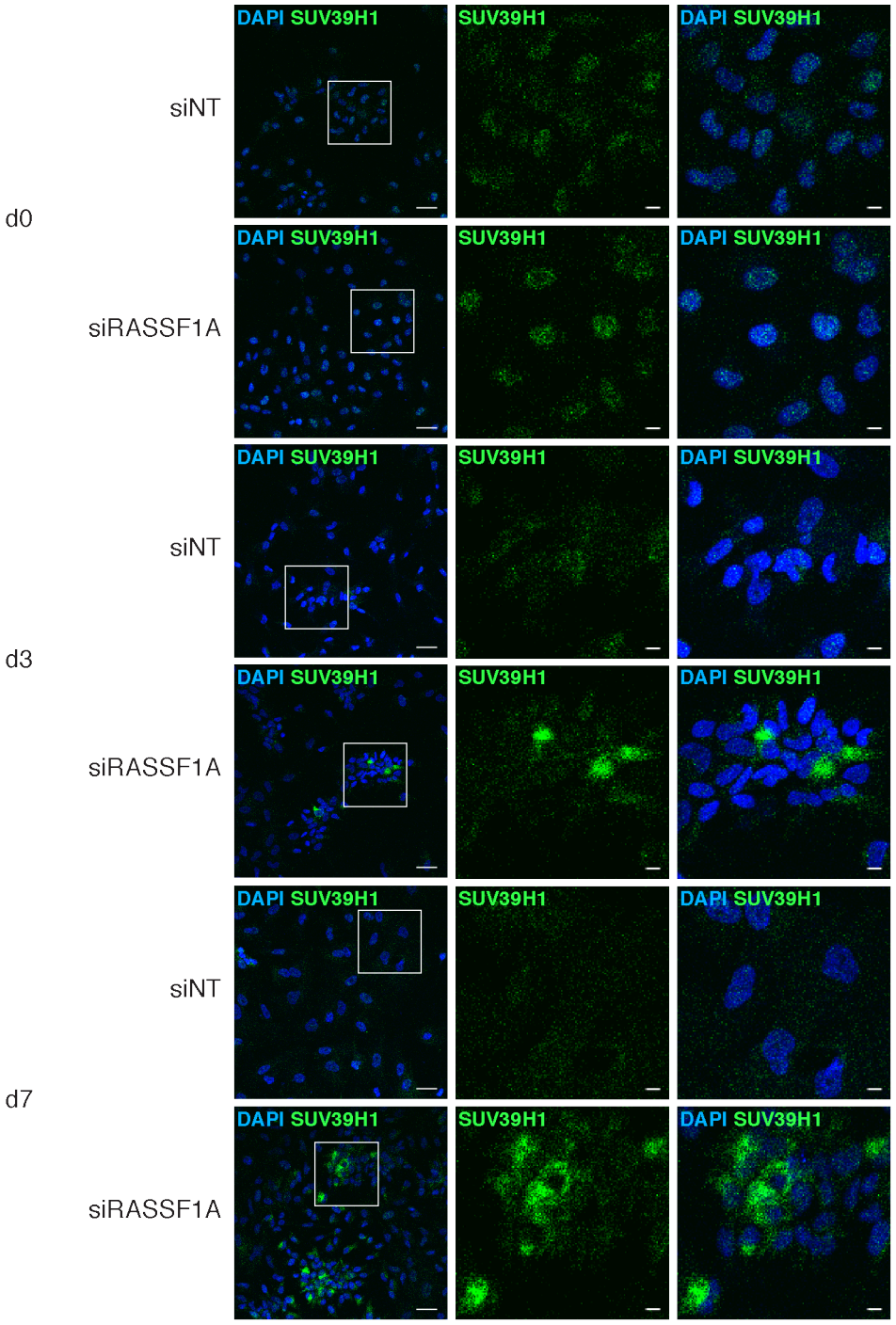


Figure 9.7: SUV39H1 localisation changes with RASSF1A Knock-down in CX cells: Differentiation- and RASSF1A-dependent SUV39H1 localisation was assessed in CX cells by immunofluorescence. Cell were transfected with siNT or siRASSF1A and differentiated for seven days. Scale bar represents 50 µm and 10 µm for zoomed images.

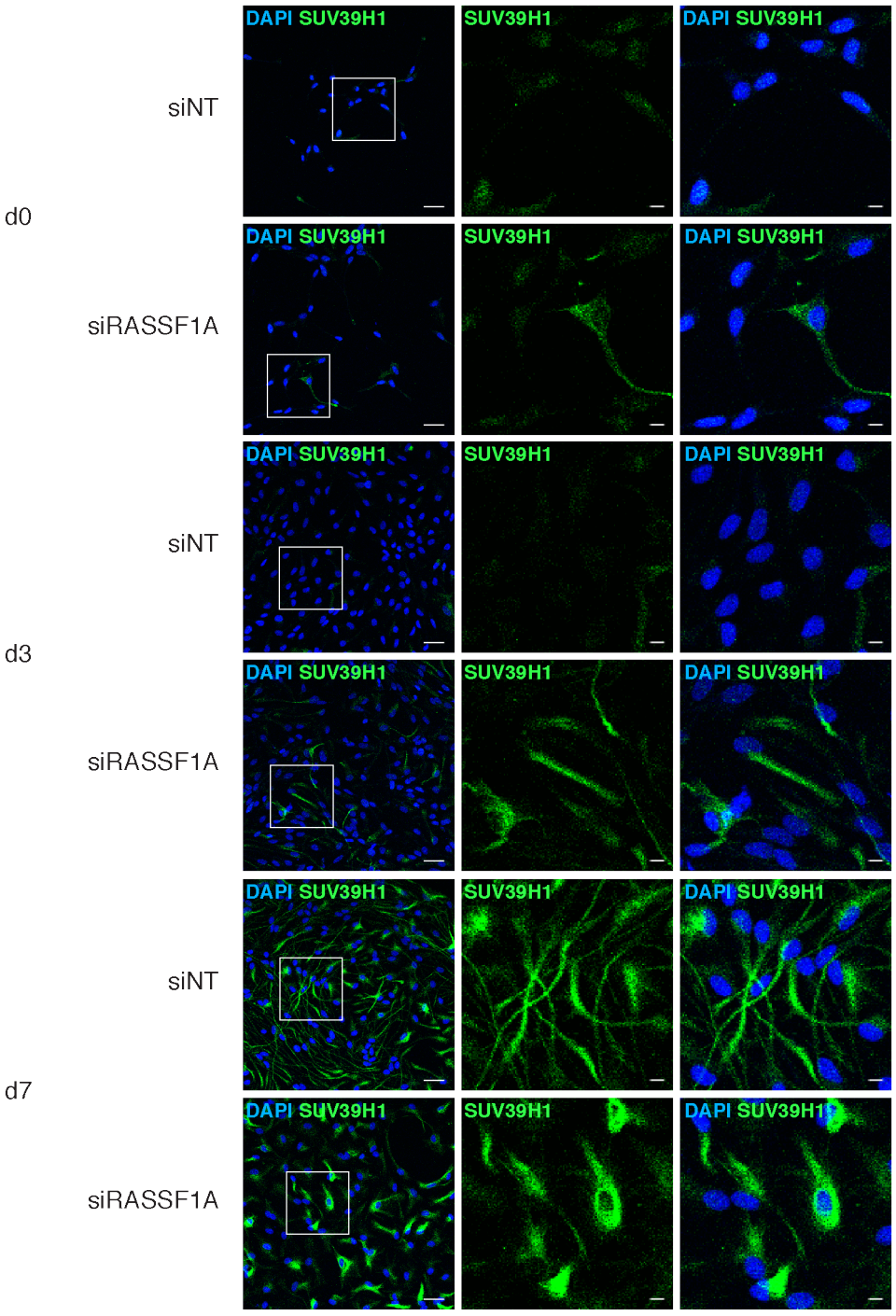


Figure 9.8: SUV39H1 localisation changes with RASSF1A Knock-down in VM cells: Differentiation- and RASSF1A-dependent SUV39H1 localisation was assessed in VM cells by immunofluorescence. Cell were transfected with siNT or siRASSF1A and differentiated for seven days. Scale bar represents 50 µm and 10 µm for zoomed images.

Immunofluorescent staining in RASSF1A depleted cells confirms increased SUV39H1 expression (Fig. 9.7 and Fig. 9.8). Intriguingly, with differentiation, SUV39H1 is largely localised to the cytoplasm. Additionally, the cytoplasmic localisation of SUV39H1 seems further increased with RASSF1A knock-down. Since the RASSF1A-dependent increase of SUV39H1 expression does not affect H3K9me3, we hypothesized that RASSF1A modulates SUV39H1 localisation.

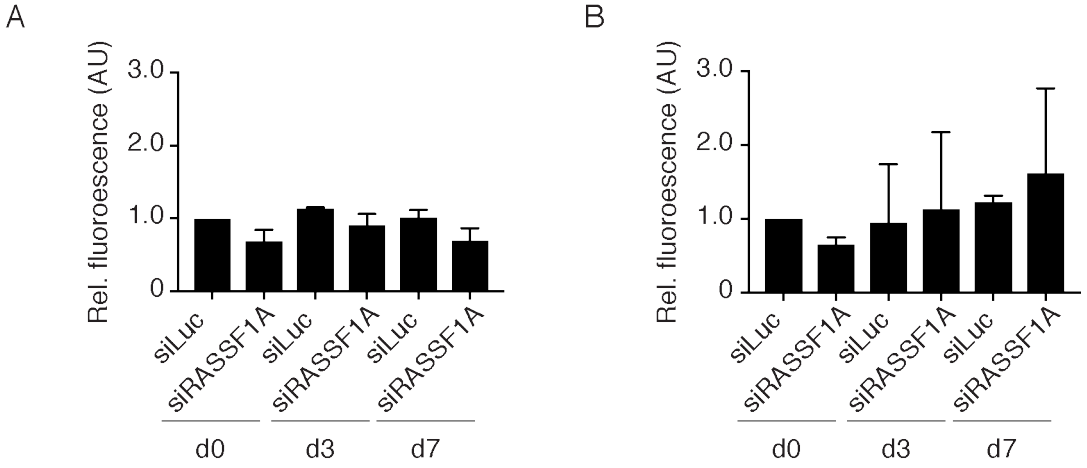


Figure 9.9: Quantification of SUV39H1 localisation with RASSF1A Knock-down in hNSCs: The immunofluorescence of nuclear and total SUV39H1 was quantified relative to cell number. Cytoplasmic SUV39H1 is shown relative to siNT/siLuc and relative to nuclear SUV39H1 at d0 in A) CX and B) VM cells. Error bars represent n=2 biological replicates.

Interestingly, quantification of changes in cytoplasmic SUV39H1 relative to nuclear SUV39H1 showed no increase with RASSF1A knock-down or differentiation in CX cells (Fig. 9.9A). In VM cells, cytoplasmic SUV39H1 seems to be increase compared to nuclear SUV39H1 with RASSF1A knock-down at day 3 and 7. Noteworthy, cytoplasmic marker, e.g. phalloidin, should have been included in SUV39H1 immunofluorescence analysis for exact nuclear/cytoplasmic distinction.

Next, we assessed H3K9 trimethylation by immunofluorescence.

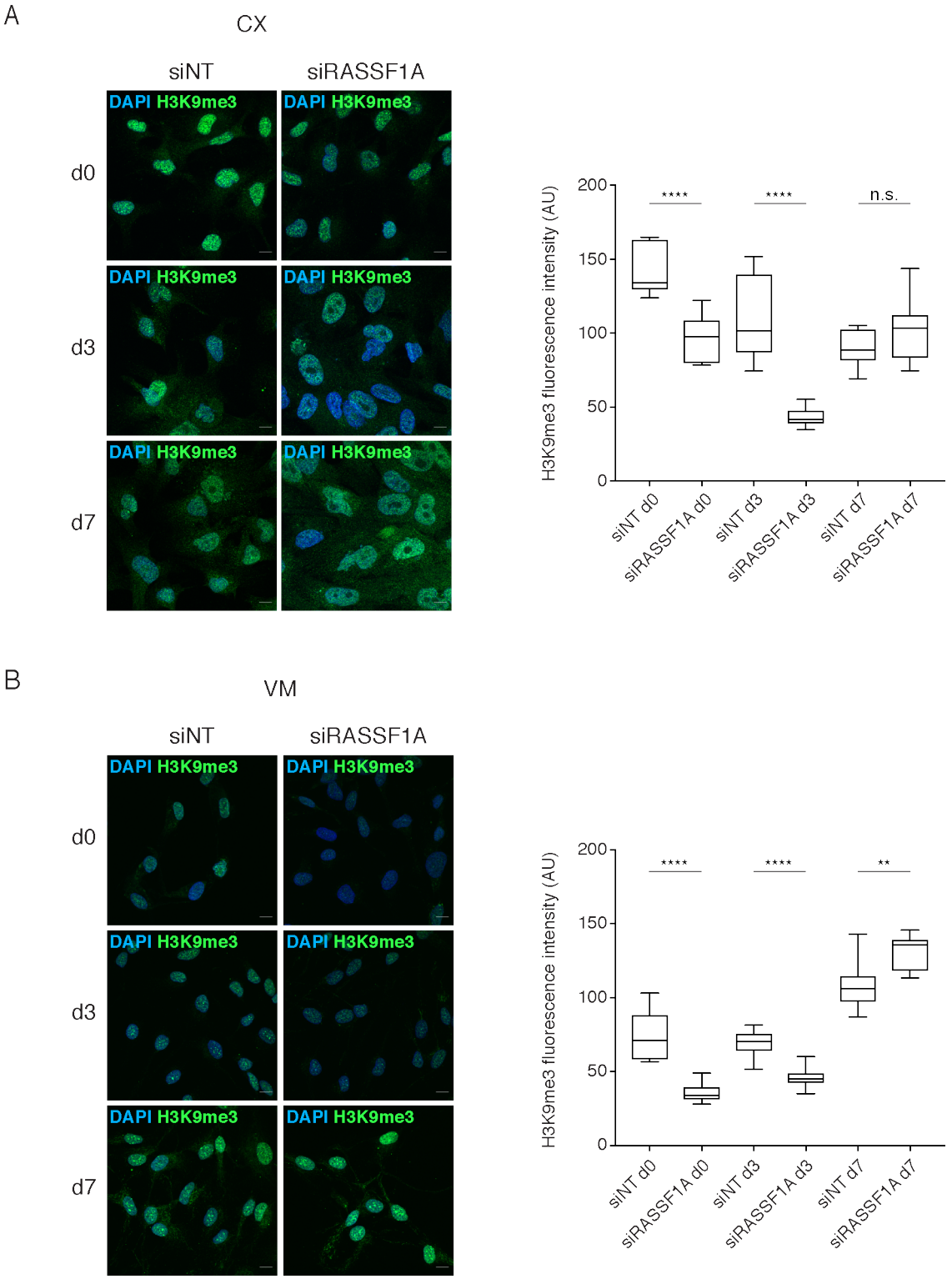


Figure 9.10: H3K9me3 is reduced with RASSF1A knock-down: H3K9 trimethylation was assessed in RASSF1A-depleted and differentiated A) CX and B) VM cells by immunofluorescence with the indicated antibody. Plot represents quantifications of representative images of A) and B). Image field medians (lines in boxes), 25th - 75th percentiles (boxes) and 1th - 99th percentiles (whiskers) are shown. Scale bar represents 10 µm. Statistical significance was determined by a unpaired t-test, n.s. $P > 0.05$, ** $P < 0.01$, **** $P < 0.0001$

The histone mark H3K9me3 was significantly reduced with RASSF1A depletion at day 0 and day 3 in CX (Fig. 9.10A) and at day 0, day 3 and day 7 in VM cells (Fig. 9.10B). At day 7, differences in H3K9 trimethylation might have been lost due to the transient nature of siRNA-mediated knock-down as knock-down of RASSF1A at d7 of differentiation was no longer evident (Fig. S6).

As we postulate that RASSF1A alters the activity of SUV39H1 towards its substrate H3K9me3, we ask if direct interaction of RASSF1A and SUV39H1 leads to a translocation of the complex into the cytoplasm.

Firstly, to test the proposed model, we evaluated if RASSF1A and SUV39H1 are interacting in hNSCs. Therefore, we overexpressed FLAG-tagged wildtype RASSF1A (FLAG-RASSF1A) and the unphosphorylated mutant FLAG-RASSF1A-S131A in VM cells, and immunoprecipitated with an anti-FLAG antibody.

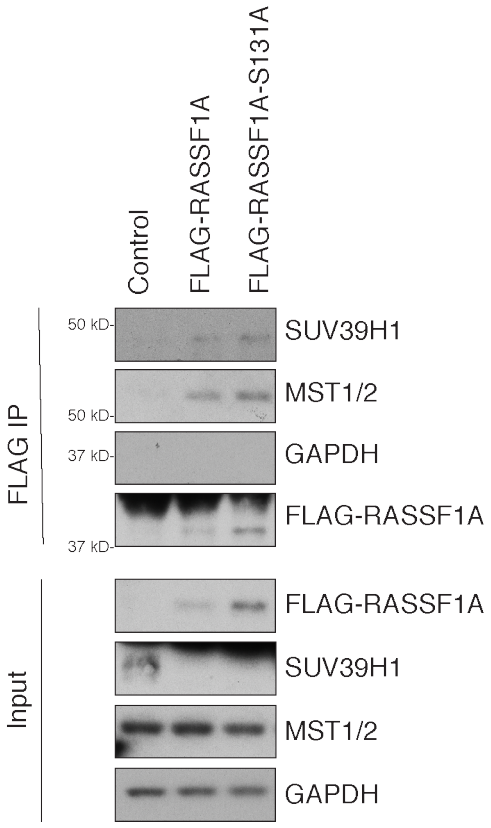


Figure 9.11: Interaction of RASSF1A and SUV39H1: VM cells were transiently nucleofected with pcDNA3.1, FLAG-RASSF1A and FLAG-RASSF1A-S131A and differentiated 24h post-transfection. FLAG was immunoprecipitated and assessed for co-immunoprecipitation of SUV39H1 and MST1/2. GAPDH was used as a loading control.

Indeed, SUV39H1 identified as a binding partner of RASSF1A in VM cells. Interestingly, this interaction was not disrupted with the RASSF1A mutant and therefore the interaction was not dependent on the activation of RASSF1A. Additionally, MST1/2 were found as a complex partner of RASSF1A and SUV39H1. This suggests that RASSF1A mediates MST1/2 function towards SUV39H1.

To evaluate if the RASSF1A-dependent altered localisation of SUV39H1 contributes to impaired differentiation, we performed a knock-down experiment of SUV39H1 and assessed the expression of the differentiation markers GFAP and TUJ1. Initial knock-down (d0) was confirmed by Western Blot (Fig. 9.1 and Fig. 9.3).

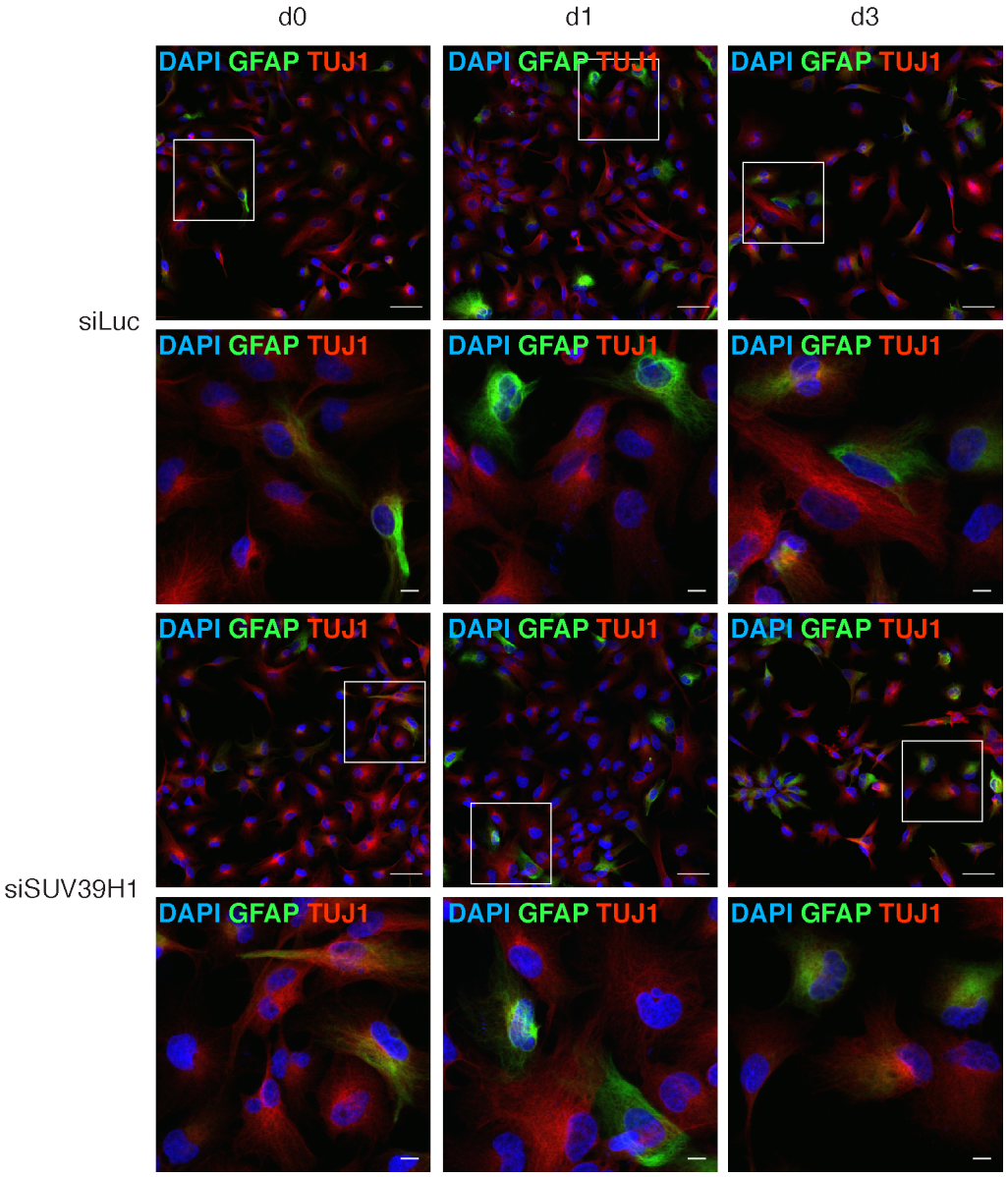


Figure 9.12: SUV39H1 knock-down in CX cells: Transient knock-down of SUV39H1 was performed in CX cells. Differentiation was assessed by immunofluorescent staining of the marker with the antibodies indicated. Scale bar represents 50 µm and 10 µm for zoomed images.

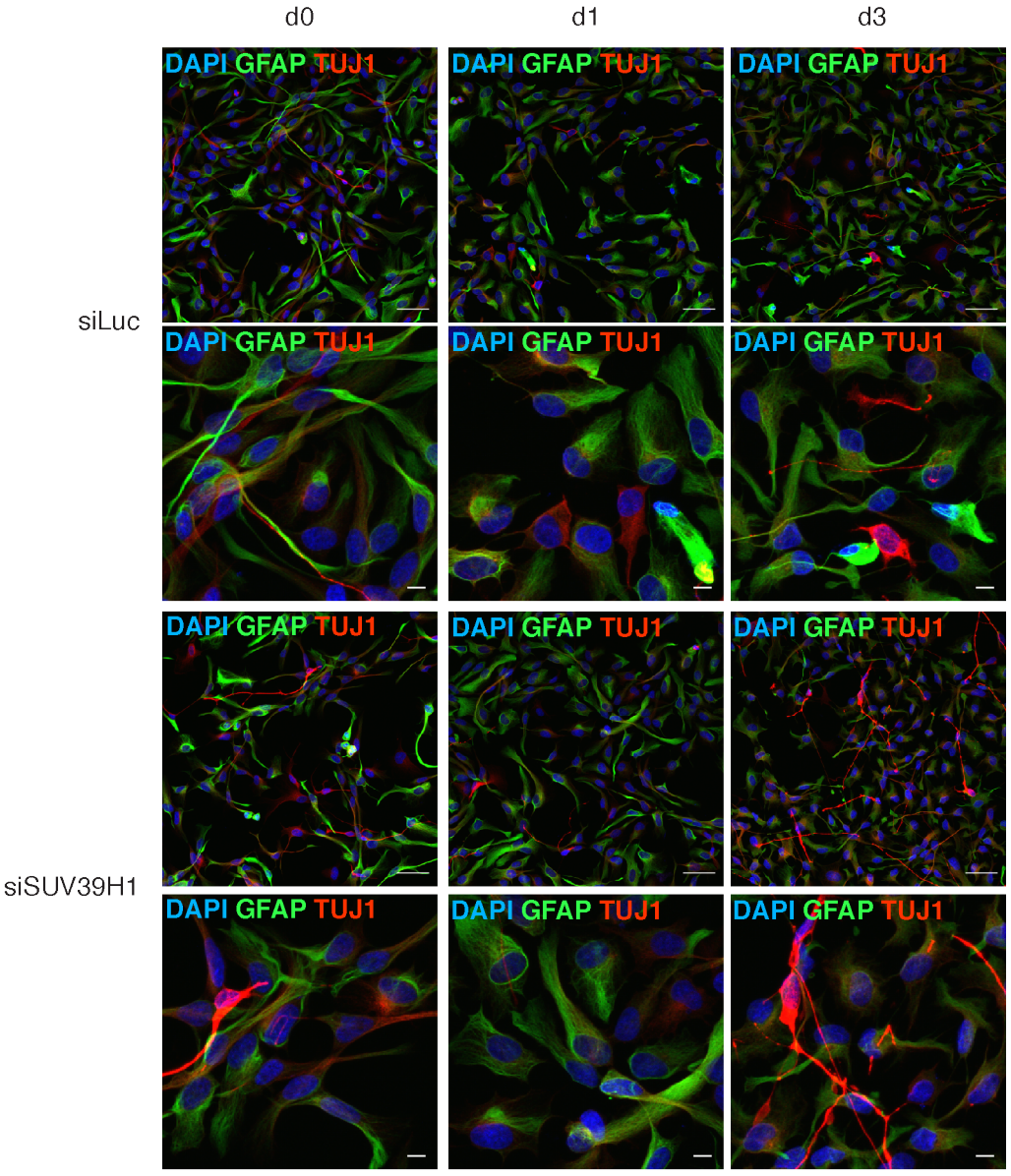


Figure 9.13: SUV39H1 knock-down in VM cells: Transient knock-down of SUV39H1 was performed in VM cells. Differentiation was assessed by immunofluorescent staining of the marker with the antibodies indicated. Scale bar represents 50 µm and 10 µm for zoomed images.

No differences in expression of GFAP and TUJ1 was observed in CX cells (Fig. 9.12), indicating no difference in the differentiation potential of these cells in the absent of SUV39H1. Intriguingly, SUV39H1-depleted VM cells formed more neurons (TUJ+ cells) and less glial (GFAP+) cells, suggesting the reduced SUV39H1 levels favour the neuronal lineage in VM cells. The knock-down experiments revealed that RASSF1A-

dependent localisation of SUV39H1 and knock-down of SUV39H1 do not affect hNSC differentiation in a similar manner.

9.4 Discussion

In the third chapter, we are investigating the effects of RASSF1A on SUV39H1 in hNSCs regarding their differentiation potential.

First, we investigated SUV39H1 during neuroectodermal differentiation of hESCs (Fig. 9.2). SUV39H1 and the chromatin mark H3K9me3 are not changing with differentiation. H3K9me3, as a gene silencing chromatin mark, is established in cells differentiating towards mature cells. We confirm that the mark is not increased during early embryonic development towards neuroectodermal differentiation. Interestingly, the global trimethylation of H3K27 and H3K4, the bivalent histone mark, are both increased with neuroectodermal differentiation. The repressive H3K27me3 mark, increases from day 3 to day 7, when the cells start expressing high levels of NESTIN, confirming differentiation. Additionally, YAP is activated and its expressions is increased, which further confirms activation of the Hippo Pathway during neuroectodermal differentiation. The Hippo pathway is essential for *Drosophila* NSCs to go into quiescence (Ding et al., 2016). A loss of Hippo pathway results in increased proliferation of the NSCs. This suggests that activation of the pathway with neuroectodermal differentiation in hESCs is essential to maintain balance between proliferation and differentiation. We then wanted to assess the specificity of the SUV39H1 antibody during differentiation (Fig. 9.3). Due to the transient nature of the knock down, we differentiated the cells for 24 hours and collected the samples for Western blotting. Knock-down of SUV39H1 confirmed the specificity of the antibody in differentiated hNSCs. Interestingly, the H3K9me3 mark did not decrease until day 1. This suggests a possible delay in demethylation of H3K9me3.

Next, hNSCs were assessed for SUV39H1 levels during differentiation for 14 days (Fig. 9.4). Undifferentiated CX express SUV39H1 and downregulate the expression with differentiation. It is possible that the CX cells represent NSCs at a later developmental or differentiation stage and hence do not require further transcriptional

shutdown of genes. However, the global H3K9me3 mark is decreased as well, which might suggests a remodelling of the chromatin. During differentiation of the VM cells, SUV39H1 expression and H3K9 trimethylation are increased. This confirms differentiation of the cells towards mature cell types. Since the VM cells are the cells which differentiate into mature neurons, it might implicate that SUV39H1 is specifically needed for differentiation into neurons. SIRT1 deacetylates SUV39H1 and enhances its activity towards H3K9me3. In both of the hNSCs, SIRT1 is downregulated with differentiation. In VM cells this could be a regulatory mechanism to restrict SUV39H1 activity, as upregulation of the protein might be sufficient to establish H3K9me3. The upregulation of SUV39H1 during differentiation was not observed on transcriptional level (Fig. 9.5). Indeed, in VM cells SUV39H1 transcription seems to be downregulated. A discrepancy between mRNA and protein levels is nowadays widely investigated (previously discussed in Chapter 7). With the development of high-throughput gene expression microarrays there is a need to understand how mRNA and protein expression correlate, since functional assumptions are made based on the regulation of gene transcription.

Since SUV39H1 is upregulated with RASSF1A knock-down in hESCs, we asked if similar regulatory mechanism can be observed in hNSCs. Indeed, SUV39H1 was upregulated in both hNSC cell lines after RASSF1A depletion (Fig. 9.6). In western blot we did not observe changes in H3K9me3, however immunofluorescence showed a significant decrease in the mark (Fig. 9.10). Interestingly, RASSF1A depletion resulted in a mislocation of SUV39H1 in differentiated cells (Fig. 9.7), suggesting that this mislocation is affecting SUV39H1 activity towards H3K9me3. We speculated that SUV39H1 is retained in the cytoplasm in RASSF1A depleted cells and therefore its function to trimethylate H3K9 is diminished. Immunoprecipitation showed that RASSF1A interacts with SUV39H1 in VM cells (Fig. 9.11). Due to technical limitations we were not able to determine if RASSF1A interacts with SUV39H1 in CX cells. Interestingly, MST1/2 was found to be part of the RASSF1A/SUV39H1 com-

plex, suggesting that MST1/2 has an additional role in SUV39H1 regulation. Since SUV39H1 shows an increased cytoplasmic localisation with RASSF1A knock-down, we asked whether the mislocation effects differentiation. Interestingly, knock-down of SUV39H1 resulted in increased neuronal differentiation of VM cells, suggesting that SUV39H1 might mediate the silencing of pro-neuronal cells. However, SUV39H1 knock-down experiments need to be repeated for a final conclusion.

Part IV

Discussion

Chapter 10

RASSF1A and SUV39H1 in Differentiation

In this study, we investigated the biological significance of RASSF1A during differentiation of human embryonic and neural stem cells. RASSF1A depletion resulted in an upregulation of SUV39H1, however the SUV39H1-dependent chromatin mark H3K9me3 was decreased in hESCs and hNSCs. Additionally, glial and neuronal differentiation was impaired in hNSCs with RASSF1A depletion, suggesting that the loss of RASSF1A contributes to a dedifferentiated phenotype.

In the first chapter, we focussed on early human embryonic development. We established the hESCs stem cell line H9, validated differentiation protocols and studied RASSF1A in human pluripotent stem cells and early lineage commitment. The pluripotency of the hESCs was regularly monitored by morphology and expression of the pluripotency markers OCT4, SOX2 and NANOG. Spontaneous differentiation of hESCs has previously been described in *in vitro* hESC cultures, and occur particularly at the edge of the colonies (Rosowski et al., 2015). The morphology of these differentiated cells changes from a rounded dome-shaped cell formation to a two-dimensional adherent cell layer. This implies significant changes in cell-cell-adhesion and mechanotransduction. The Hippo pathway plays an important role in sensing mechanical stimuli, i.e. cell density and matrix stiffness (reviewed in

Halder et al., 2012) and cell polarity. These are important for cell fate decisions, particularly towards TE and ICM formation, and embryo remodelling (Hirate et al., 2013). Since RASSF1A protein levels are upregulated with hESC differentiation into all three germ layers (Fig. 7.5), it suggests that RASSF1A is crucial for these processes. Interestingly, on a molecular level, hESC maintenance is supported by FGF2 and TGFβ signalling (James et al., 2005). In response to TGFβ signalling, RASSF1A is recruited to the TGFβRI/II complex and following ubiquitination by the E3 ubiquitin-protein ligase Itchy homolog (ITCH), RASSF1A is degraded (Pefani et al., 2016). Induction of ectodermal differentiation requires active TGFβ inhibition (additionally to BMP signalling inhibition), thus allowing ectodermal cells to acquire sufficient RASSF1A levels. Intriguingly, the TGFβ pathway is a developmental pathway, often 'hijacked' by cells in pathological conditions where it promotes tumourigenesis (reviewed in Massague, 2012). RASSF1A transcript levels appeared to be downregulated with neuroectodermal differentiation (Fig. 7.8C). In order to test the assays in a cell line that is easier to transfect, we depleted and overexpressed RASSF1A in an independent cancer cell line (SH-SY5Y) and determined the RASSF1A transcription levels with the RASSF1A-specific TaqMan® assay (Fig. S1). QRT-PCR confirmed the specificity of the RASSF1A TaqMan® probes in SH-SY5Y cells. The RASSF1A probes are specifically designed to detect the isoform A of the RASSF1 gene by targeting the exon 1 not present in the other abundant isoform RASSF1C. The deviations between mRNA and protein levels suggests that posttranscriptional regulations might influence mRNA stability or limits mRNA detection. RASSF1A is also induced in the endoderm and mesoderm lineage. Initiation of differentiation to both lineages requires TGF β signalling, which demonstrates that the upregulation and activation of RASSF1A is differentiation-dependent.

In human fetal NSCs, RASSF1A protein expression is induced with differentiation in cells derived from the midbrain, but not in cortical-derived cells (Fig. 8.4). We reasoned that CX and VM cells might reflect various differentiation states. Con-

sidering that RASSF1A increases with maturation of hESCs and undifferentiated CX cells express high levels of RASSF1A, we speculate that the VM cells are less mature than the CX cells and the CX cells are more prime to the glial lineage. When we depleted RASSF1A, differentiation is impaired (Fig. 8.9), which demonstrates the significance of RASSF1A in neural differentiation.

We tested if RASSF1A might be evolutionary repressed by DNA methylation in hNSCs (Fig. 8.6). Since the promoter of RASSF1A is frequently methylated, we speculated if this is a non-pathological mechanism to suppress RASSF1A in stem cells. We found no evidence, that initiation of RASSF1A expression during differentiation is due to promoter demethylation. To date, little is known about the transcriptional regulation of RASSF1A and therefore the mechanism for RASSF1A transcriptional activation during differentiation remains elusive. Particularly, the increased RASSF1A protein expression in differentiated hECSs suggests that up-regulation of the expression is not due to induction of transcriptional expression. It would be of interest, if protein stabilising mechanisms play a role in RASSF1A induction with differentiation.

In the following model we summarise our observations in hESCs and hNSCs (Fig. 10.1).

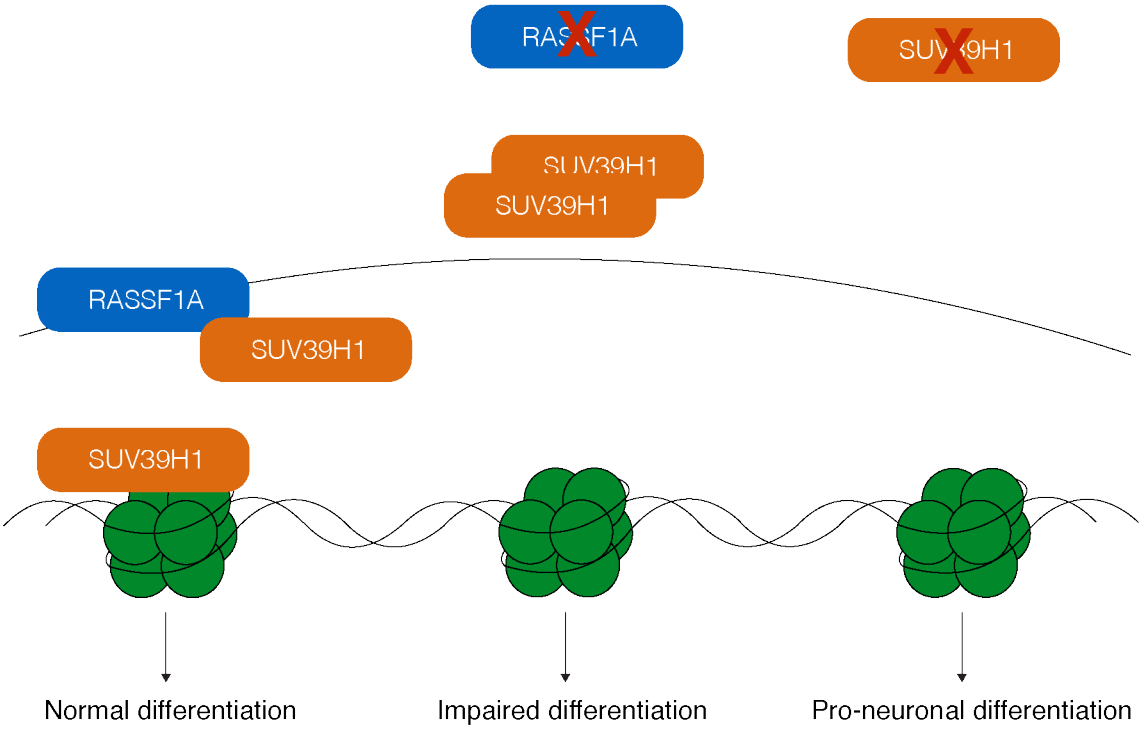


Figure 10.1: Model of RASSF1A-dependent SUV39H1 regulation: The potential outcomes for SUV39H1 localisation, stability and activity and potential involvement of RASSF1A.

RASSF1A mediates SUV39H1 localisation, and a loss of RASSF1A is negatively effecting the trimethylation of H3K9. This suggests that in a non-pathological situation, when RASSF1A and SUV39H1 levels are balanced, differentiation is not effected. However, with loss of RASSF1A, SUV39H1 is increased. Increased SUV39H1 levels have been reported for various GBM cell lines and tissue, when compared to normal tissue (Spyropoulou et al., 2014). It would be of great interest if in these elevated SUV39H1 GBMs, RASSF1A is still expressed or if, as often described in cancer, RASSF1A is silenced due to promoter methylation. Additionally, the question remains, which mechanism leads to increased SUV39H1 levels. Potentially, RASSF1A could play a role in SUV39H1 protein degradation, but a transcriptional upregulation of SUV39H1 as a response to decreased H3K9me3 levels can not be excluded. Such feedback loop mechanisms could be initiated by the cell to ensure proper differentiation capacities. It is intriguing that SUV39H1 localises to

the cytoplasm in differentiated cells. It might be possible that in terminal differentiated cells SUV39H1 acquires different functions than trimethylation of H3K9. Indeed, Kudithipudi *et al.* have identified non-histone proteins that are methylated by SUV39H1 (Kudithipudi et al., 2017). Different SUV39H1 targets could also be involved in the establishment of the SUV39H1 knock-down phenotype, which includes pro-neural differentiation (shown in this work), neuroprotection (Schweizer et al., 2015) and neurite outgrowth (Sen and Snyder, 2011).

Chapter 11

Remaining Aspects

Our data demonstrate that decreased RASSF1A levels result in increased SUV39H1 levels but reduced trimethylation of H3K9. Potential mechanisms include cytoplasmic retention, increased protein stabilisation and deregulated activity of SUV39H1, summarised in Fig. 11.1.

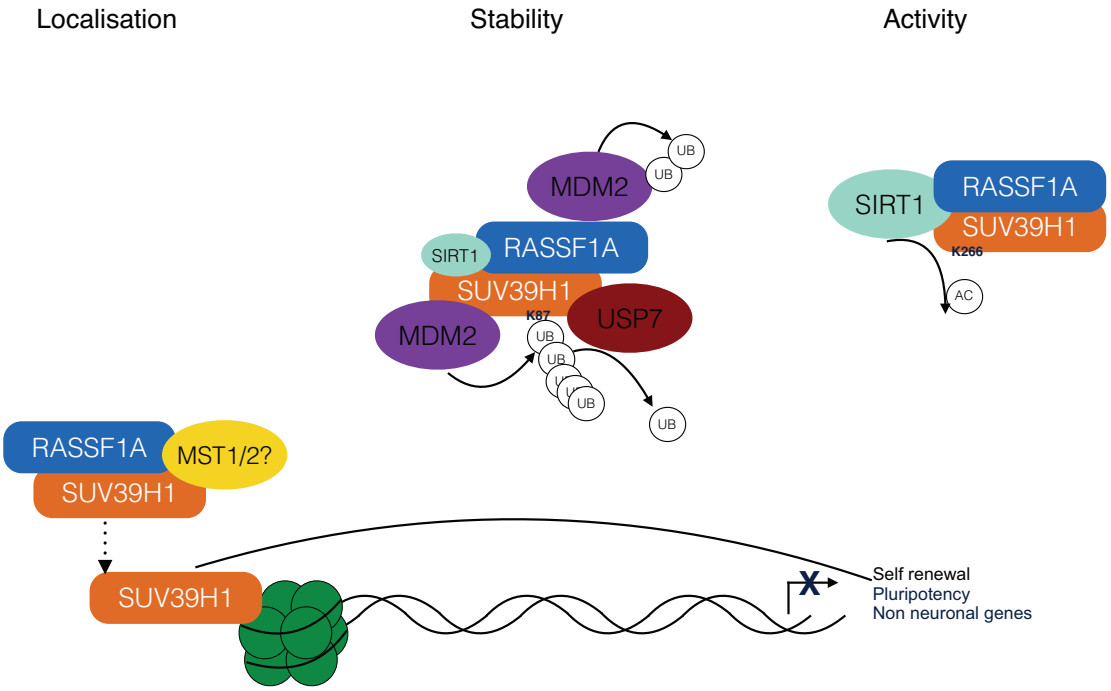


Figure 11.1: Hypothesis of RASSF1A-dependent SUV39H1 regulation: Potential mechanisms for SUV39H1 localisation, stability and activity and potential involvement of RASSF1A.

We hypothesise that RASSF1A stabilisation during differentiation and consequently active Hippo Pathway might modulate SUV39H1 protein levels to promote differentiation. SUV39H1 mediates transcriptional shut-down by establishing heterochromatin through trimethylation of H3K9. MDM2 has been shown to target SUV39H1 for proteolytical degradation (Bosch-Presegue et al., 2011) and to stabilise p73 upon binding (Ongkeko et al., 1999). Since p73 promotes neuronal differentiation (De Laurenzi et al., 2000; Velletri et al., 2013) and the p73/YAP complex regulates transcription (Strano et al., 2001), apoptosis (Matallanas et al., 2007) or differentiation (Papaspyropoulos et al., 2018), we speculate that RASSF1A might scaffold for MDM2-dependent protein stabilisation of SUV39H1 and p73 to promote differentiation. To elucidate the mechanism for RASSF1A-dependent regulation of SUV39H1, the following questions need to be addressed.

11.1 Localisation of SUV39H1

Watson *et al.* showed that SUV39H1 has been predicted to have a nuclear localisation signal (NLS) from amino acid 391 to 399 (391-SPKKRVRIE-399, Watson et al., 2014). They shows that SUV39H1 localises to the cytoplasm in response to sulforaphane, a drug with cancer-suppressive properties. To test whether the NLS is involved in nuclear translocation of SUV39H1 and if the phosphorylation of serine 391 is mediated by RASSF1A/MST1/2 we planned to GFP-tag SUV39H1 and to mutate serine 391. To prevent phosphorylation, serine is mutated to alanine (S391A) and to mimic phosphorylation serine is exchanged for glutamic acid (S391E). Additionally, we planned to delete the NSL (SUV39H1 Δ391-399) and inactivate its methyltransferase activity by mutating lysine 266 to glutamine (K266Q). Localisation of SUV39H1 could be assessed by GFP visualisation. We attempted to subclone SUV39H1 into a GFP vector, which to date has been unsuccessful.

11.2 SUV39H1 regulation

Since depletion of RASSF1A results in increased SUV39H1 protein levels, it would be of interest if RASSF1A has a role in SUV39H1 protein degradation. As previously mentioned, RASSF1A has been described to promote MDM2 self-ubiquitination by disrupting the complex formation of MDM2/USP7/DAXX (Song et al., 2008b). Interestingly, the E3 ligase MDM2 and deubiquitinase USP7 have been described to regulate SUV39H1 (Mungamuri et al., 2016). Therefore, it would be of interest if the ubiquitin levels of SUV39H1 in the pre- and absence of RASSF1A are altered. Experiments to co-immunoprecipitate SUV39H1 in RASSF1A depleted cells have been attempted but failed due to technical limitations. Additionally, cycloheximide (CHX) chase experiments in RASSF1A depleted cells would clarify if RASSF1A is involved in SUV39H1 protein degradation. CHX is a drug that inhibits protein synthesis. Chase experiments in the pre- and absence of RASSF1A to assess turnover of SUV39H1 were attempted and failed due to nucleofection failure. Furthermore, SIRT1 regulates the activity of SUV39H1 by deacetylation of the SET domain (Vaquero et al., 2007). Additionally, RASSF1A has been shown to mediate SIRT1 activity towards other proteins (i.e. XPA, Donninger et al., 2015), therefore it would be of interest if RASSF1A can mediate SIRT1 activity towards SUV39H1. If so, a loss of RASSF1A would subsequently lead to a reduced activity of SUV39H1 towards H3K9me3.

11.3 RASSF1A dependent changes of chromatin marks

It remains as a key question which specific genes are silenced by H3K9me3 in human ESCs and NSCs. Chromatin-immunoprecipitation (ChIP) experiments of the

H3K9me3 mark would reveal SUV39H1 targets by either sequencing or PCR for appropriate candidates (pluripotency and neural differentiation genes).

Chapter 12

Conclusion

In a non pathological situation, balanced levels of RASS1A and SUV39H1 allow normal differentiation. Loss of RASSF1A, as observed in various tumours, leads to an upregulation, but mislocalisation of SUV39H1 resulting in a decreased H3K9me3 mark. This subsequently impairs differentiation contributing to the initiation of transforming stem into cancer cells. Whenever SUV39H1 expression is lost, normal hNSCs have a higher capacity to differentiate into neurons. Inhibition of SUV39H1 has been shown to have neuroprotective functions in hypoxic conditions (Schweizer et al., 2015) and SUV39H1 depletion resulted in increased dendritic length and neurite outgrowth (Sen and Snyder, 2011). Epigenetic silencing of RASSF1A by promoter methylation has been identified as a marker for high grade brain cancer and poor survival (Grawenda and O'Neill, 2015; Misawa et al., 2009). Interestingly, high expression levels of SUV39H1 have been found in various glioma cell lines and tissue compared to normal tissue (Spyropoulou et al., 2014), suggesting that SUV39H1 plays a role in glioma pathogenesis. Since SUV39H1 protein levels are increased with RASSF1A depletion and RASSF1A has been shown to interact with SUV39H1 in two independent assays, we conclude that the RASSF1A-dependent regulation of SUV39H1 contributes to the clinical phenotypes described above.

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Appendices

I RASSF1A Transcription levels

To test the RASSF1A TaqMan assay for specificity, we transfected the human neuroblastoma cell line SH-SY5Y with a RASSF1A overexpression plasmid and an RASSF1A specific siRNA and performed qRT-PCR.

To test the RASSF1A TaqMan® assay for specificity, RASSF1A was overexpressed and depleted in the neuroblastoma cell line SH-SY5Y using nucleofection. Since transfection of cancer cells is more efficient than transfection of stem cells, SH-SY5Y cells were used in this experiment.

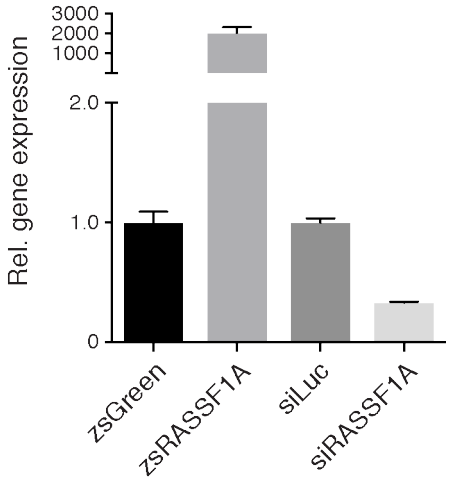


Figure S1: Transfection of the neuroblastoma cell line SY5Y: SY5Y cells were transfected with zsRASSF1A (control zsGreen) and siRASSF1A (control siLuc) using Lipoectamine 3000. After 24h, cells and supernatant was collected for qRT-PCR. Error-bars represent n=3 technical replicates.

II Additional NSC Marker

CX and VM cells express SOX2.

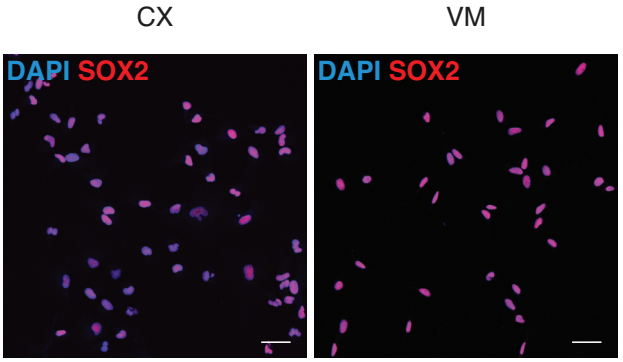


Figure S2: CX and VM express pluripotency marker SOX2: Undifferentiated CX and VM cells were immunofluorescent stained with the antibody indicated. Scale bars represent 50 µm.

III Optimisation of hNSC Transfection

In order to establish a transfection protocol for the human NSC lines, we attempted to use Lipofectamine 3000 to induce RASSF1A expression.

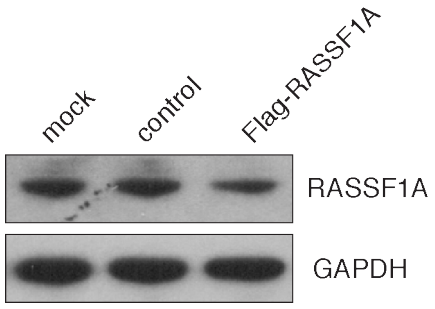


Figure S3: Transfection of CX cells using Lipofectamine 3000: CX cells were transfected with pcDNA3-Flag-RASSF1A using Lipofectmaine 3000. As controls, cells were either untreated (mock) or transfected with the pcDNA3 empty vector. Overexpression was assessed by western blotting.

Transfection with Lipofectamine 3000 failed as no overexpressed protein was detected. We therefore decided to optimise the transfection efficiency of CX and VM cells using electroporation, specifically Lonza's Nucleofector™ technology in combination with the Nucleofector™ 2b device. Nucleofection was performed on both cell lines using two different nucleofection buffers (solution L and V) and eight different nucleofection programmes suggested by Lonza. Cells were transfected with GFP for transfection visualisation. Expression of GFP was allowed for 24h before imaging.

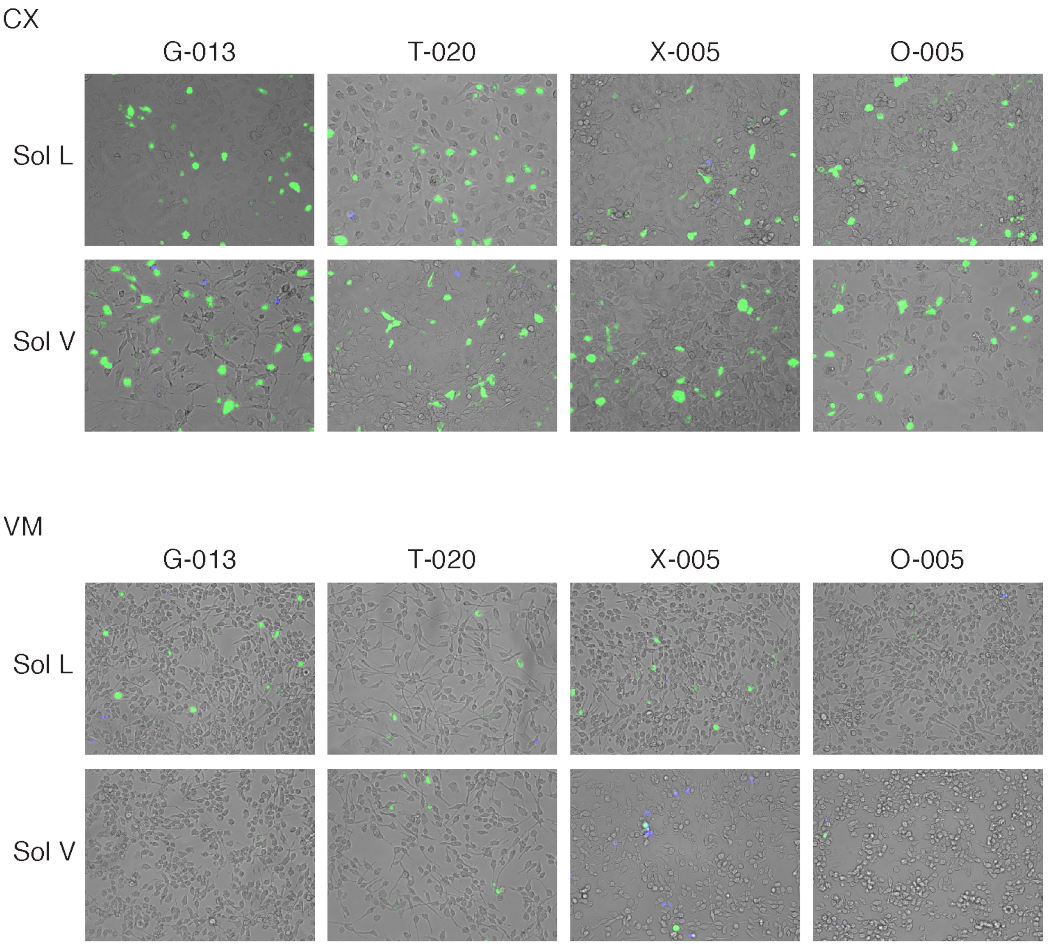


Figure S4: Optimisation of Nucleofection of CX and VM using Nucleofector 2b: Using the nucleofector 2b device, CX and VM were nucleofected with GFP in 2 different transfection buffers (L and V) using four different transfection programmes. After 24h, images were taken with Floid Cell Imaging Station.

Nucleofection was re-optimised for the Nucleofector 4D device. Overexpression was carried out using DC100 (CX) and CM162 (VM) in SF buffer.

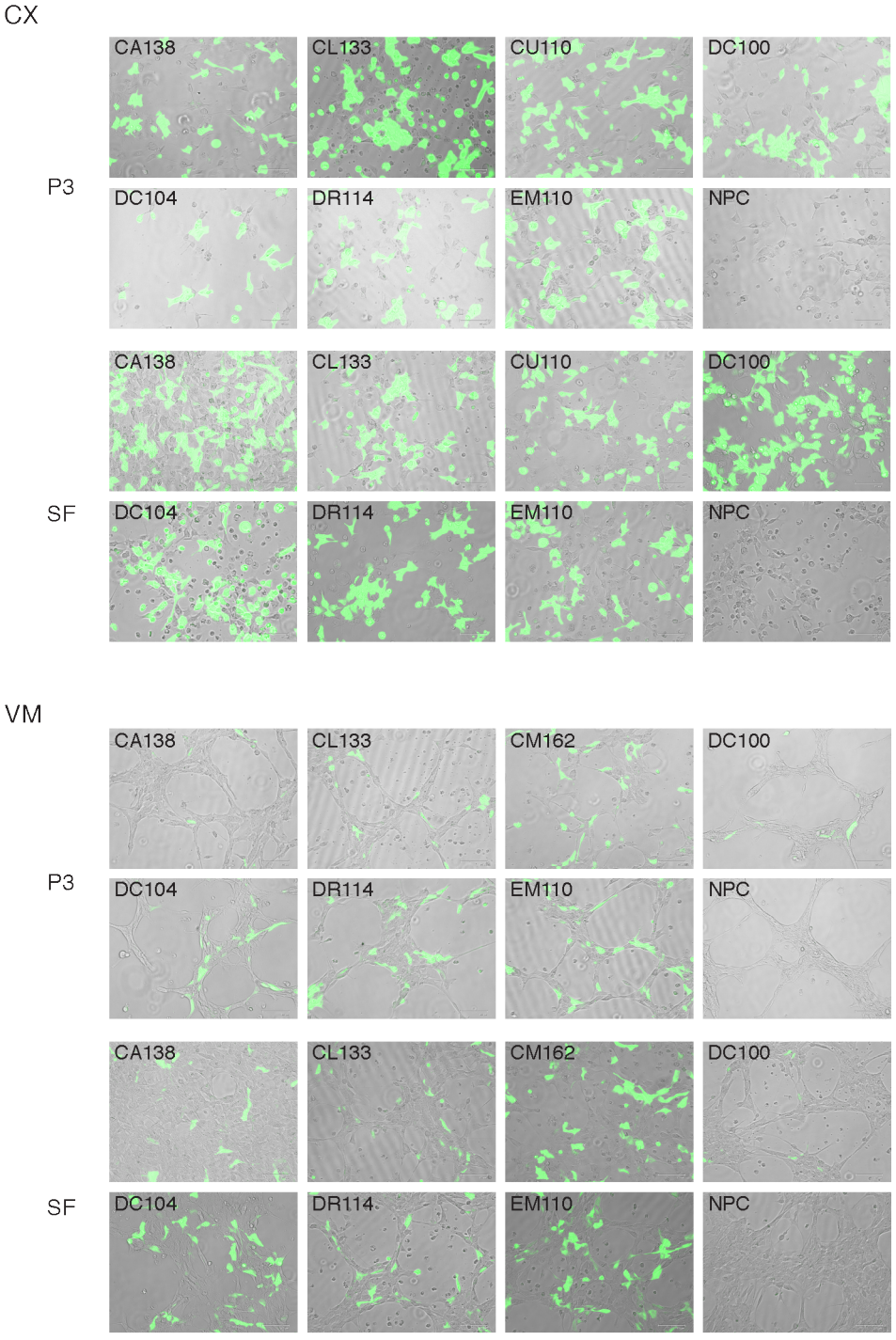


Figure S5: Optimisation of Nucleofection of CX and VM using Nucleofector 4D: Using the nucleofector 4D device, CX and VM were nucleofected with GFP in 2 different transfection buffers (P and SF) using eight different transfection programmes. After 24h, images were taken with Floid Cell Imaging Station. Photos are representative for two biological replicates, n=2

IV RASSF1A Knock-down at Day 7

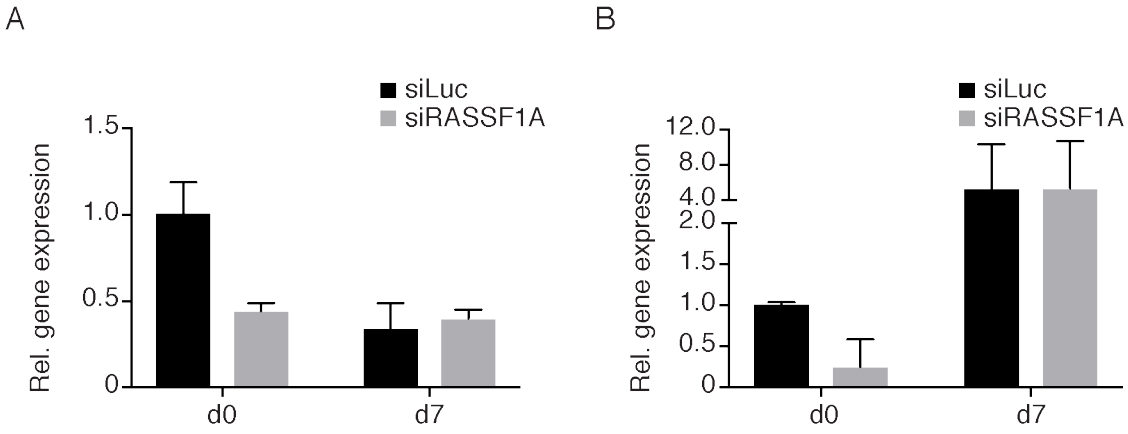


Figure S6: Knock-down of RASSF1A is lost at Day 7 of Differentiation: QPCR confirms initial knock-down at d0 (24h post transfection) in A) CX and B) VM cells. At d7 of differentiation knock-down of RASSF1A is lost. Error bars represent n=3 technical replicates for CX and n=2 biological replicates for VM.

V Regulation of SIRT1 with Neural Differentiation

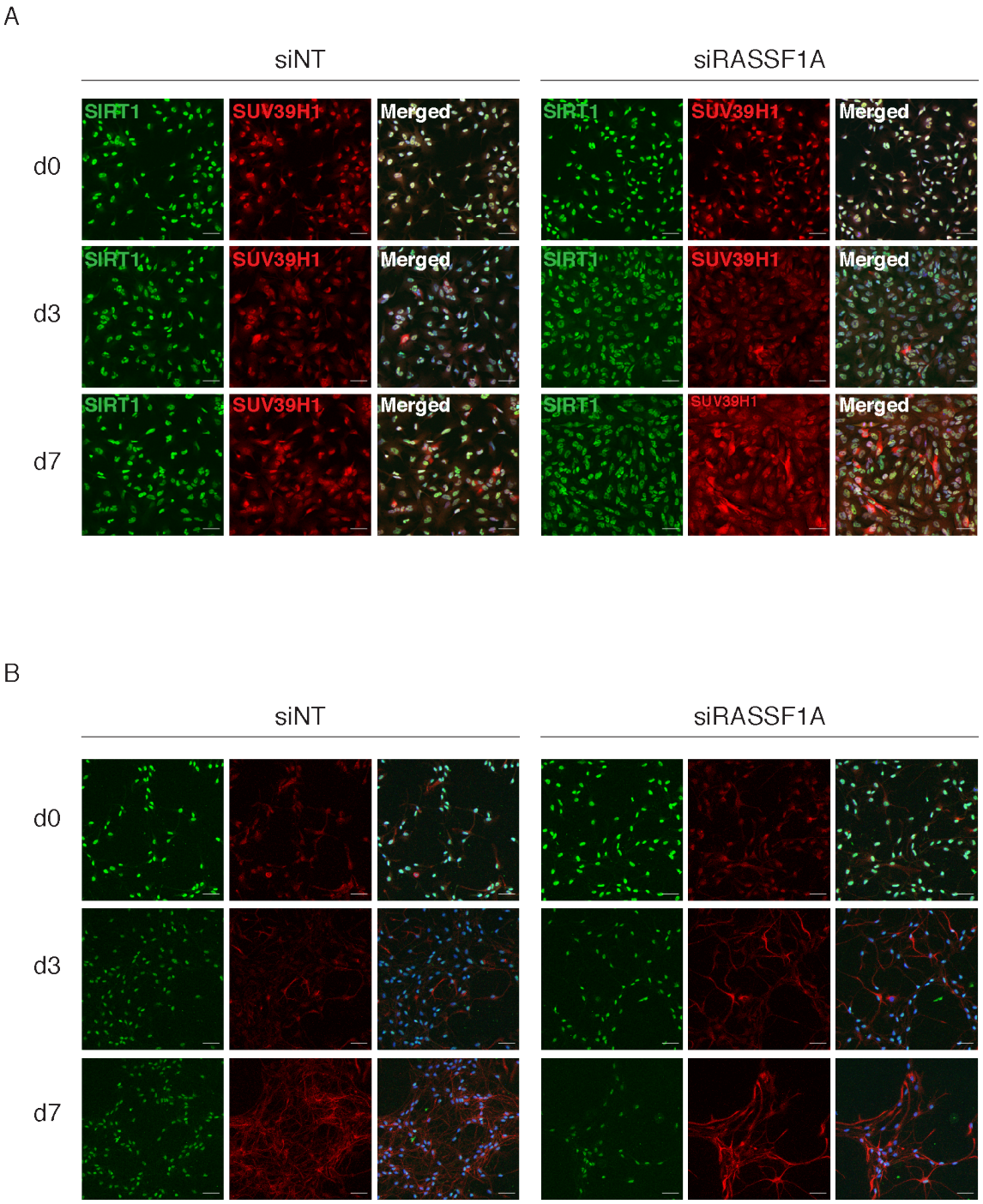


Figure S7: Regulation of SIRT1 with Differentiation and RASSF1A depletion:

VI Generation of CRISPR Cell Lines

Due to the nature of transient knock-down, the effects of protein depletion is diminished by day 7. To investigate the effects of RASSF1A on SUV39H1 in differentiated hNSCs at later stages of differentiation we employed the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated protein 9 (Cas9) gene editing tool to delete RASSF1A in hNSCs (Ran et al., 2013).

To create stable and specific RASSF1A KO lines from CX and VM cells, we applied the CRISPR-Cas9 technology to delete exon 1 of the RASSF1A gene.

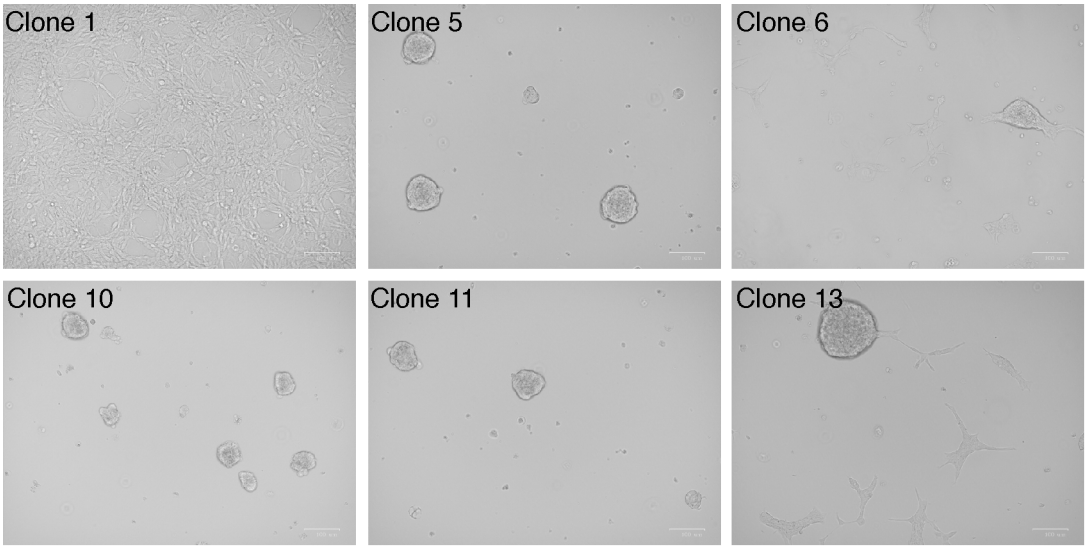


Figure S8: RASSF1A Knock-out CRISPR Clones: Brightfield photos of selected RASSF1A CRISPR clones of the VM cell line. Clone 1 represents wildtype morphology. Scale bars represent 100 µm.

In CX cells, no clones survived when RASSF1A was deleted using the CRISPR-Cas9 technology. Since CX express high RASSF1A levels, this could suggest a hypersensitivity to complete deletion of RASSF1A in these cells. In the VM cells, 16 clones were able to survive, of which the knock-out needs to be confirmed by genotyping. Interestingly, 6 out of the 16 clones showed substantial changes in morphology (here

shown 5 clones in comparison to clone 1, which shows wildtype morphology). The cells grow in large colonies, a hallmark of stemness and pluripotency, implying that the RASSF1A knock-out NSCs are more stem-like.

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