

This electronic thesis or dissertation has been downloaded from the King's Research Portal at <https://kclpure.kcl.ac.uk/portal/>



HUWE1 suppresses neurogenesis through degradation of ASCL1 in human neural stem cells

Tan, Yu Xuan

Awarding institution:
King's College London

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

END USER LICENCE AGREEMENT



Unless another licence is stated on the immediately following page this work is licensed

under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International

licence. <https://creativecommons.org/licenses/by-nc-nd/4.0/>

You are free to copy, distribute and transmit the work

Under the following conditions:

- Attribution: You must attribute the work in the manner specified by the author (but not in any way that suggests that they endorse you or your use of the work).
- Non Commercial: You may not use this work for commercial purposes.
- No Derivative Works - You may not alter, transform, or build upon this work.

Any of these conditions can be waived if you receive permission from the author. Your fair dealings and other rights are in no way affected by the above.

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

HUWE1 suppresses neurogenesis through degradation of
ASCL1 in human neural stem cells

Yu Xuan Tan

King's College London
and
The Francis Crick Institute

PhD Supervisors:
Francois Guillemot
Deepak Srivastava

A thesis submitted for the degree of
Doctor of Philosophy
King's College London

Mar 2023

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

Declaration

I, Yu Xuan Tan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The E3 ubiquitin ligase HUWE1 (HECT, UBA And WWE Domain Containing E3 Ubiquitin Protein Ligase 1) regulates neural stem cells' (NSCs) differentiation during neurogenesis in mice through the ubiquitination and degradation of several key proneural transcription factors. In human neurogenesis, HUWE1 remains poorly characterised, but mutations in HUWE1 cause Turner-type X-linked syndromic intellectual developmental disorder through unstudied mechanisms (MRXST, #OMIM 309590, though patient families prefer the term "HUWE1-related genetic condition"). Prior work in the Guillemot lab (Urban et al. 2017) showed the master proneural factor ASCL1 to be a HUWE1 substrate during adult hippocampal neurogenesis in mice, opening a new avenue for exploration into how HUWE1 might regulate neurogenesis in humans during development. I thus sought to see if HUWE1 similarly controls ASCL1 activity in human NSCs, and how this regulation might affect human neurogenesis.

Through CRISPR-Cas9 editing to generate clonal lines, I found that constitutive HUWE1 mutant iPSCs failed to survive early cortical neural differentiation (modelled using the 2D dual-SMAD inhibition protocol of Shi et al. 2012) - with potential involvement of the known HUWE1-substrate, p53. This was highly similar to work (Aprigliano et al, 2021) published independently on patient-derived iPSCs during my PhD, but is a phenotype likely incompatible with life, and therefore of unknown validity. However, by generating a 4-hydroxytamoxifen (4OHT) inducible knockout line (HUWE1cKO) through successive CRISPR-Cas9 edits, I show the early differentiation block can be circumvented, and HUWE1 knockout NSCs and neurons obtained, albeit as a mosaic culture. This mosaicism unfortunately diluted most population-level phenotypes (other than a robust protein-level increase in ASCL1), and necessitated the use of single-cell resolution analyses.

Using single-cell RNA sequencing, I discovered an ASCL1-enriched transitional-progenitor that produces potentially neurons with a GABAergic signature, and whose unique transcripts are upregulated in 4OHT-treated HUWE1cKO cultures. Unfortunately, HUWE1 knockout cells could not be definitively identified through scRNAseq. This work, however, led us to perform scRNAseq on neurally-differentiated ASCL1-knockout human iPSCs (Păun et al. 2023) which showed the NSCs require ASCL1 to differentiate into transitional-progenitors and subsequently immature

neurons. Taken together, I hypothesised that HUWE1-KO NSCs might undergo the opposite phenotype.

Owing to the dense stacking of cells in cultures, immunofluorescence approaches were unsuccessful, even after a Cre-reporting line was generated, hindering further single-cell investigation. This was finally addressed through flow cytometry, where I found that HUWE1-KO NSCs had increases in ASCL1 accompanied by cell-cycle exit and premature differentiation into differentiating-progenitors and neurons - phenotypes not seen in their WT counterparts in the mosaic cultures - with no involvement of p53.

Attempts to address possible non-cell-autonomous contributions to the premature differentiation phenotype by the co-cultured WT cells were unsuccessful but established a high-efficiency electroporation-based method compatible with the 2D differentiation that could prove useful in future studies.

Our results demonstrate for the first time an ASCL1-dependent transitional-progenitor in humans, which I show likely prematurely forms upon loss of HUWE1 due to ASCL1 stabilization. My results also show that HUWE1 regulates ASCL1 levels on a post-translational level in human NSCs, with hints that ASCL1 levels also need to be reduced, after stabilization, for neuronal maturation. These results expand on HUWE1's function in humans, suggesting it has dual roles during neurogenesis (regulating p53 and survival early, but ASCL1 and terminal differentiation later), and also provide a mechanism for HUWE1-related genetic condition worth investigating.

COVID-19 Impact Statement

I returned to Singapore from Mar-Jul 2020 and was unable to perform any experiments or analyses related to my project as my work was entirely wet-lab then. Upon my return, the Crick Institute also had a daily personnel limit through to Mar 2021, which restricted the sizes of experiments that could be carried out, given the labour-intensive nature of stem cell culture work, with priority given to lab members whose contracts were completing.

Longstanding supply difficulties with cell-culture reagents and plasticware persisted after restrictions were lifted, and severely constrained the sizes of experiments that could be planned for.

Because of these issues, the semi-successful single-cell RNA sequencing experiment on my conditional knockouts was introduced after the first lockdown to speed up phenotype discovery and reduce reliance on wet-lab experiments. Analysing the data from this experiment kept me occupied during the Omicron lockdown, and my COVID self-isolations, and generated some leads.

Another change was to switch to much larger cell culture experiments with three concurrent, staggered, replicates, and with collection of material at multiple timepoints, to make up for lost time.

Had there been more time, I might have been able to achieve a 100% recombination efficiency through the combined use of 4OHT and electroporation of a Cre plasmid, since I had run out of time just after successfully validating the electroporation method. This could have addressed the question I had on whether my phenotypes would still be present in a homogenous culture.

Acknowledgement

These were trying times for everyone around the world, much less for me trying to complete a PhD project. I owe much to many for seeing me through to the end, and would first like to thank my supervisors Francois Guillemot, Deepak Srivastava, and my day-to-day supervisor Siew-Lan Ang. Without their patience, insights and suggestions, and occasional nudging, I could not have come this far. There were so many times when I felt so stuck and at a loss because nothing was working and time was wasting away, but it always felt safe to share my difficulties with them and ask for help.

I would also like to thank members of the Guillemot lab, past and present. I would especially like to thank: Oana Păun for her constant encouragement and support; Berta Terre Torras for recognizing when I was at my lowest and being there for me then; Michael Lattke, Thomas Kerloch and Lachlan Harris for starting me down the single-cell RNA sequencing path and its analysis in R; Piero Rigo, Alice Rossi, and Sara Ahmed de Prado, the lunch gang for creating a lab environment worth going in for; Clementina Cobolli-Gigli for her hard work in bringing the differentiation protocol into the lab and for training me; and Cristina Dias – the high standards she set meant I learnt so much working with her and Oana on the paper. Thanks also go out to the other 5th floor PhD students: Maddy Demuth, Isabel Zhang, Oliver Inge, and Joe Padgett for many an interesting conversation and sharing in commiserations.

Much of the work here would also not have been possible without the help of the Crick's facility staff. In particular: Ana Agua-Doce of the Flow Cytometry facility who practically saved my project single-handedly by introducing me to flow cytometry and making it accessible to this scared newbie – I would not have become confident enough to rely on it without her initial help; Stephanie Strohbuecker of BABS for her help on both single-cell RNA sequencing projects' analyses and for giving me the confidence to run my own analyses; Lyn Healy and Liani Devito of HESCU for providing a well-run environment for stem cell culture work – one that maintains high standards and makes our work so much easier, but one which we take for granted;

and Molly Strom of the Viral Vector Core – any construct was possible with her help, and she was just so helpful.

I am also grateful to my thesis committee: Rickie Patani, Vassilis Pachnis, Corinne Houart, and Selina Wray for their encouragement and faith in me and my progress. I may never see what they saw in me, but I am glad that they did.

I also owe much of my scientific training to the wide exposure and opportunities given to me in my previous lab, the Reversade lab, without which this project would have been so much harder. Thank you especially to Nathalie Escande-Beillard, Abigail Loh, and Bruno Reversade for helping me find my footing in academic research.

This was a journey that I did not take alone. I am deeply indebted to my partner, Christine Chang, for sticking with me through all my pain. I know it was not easy and must have been hard on you. Thank you for bringing colour to my life when all was grey. I am also thankful to my flatmates and friends, Sandra Chong, and Josiah Tan, for all the fun and games, and the many holidays we took together – thank you for making life in London more than just about work. I probably never would have visited Slovenia, Barcelona, and St Gervais if not for you all. Thank you also to Sandra's parents for their generosity in opening up their flat to us. I would also like to mention the Simons for their emotional support. A final special thank you goes out to my parents for all the care and opportunities you provided me that led me to where I am today.

Thank you to everyone who believed in me when I lost that belief myself – this project could not have been completed without you.

Table of Contents

Abstract	3
COVID-19 Impact Statement	5
Acknowledgement	6
Table of Contents	8
Table of figures	10
List of tables	13
Abbreviations	14
Introduction	17
1.1 Mammalian cortical development	18
1.1.1 Stem cells, progenitors, and neurons in cortical neurogenesis	18
1.1.2 Factors regulating proliferation and differentiation	21
1.2 Modelling cortical neurogenesis	30
1.2.1 2D and 3D <i>in vitro</i> models	30
1.2.2 CRISPR-Cas9 and differentiation models	33
1.3 HUWE1 and neurogenesis	34
1.3.1 HUWE1-related genetic conditions	34
1.3.2 HUWE1 in developmental neurogenesis	35
1.3.3 Molecular regulation of HUWE1 activity	37
1.3.4 HUWE1 dysregulation may affect mature neuronal function	40
1.4 Project Aims	41
Materials & Methods	43
2.1 Cell Culture	43
2.1.1 Routine maintenance of hPSCs	43
2.1.2 Gene-editing of hPSCs	44
2.1.3 Quality Control	48
2.1.4 2D neuronal differentiation and 4OHT treatment	48
2.1.5 Cre-dependent reporter generation	50
2.1.6 Lentiviral transduction	50
2.1.7 Nucleofection during differentiation	51
2.2 Molecular Biology	52
2.2.1 PCR, genotyping, and gel electrophoresis	52
2.2.2 Western Blotting	53
2.2.3 RNA extraction, cDNA synthesis and qRT-PCR	55
2.2.4 Single-Cell RNA Sequencing and analysis	56
2.3 Microscopy	59
2.3.1 Live imaging in culture	59
2.3.2 Immunofluorescence microscopy	60
2.3.3 Image analysis and quantification	60
2.4 Flow cytometry	61
Results 1: Constitutive <i>HUWE1</i> mutant iPSCs fail to survive neural differentiation	63
3.1 <i>HUWE1</i> is expressed throughout <i>in vitro</i> cortical neurogenesis	64
3.2 <i>HUWE1</i> is necessary for survival during early neurogenesis	67
Results 2: Acute <i>HUWE1</i> knockouts undergo neural differentiation with stabilization of <i>ASCL1</i>	74

4.1 HUWE1cKO: a 4OHT-inducible HUWE1 knockout hESC line	75
4.2 HUWE1cKOs show stabilized ASCL1, but not p53, despite mosaicism	88
4.3 HUWE1cKOs show no population-level differentiation phenotypes	95
Results 3: Single-cell RNA sequencing identifies an ASCL1-dependent transitional progenitor	102
5.1 scRNAseq reveals an ASCL1-defined transitional progenitor but cannot identify HUWE1-knockouts	103
5.2 ASCL1 drives differentiation of cycling progenitors into transitional progenitors and neurons	112
5.3 HUWE1 knockout phenotypes may involve transitional progenitors 120	
Results 4: Flow cytometry reveals premature differentiation of HUWE1- knockouts following ASCL1 stabilization	124
6.1 Labelling HUWE1-knockouts through reporting Cre-activity	125
6.2 Flow cytometric detection of HUWE1-knockouts	131
6.3 HUWE1-knockouts prematurely differentiate following ASCL1 stabilization	136
6.4 HUWE1-knockouts show premature differentiation following ASCL1 stabilization	141
Results 5: Unsuccessful attempts to address questions on cell autonomy and ASCL1	154
7.1 HUWE1 small molecule inhibitor BI8622 did not affect neural differentiation	155
7.2 TAT-Cre is toxic to neural cultures	162
7.3 HUWE1-knockouts are vulnerable to lentivirus treatment	164
Discussion	170
8.1 HUWE1 degrades p53 and ASCL1 at different stages of neurogenesis to expand the neural progenitor pool	170
8.2 Implications for the aetiology of HUWE1-related genetic disorders 173	
8.3 Non-cell-autonomous contribution of ASCL1 activity to HUWE1 function	176
8.4 Mosaicism in HUWE1 conditional knockouts during differentiation 179	
8.5 4-hydroxytamoxifen-specific induction of p53 in neural progenitors 180	
8.6 Increase in ASCL1 levels in transitional progenitors	181
8.7 Identity of ASCL1-driven transitional progenitors and neurons	182
8.8 Workarounds for addressing cell-autonomy and demonstrating causality of ASCL1	185
8.9 Conclusions	186
Reference List	188

Table of figures

Figure 1. Neural progenitor types and their lineages during cortical development.	20
Figure 2. Signalling pathways and proneural genes regulate proliferation and differentiation decisions.	22
Figure 3. Lateral inhibition of Notch pathway intersects with proneural activity.	29
Figure 4. Summary of HUWE1 domains and some of its targets.	38
Figure 5. Summary of neural differentiation protocol.....	49
Figure 6. Characterisation of <i>HUWE1</i> mRNA expression during <i>in vitro</i> cortical neurogenesis.	66
Figure 7. Generation and verification of <i>HUWE1</i> E81-82Del mutants.	69
Figure 8. HUWE1 E81-82Dels are pluripotent and have reduced HUWE1 stability.	71
Figure 9. HUWE1 E81-82Dels fail to survive early neural differentiation, with potential involvement of p53.....	72
Figure 10. Generation, verification, and validation of a floxed <i>HUWE1</i> exon 81 in H1 hESCs.	76
Figure 11. Integration of CreERT2 into the <i>AAVS1</i> locus of HUWE1 E81-fl.....	78
Figure 12. HUWE1cKOs are pluripotent and possess Cre activity.....	81
Figure 13. 4OHT-dependent deletion of <i>HUWE1</i> in HUWE1cKO hESCs results in loss of HUWE1 on both protein and transcript levels, with increase in p53.	83
Figure 14. Validation of HUWE1 antibodies by immunofluorescence.....	85
Figure 15. HUWE1cKO also fails to differentiate following early 4OHT treatment..	87
Figure 16. HUWE1cKO can bypass early differentiation failure but undergo mosaic recombination.	90
Figure 17. HUWE1cKO show stabilization of ASCL1 but not p53 despite mosaic recombination.	92
Figure 18. HUWE1cKO shows no difference in differentiation markers by qRT-PCR.	96
Figure 19. Immunofluorescence cannot reliably identify HUWE1 KO cells in mosaic neuronal cultures.	98
Figure 20. Immunofluorescence shows no change in differentiation markers in HUWE1cKO.....	101

Figure 21. Single-cell RNA sequencing of Day 20 cultures identifies 4 major cell types.	104
Figure 22. Transitional Progenitors are differentiating progenitors defined by <i>ASCL1</i> expression.	106
Figure 23. Expression of selected markers in Day 20 cultures.	108
Figure 24. Expression of more selected markers in Day 20 cultures.	109
Figure 25. Single-cell RNA sequencing cannot identify HUWE1 KO cells.	111
Figure 26. scRNAseq of wildtype KOLF2 at Day 24 also identifies transitional progenitors.	114
Figure 27. <i>ASCL1</i> is required to drive differentiation of cycling progenitors into transitional progenitors.	116
Figure 28. Flow cytometry analysis of <i>ASCL1</i> -high cells support its role as a marker of transitional progenitors.	119
Figure 29. HUWE1-knockouts may be forming transitional progenitors at the expense of wildtype cells.	121
Figure 30. Generation of HUWE1cKO-reporter, a Cre activity-reporting cell line to identify HUWE1 knockouts.	127
Figure 31. Cre-reporter lines express EGFP and not tagRFP during early neural differentiation.	128
Figure 32. HUWE1cKO-reporter may label HUWE1 knockouts imperfectly.	130
Figure 33. HUWE1cKO-reporter preferentially labels HUWE1 knockouts, but flow cytometry can identify knockouts.	132
Figure 34. HUWE1 antibodies discriminate knockouts only in conjunction with bright fluorophores.	135
Figure 35. Flow cytometry enables limiting analyses to HUWE1-knockouts within mosaic HUWE1cKO cultures.	137
Figure 36. HUWE1 expression increases with differentiation.	140
Figure 37. HUWE1-knockouts specifically upregulate <i>ASCL1</i> and not p53.	142
Figure 38. HUWE1-knockout cells differentiate prematurely following increase in <i>ASCL1</i> .	145
Figure 39. HUWE1-knockouts have reduced proliferation but are not more apoptotic.	148
Figure 40. <i>ASCL1</i> overexpression is restricted to progenitors in HUWE1-knockouts.	151

Figure 41. 4OHT-treated HUWE1cKO differentiates similarly to vehicle-controls overall.	153
Figure 42. BI8622 is toxic to neural progenitors at 10 μ M, and results in a change of media colour.	156
Figure 43. Western Blot for BI8622-treated neural differentiations at Days 16 and 20.	157
Figure 44. 2 μ M BI8622 has no effect on neuronal differentiation.	159
Figure 45. BI8622 did not affect cell proliferation.	161
Figure 46. TAT-Cre treatment is toxic to neural progenitors.	163
Figure 47. Lentivirus-delivered shRNA causes selective loss of HUWE1-knockouts.	165
Figure 48. Electroporation delivers shRNA into HUWE1cKO successfully, but shASCL1 sequences are ineffective.	168
Figure 49. Proposed functions of HUWE1 during human neurogenesis.	172
Figure 50. Increased lateral inhibition in wildtype cells may contribute to premature differentiation of HUWE1 knockouts.	177

List of tables

Table 1. Oligo sequences for gene-editing of hPSCs.....	45
Table 2. Primer sequences for clone-screening and CRISPR off-target verification.	48
Table 3. N2 and B27 media composition.	49
Table 4. shRNA sequences used in pLKO.1 zsGreen.	51
Table 5. All primary antibodies used, and applications tested.	54
Table 6. All secondary antibodies used.....	54
Table 7. Taqman probes used in this study	56
Table 8. Primers for SYBRGreen-based qRT-PCR.....	56

Abbreviations

4OHT: 4-Hydroxy-Tamoxifen

AF: Alexa-Fluor

ASCL1: Achaete-Scute Family BHLH Transcription Factor 1

ASF: Advanced Sequencing Facility

BABS: Bioinformatics and Biostatistics STP

BCL11B: BCL11 Transcription Factor B

BMP: Bone Morphogenetic Protein

CCND1: Cyclin D1

cKO: conditional knock-out

CP: cortical plate

CreERT2: 4OHT-inducible Cre recombinase

crRNA: CRISPR RNA

DAPT: Notch inhibitor, gamma-secretase inhibitor, N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-s-phenylglycine-butyl ester

DLL: Delta Like Canonical Notch Ligand

DLX: Distal-Less Homeobox

EdU: 5-ethynyl-2'-deoxyuridine

EMX: Empty Spiracles Homeobox

EOMES: Eomesodermin

FABP7: Fatty Acid Binding Protein 7

FACS: fluorescence-activated cell-sorting

FGF: Fibroblast Growth Factor

flox: flank by loxPs

FOXP1: Forkhead Box G1

GABA: Gamma-Aminobutyric Acid

GAD2: Glutamate Decarboxylase 2

GADD45G: Growth Arrest And DNA Damage Inducible Gamma

GFAP: Glial Fibrillary Acidic Protein

GLI: GLI Family Zinc Finger

GSK3: Glycogen Synthase Kinase 3

gRNA: guide RNA

H1Cre: control line generated in this study from H1 hESCs that contains a CAG-promoter driven CreERT2 integrated into the AAVS1 locus

HDR: homology-directed repair

HES: Hes Family BHLH Transcription Factor

hESCs: human embryonic stem cells

hiPSCs: human induced-pluripotent stem cells

hPSCs: human pluripotent stem cells (hESCs + hiPSCs)

HUWE1: HECT, UBA And WWE Domain Containing E3 Ubiquitin Protein Ligase 1

HUWE1cKO: 4OHT-inducible HUWE1 knockout line generated in this study, in the H1 hESC background

ID: Intellectual Disability

ID protein: Inhibitor Of DNA Binding (protein)

IF: Immunofluorescence microscopy

IKNM: interkinetic nuclear migration

IPC: intermediate progenitor cell

KO: knockout

LHX: LIM Homeobox

LOF: loss of function

MAPK: Mitogen-Activated Protein Kinase

NANOG: Nanog Homeobox

NE: neuroepithelium/neuroepithelial cell

NES: Nestin

NEUROD: Neuronal Differentiation (gene)

NEUROG: neurogenin

NICD: Notch intracellular domain

NKX2.1: NK2 Homeobox 1

OCT4: POU Class 5 Homeobox 1

OLIG2: Oligodendrocyte Transcription Factor 2

PAX6: Paired Box 6

PLXNA2: Plexin A2

PROX1: Prospero Homeobox 1

qRT-PCR: Quantitative Real-Time PCR

RA: retinoic acid

RBPJ: Recombination Signal Binding Protein For Immunoglobulin Kappa J Region

RG: radial glia
S100 β : S100 Calcium Binding Protein B
SATB2: SATB Homeobox 2
scRNAseq: single-cell RNA sequencing
SHH: Sonic Hedgehog
shRNA: short hairpin RNA
SLC1A3: Solute Carrier Family 1 Member 3
SMAD: SMAD Family Member
SMO: Smoothed, Frizzled Class Receptor
SOX: SRY-Box Transcription Factor
ssODN: single-stranded oligodeoxynucleotide
STP: Service and Technology Platform
SVZ: subventricular zone
TBR1: T-Box Brain Transcription Factor 1
TCF/LEF: Lymphoid Enhancer Binding Factor
TF: transcription factor
TGF β : Transforming Growth Factor Beta
tracrRNA: trans-activating crRNA
TUBB3: Tubulin Beta 3 Class III
Veh: Vehicle
VIM: Vimentin
VZ: ventricular zone
WNT: Wnt Family Member
WT: Wild-type

Introduction

The mammalian cerebral cortex is a complex and highly organized structure. As the outermost layer of the brain, and a feature uniquely expanded in mammals¹, it is thought to be responsible for a wide range of cognitive functions such as perception, attention, memory, language, and decision-making². Understanding the development of the mammalian cerebral cortex is therefore essential for unravelling the mysteries of brain function, and its dysfunction.

Cortical development is a complex process involving several sequences of events that ultimately lead to the formation of a diverse array of cell types and the establishment of intricate patterns of connectivity³. A series of proneural transcription factors and signalling pathways is thought to underlie control of the individual decisions that eventually give rise to this diversity^{4–6}. Abnormalities in these processes can lead to a range of neurodevelopmental disorders, such as intellectual disability, autism, and schizophrenia⁷, highlighting the importance of understanding them.

Unfortunately, the study of human cortical development is challenging as access to foetal brain tissue is understandably unreliable and limited, and such tissue is also not amenable to genetic manipulation. Fortunately, advances in stem cell technology, genetic-editing, and differentiation protocols, over the years have allowed researchers to model and study cortical neurogenesis *in vitro*^{8–10}. Consequently, much insight has been gained on how specific genes, signalling pathways, or environmental factors, influence the development of neurons^{9,11}.

In this chapter, I will discuss each of the above in-depth, starting with an overview of mammalian cortical development, focussing on the diversity and differentiation of stem cells, progenitors, and neurons. I will then discuss the mechanisms underlying this differentiation, with a focus on proneural transcription factors (particularly ASCL1), and Notch signalling. Next, I will discuss how human pluripotent stem cell (hPSC) technology has been combined with gene-editing and differentiation protocols to model neurodevelopmental processes and dysfunction *in vitro*. Finally, I

will describe the E3 ubiquitin ligase HUWE1, its links to neurogenesis, and how I aim to leverage *in vitro* models to study HUWE1's roles in human cortical neurogenesis.

1.1 Mammalian cortical development

Anatomically, the developing mammalian cerebral cortex (or neocortex) is arranged into interconnected “radial columns” – 6 horizontal layers of projection neurons and interneurons (collectively termed the Cortical Plate, CP) that become extensively connected vertically across layers, and to other radial columns². These columns are found overlying the ventricular (VZ) and subventricular (SVZ) proliferative zones located at the lateral ventricles' surfaces, from which new-born neurons arise and migrate^{2,12}. The earliest-born neurons migrate radially outwards from the VZ to form Layer I⁷. The next wave forms Layer VI, initially settling directly under Layer I, but as each subsequent generation of neurons migrates over the last, stopping under Layer I in an “inside-out” manner, the previous layer gets spaced further away from the basal surface and Layer I¹³. Understanding the sequence of events leading to this organisation and complexity is key to understanding neurodevelopmental disorders⁷.

1.1.1 Stem cells, progenitors, and neurons in cortical neurogenesis

The neocortex starts off as a single-cell-thick sheet of columnar neuroepithelial cells (NEs) at the rostral neural plate, that undergoes rapid symmetrical divisions to produce more NEs, resulting in a lateral expansion¹⁴. Lateral expansion, coupled with apical constriction (among many mechanisms, reviewed in Nikolopoulou et al. 2017¹⁵), result in the neural plate folding and eventually closing to form the neural tube, with the apical surface closest to the lumen. The cortex then arises from the dorsal side of the neural tube.

Proliferation of NEs occurs via interkinetic nuclear migration (IKNM), whereby the nuclei of NEs migrate basally during G1 and S phases, but return to the apical surface prior to division, resulting in a pseudostratified appearance¹⁵. Neurogenesis begins when individual NEs first switch to asymmetric divisions, 3 days (at embryonic

day (E) 33) after neural tube closure (at E30) in humans, giving a daughter progenitor cell and a daughter postmitotic cell destined to become a neuron¹³. The daughter progenitor cells undergo radial glia transition¹³, gradually losing epithelial characteristics while taking on astroglial ones, to give rise to the distinct, but highly-similar, apical radial glia (aRGs) that eventually replace all NEs¹⁴.

aRGs retain the NE marker Nestin (*NES*), apico-basal polarity and contact between both the apical and basal surfaces, and continue to exist as a pseudostratified epithelium and proliferate via IKNM¹⁴. They however lose the apical tight junctions, and start expressing the astroglia markers Vimentin (*VIM*), *GLAST* (*SLC1A3*), *GFAP*, *BLBP* (*FABP7*), *S100β*, and in the case of cortical radial glia, *PAX6*^{13,14}. Additionally, aRGs are morphologically distinct from NEs and possess a microtubule-rich radial process directed at the basal pial surface¹⁶. It is along these processes that new-born neurons and intermediate progenitor cells (IPCs) migrate radially and expand the neural tube to give rise to the telencephalon¹⁶.

The earliest new-born neurons and IPCs arise from cell divisions of aRGs. This occurs via IKNM throughout neurogenesis, with the aRG cell-body restricted to the VZ even as the cortex expands radially¹⁶. Divisions can be symmetric and proliferative, giving rise to two daughter aRGs, but are more frequently asymmetric and neurogenic, giving rise to a daughter aRG and a neuron directly, or an IPC^{1,14} (Figure 1). Division type is controlled by intrinsic and extrinsic factors that will be discussed later. The earliest IPCs are found in the VZ (hence termed apical IPCs (aIPCs)) and typically undergo a final round of self-consuming, symmetrical, neurogenic division to give two neurons^{17,18} (Figure 1). These neurons form Layer I and the deep-layers (Layers V and VI) of the cortex.

Later in development, a second proliferative zone emerges basal to the VZ (termed the subventricular zone (SVZ)), formed of basal progenitors. Two distinct types of basal progenitors have been identified – basal radial glia (bRGs, also known as outer radial glia) and basal intermediate progenitor cells (bIPCs) – both of which are interconvertible and arise from aRGs¹ (Figure 1). Both bRGs and bIPCs can self-renew via symmetric proliferative divisions to amplify their numbers or asymmetrically to generate neurons^{1,19} (Figure 1). They however differ

morphologically and molecularly, with the monopolar, bRGs unlike bIPCs, possessing a basal process^{19,20} and retaining *PAX6* and astroglial marker expression. Both differ from the apical progenitors for example, by expression of the transcription factor *TBR2* (*EOMES*), with bIPCs additionally losing *PAX6* expression²¹, and bRGs upregulating *HOPX*¹⁹. Subtypes of bRGs have been classified based on differences in the orientation and number of processes²¹, but it is unknown whether they represent distinct subtypes³. Neurons arising from basal progenitors similarly migrate radially, past deep-layer neurons to establish the upper-layers (Layers II-IV)²².

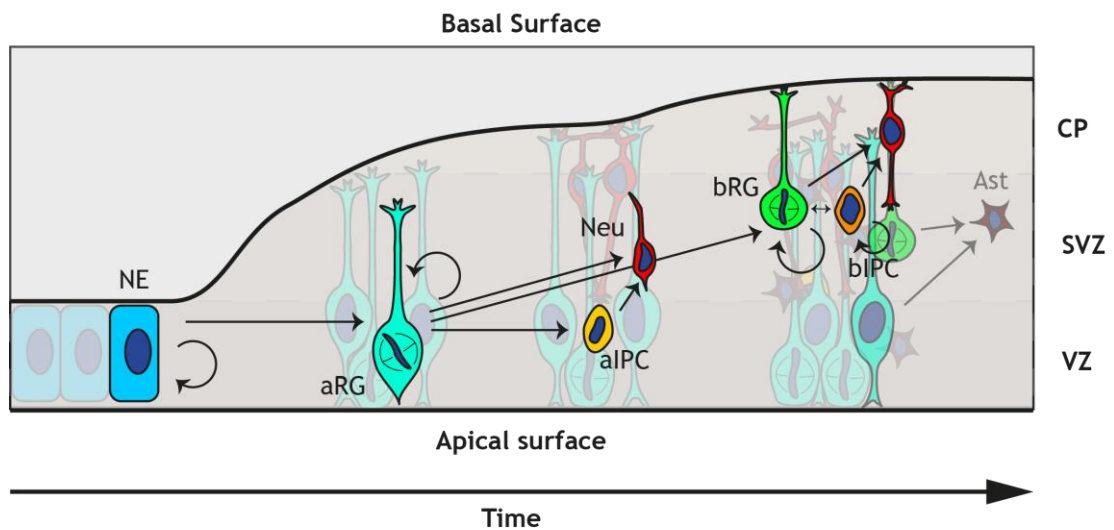


Figure 1. Neural progenitor types and their lineages during cortical development. Embryonic cortical neurogenesis begins from neuroepithelial cells at the rostral neural tube that can self-renew. Neuroepithelial cells transition into apical radial glia that can undergo self-renewal, but more frequently undergo asymmetric divisions to give a daughter aRG and a daughter aIPC or neuron (Neu). aIPCs divide once more to give two daughter Neurons. aRG also give rise to bRGs and bIPCs later in development, that populate the SVZ. Both bRGs and bIPCs can interconvert, self-renew, and undergo neurogenic divisions to give rise to upper-layer neurons. After the peak of neurogenesis, radial glia undergo gliogenesis to produce astrocytes. Illustration: Yu Xuan Tan.

VZ: Ventricular Zone; **SVZ:** Subventricular Zone; **CP:** Cortical Plate; **NE:** Neuroepithelial cell; **aRG:** apical Radial Glia; **aIPC:** apical Intermediate Progenitor Cell; **Neu:** Neuron; **bRG:** basal Radial Glia; **bIPC:** basal Intermediate Progenitor Cell; **Ast:** Astrocyte.

Basal progenitors are highly proliferative only in gyrencephalic species with expanded neocortices (like ferrets and primates) but primarily neurogenic, and hence rare, in lissencephalic rodents^{1,17,20,21,23}. Consequently, this difference has been shown to underlie the differences in upper-layer neuron numbers, thickness of the

cortical upper-layers, and even the folding, between gyrencephalic and lissencephalic species^{23–26}.

Besides the radial migration of new-born neurons and IPCs from the VZ and SVZ to populate the neocortical CP, there is another source of neurons to the neocortex. Specifically, the GABAergic interneurons are thought to primarily arise through tangential migration of IPCs from the ventral neural tube's derivatives – the ganglionic eminences of the ventral telencephalon¹². Such IPCs express combinations of the ventrally-associated *DLX*¹² genes, *ASCL1*²⁷, *PROX1*²⁸, *SP8/9*²⁹, *NKX2.1*, *OLIG2*, and *LHX6/8*³⁰. It should however be noted that emerging evidence suggests that, uniquely in humans, the dorsal telencephalon/early neocortex RG and IPCs can also produce interneurons with similar signatures³¹.

Neurogenesis ends when radial glia undergo a neurogenic-to-gliogenic switch, and self-consume to produce astrocytes³², though some radial glia are reserved in a quiescent state in the subventricular zone of the lateral ventricles and dentate gyrus of the hippocampus, for adult neurogenesis^{16,33}.

1.1.2 Factors regulating proliferation and differentiation

As implied in the previous section, cortical neurogenesis involves a series of decisions, made by neural progenitors (NE, RG, IPCs), between self-renewal, and differentiation into fate-restricted or postmitotic cell-types. This decision-making process is heavily influenced by extrinsic secreted proteins (morphogens such as FGF, WNT, SHH, BMP, and RA), proneural transcription factors, and Notch signalling – all of which will be discussed next. These are summarised in Figure 2.

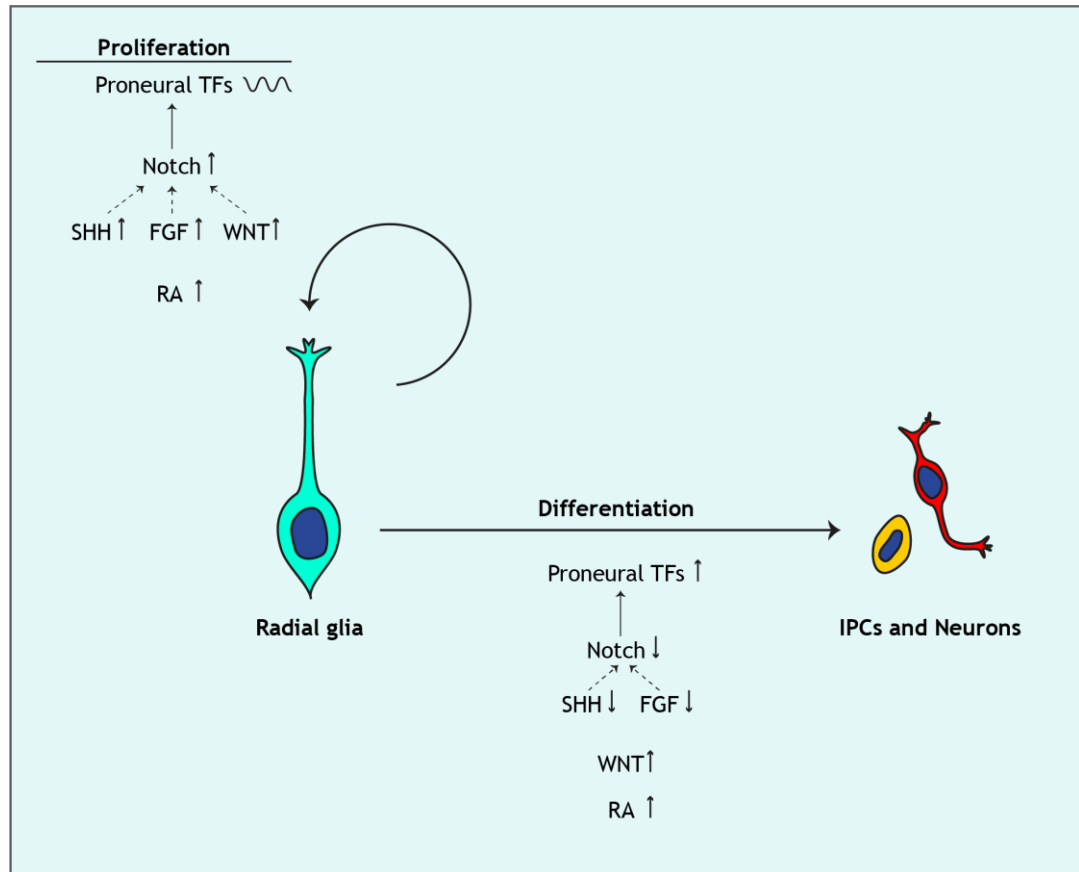


Figure 2. Signalling pathways and proneural genes regulate proliferation and differentiation decisions. Summary of the factors controlling proliferation and differentiation decisions during mammalian cortical neurogenesis. Illustration: Yu Xuan Tan.

1.1.2.1 Morphogens

Throughout development of the telencephalon, cells at the most rostral, dorsal, and ventral regions of the forebrain secrete signalling molecules that influence adjacent and underlying progenitors, conferring positional identities (and thus daughter cell-types and lineage identities¹⁶) while also promoting proliferation or differentiation in concentration-dependent manners². Due to these characteristics, such signalling molecules are termed morphogens³⁴. Three such sources, or patterning centres, have been identified – the rostral patterning centre secreting FGFs; the dorsal patterning centre for WNTs and BMPs; and the ventral patterning centre for SHH – all of which create gradients of their respective morphogens with concentrations highest at their source⁷. Consequently, progenitors experience differing levels of each morphogen depending on their position in the telencephalon and respond

accordingly, with cortical progenitors experiencing higher levels of WNTs and BMPs, followed by FGFs, then SHH.

Canonical WNT (Wingless-type MMTV integration site family signals) signalling involves the binding of WNT ligands to Frizzled receptors, the inhibition of GSK3 to release β -catenin from its GSK3 destruction complex, and translocation of β -catenin to the nucleus where it associates with TCF/LEF to initiate the transcription of downstream genes³⁵. WNTs have been shown to be able to both promote self-renewal of aRGs^{36,37} and paradoxically their maturation into basal progenitors³⁸ (bRGs, bIPCs) and even differentiation into neurons^{32,37}, with suggestions that WNT's influence differs based on the responding cell-type³⁷. More recently, this discrepancy was demonstrated to be through non-canonical WNT/STOP signalling in aRGs and basal progenitors that promotes their differentiation in contrast to canonical WNT/ β -catenin signalling in aRGs that promotes their self-renewal³⁹.

BMPs (Bone Morphogenetic Proteins) signal by binding to serine/threonine kinase receptors that in turn phosphorylate SMAD proteins allowing their association with SMAD4 to initiate transcription of downstream target genes³⁵. Prior to neurogenesis, the BMP pathway in cells is suppressed by FGF signalling⁴⁰ to allow for neural induction and formation of the neuroectoderm/neural plate⁴¹. Later, BMPs, together with WNTs, are necessary for neocortex specification through upregulation of the *EMX* genes⁴². BMP signalling also promotes the differentiation of cortical progenitors into neurons⁴³. Neurons themselves then secrete BMP4, such that a sufficiently high dose of BMP results when sufficient neurons are produced, promoting astrocyte differentiation of RGs and terminating neurogenesis^{32,35,44,45}.

FGFs (Fibroblast Growth Factors) signal via receptor tyrosine kinases that autophosphorylate and activate the downstream RAS/RAF/MAPK and PI3K/AKT pathways via second-messenger proteins⁴⁶. FGFs have been shown to promote the neuroepithelial-to-radial glia transition and self-renewal of aRGs^{47,48} while inhibiting neural differentiation via activation of the Notch (to be discussed later) signalling pathway⁴⁹ and shortening cell-cycle intervals⁵⁰. Additionally, FGF8 confers ventral identities downstream of SHH signalling, and thus suppresses the size of the neocortex^{42,51}. Counterintuitively, and similar to BMPs, neurons also secrete FGF9

that at high levels signal to RGs to begin gliogenesis and terminate neurogenesis, when sufficient neurons have been produced^{32,52}. This might in-part be through FGFs' ability to promote expression of astroglial genes, which is critical for the radial glia transition, but also gliogenesis^{32,53}.

SHH (Sonic Hedgehog) is a key ventralising morphogen that binds to the PTCH1 receptor, triggering its internalisation and degradation, and relieving PTCH1's inhibition of SMO. Activated SMO in turn converts GLI transcription factors from repressor to activator forms to initiate downstream transcription events⁵⁴. GLI3, which normally occurs in its repressor form, is initially ubiquitously expressed in the telencephalon, and is necessary for establishing a dorsal identity in this form^{42,51,55}. SHH, with its high ventral concentration, restricts the dorsalization of the telencephalon by GLI3 and initiates expression of ventrally-associated genes, thereby allowing the ventral telencephalon to emerge^{42,56}. Besides its widely-studied role in ventralizing the telencephalon, SHH signalling also promotes self-renewal of bRGs and inhibits their differentiation, increasing their numbers and possibly contributing to gyrification of the cortex^{26,57}.

Another morphogen involved in patterning the developing cortex and influencing proliferation or differentiation decisions is retinoic acid (RA). RA is first secreted from the apical surface of the ventral telencephalon into the ventricular lumen⁵⁸, creating an apical-basal gradient which was found to be necessary for promoting the self-renewal of aRGs⁵⁹. Production of RA later shifts to the meninges, creating a basal-apical gradient⁵⁸ that is, counterintuitively, important in promoting the differentiation of aRG⁶⁰ and the migration⁶¹ and identity⁶² of new-born neurons.

1.1.2.2 Proneural transcription factors

Some of the downstream effectors for the morphogens are transcription factors that have been termed “proneural transcription factors” (proneural TFs or proneural genes). These are basic-helix–loop–helix (bHLH) transcription factors that are defined as necessary and sufficient to specify the identity, and induce the differentiation, of neural progenitors⁴. Additionally, all proneural TFs also upregulate

expression of the Delta ligands *DLL1* and *DLL3*, triggering Notch signalling (to be discussed later) in neighbouring cells^{5,63}.

All bHLH transcription factors contain a basic DNA-binding domain and a helix–loop–helix domain, which is responsible for protein–protein dimerization³⁵. Homodimerization, or heterodimerization with E proteins (another class of bHLHs), is necessary for proneural TFs to bind at specific DNA sequences (5'-CANNTG-3') known as E-boxes, in the promoters or enhancers of target genes^{4,5}, where they activate expression of these targets⁶⁴. Differences in the amino acid sequences of the basic domain between proneural TFs confer additional preferences for specific E-box sequences⁶⁵. Due to this requirement for dimerization, ID proteins (HLH transcription factors that lack DNA binding domains) can inhibit proneural TF activity by either sequestering E proteins, or dimerizing to proneural TFs without allowing for DNA-binding, and promoting their proteasomal degradation⁶⁶.

In the developing cortex, expression of three proneural genes in RGs has been found – *ASCL1*, *NEUROG1*, and *NEUROG2*⁶⁷ – with their expression oscillating such that only a fraction of progenitors express them at any time^{5,68}, and stabilizing while peaking in differentiating progenitors^{69,70}, resulting in a “salt-and-pepper” distribution³⁵. It should also be noted that *ASCL1*, despite also being expressed in the developing human neocortex, is much more widely-expressed ventrally, and so is considered a ventrally-associated marker⁷¹. *ASCL1* expression in progenitors is also mutually exclusive from the neurogenins (*NEUROG1* and *NEUROG2*), as they repress transcription of each other^{6,72}.

The target genes of proneural TFs, specifically *ASCL1* and *NEUROG2*, have been found to be involved in cell-cycle exit (e.g., *GADD45G*) and neuronal differentiation (e.g., *SOX4*, *NEUROD4*), but also neuronal transmission (e.g., *GAD2*) and axonal maturation (e.g., *TUBB3*, *PLXNA2*)^{65,73}, consistent with their proneural definition. Consequently, *ASCL1*, for example, promotes the general differentiation of neural progenitors in the ventral²⁷ and dorsal^{68,74} telencephalon, such that *Asc1* mouse mutants lack specific sets of neurons. Additionally, *ASCL1* also promotes a GABAergic identity⁷² in, and the migration⁷⁵ of, new-born neurons, although GABAergic specification was found to be context-dependent and not obligate⁶⁸.

NEUROG1 and NEUROG2 have similarly been found via mouse mutants to be necessary for the production of glutamatergic neurons^{68,72}.

Besides promoting neuronal differentiation in general, both ASCL1 and NEUROG2's functions are adjusted temporally during cortical neurogenesis, specifically in establishing cell-type identities. For example, they promote expression of *CTIP2* (*BCL11B*), a marker of deep-layer V neurons, while suppressing *SATB2*, an upper-layer marker⁷⁶. Later, ASCL1 and NEUROG2 instead suppress deep-layer identity by promoting expression of *FOXP1*, which represses expression of *TBR1*, leading to acquisition of an upper layer identity^{77,78}. Finally, ASCL1 and NEUROG2 delay the termination of neurogenesis, with their absence resulting in precocious gliogenesis^{68,74}.

As mentioned above, the various morphogens achieve their influences on neurogenesis partly via regulating the activity of proneural TFs. WNT signalling, via β -catenin, induces *NEUROG2* expression in aRGs to dorsalize the neuroepithelium⁷⁹. BMP signalling also promotes expression of *NEUROG2*⁸⁰ and degradation of ASCL1 by upregulating *ID1*⁸¹. Conversely, FGF signalling, via the RAS/MAPK pathway, induces expression of *ASCL1* to promote a GABAergic identity^{82,83}. Additionally, the kinases GSK3 (inhibited by WNT signalling) and MAPK (FGF signalling effector) can phosphorylate NEUROG2⁸⁴ and ASCL1⁸³ respectively to promote proliferation. Phosphorylated NEUROG2 preferentially forms NEUROG2-E47 dimers with weaker ability to induce differentiation instead of homodimers⁸⁴, while phosphorylated ASCL1 is similarly less able to induce neuronal differentiation than ASCL1SA6 – a mutant form with six serine-alanine substitutions incapable of being phosphorylated⁸⁵.

On the topic of phosphorylation, cyclin-dependent kinases have also been demonstrated to phosphorylate NEUROG2⁸⁶ and ASCL1⁸⁵ in proliferating progenitors, thereby reducing their pro-differentiation ability. Mechanistically, this was shown to be by restricting their activity to open chromatin sites not containing critical neuronal differentiation genes^{87,88}.

Counterintuitively, *ASCL1* was found to also directly promote proliferation, by upregulating expression of cell-cycle genes (e.g., *E2F1*, *CCND1*), such that acute deletion of *Ascl1* led to cell-cycle exit of neural progenitors *in vivo* and *in vitro*⁷³. *NEUROG2* similarly could induce proliferation of neural progenitors when electroporated *in vivo*⁸⁹. A solution to this paradox was proposed in seminal studies by the Kageyama lab, where it was shown, in mouse models, that *ASCL1*⁹⁰ and *NEUROG2*⁹¹ expression oscillates in 2-3hr periods in proliferating progenitors, out of phase with the oscillatory expression of the Notch signalling target genes *HES1* and *HES5*. The HES proteins were previously found to repress transcription of themselves and the proneural genes⁹², with repression of their own transcription resulting in their oscillatory expression pattern that then sets up the out-of-phase oscillation of the proneural genes^{90,93}. In terminally-differentiating progenitors, *ASCL1*⁹⁰ and *NEUROG2*⁹¹ expressions are stabilized prior to the final neurogenic division. Crucially, enforcing either oscillatory or stable expression patterns via optogenetics led to proliferation or differentiation respectively⁹⁰. Together, these results demonstrate that the proneural genes' activities control both proliferation and differentiation in neural progenitors, and that this control is also inextricably linked to Notch signalling. While these experiments have yet to be repeated in humans yet, they likely hold true, and evidence of the proneural genes' oscillations in humans might even be buried inadvertently in high read-depth single-cell sequencing experiments as more data is generated.

1.1.2.3 Notch signalling and the proneural TFs

As touched upon in the previous sections, Notch signalling greatly influences the proliferation and differentiation of neural progenitors via modulating the activity of the proneural TFs. It has also been shown to have cross-talk with the morphogen signalling pathways and to regulate differentiation in its own right.

The Notch signalling pathway involves binding of extracellular membrane-bound ligands of the Jagged (*JAG1/2*) or Delta (*DLL1/2/4*) families on a sender cell to Notch receptors on a neighbouring receiver cell⁹⁴. Ligand binding exposes a cleavage site on the Notch receptor that allows the metalloproteinase ADAM10 and γ -secretase to

act on, releasing the Notch intracellular domain (NICD), and the extracellular domain (ECD) which is endocytosed into the sender cell⁹⁴. Within the receiver cell, NICD translocates and interacts with RBPJ (CSL) and MAM in the nucleus to initiate transcription of downstream target genes. Among these target genes are those for the Hairy enhancer of split (*HES*) transcription factors, of which *HES1* and *HES5* are most relevant in neurogenesis⁹².

HES 1 and *HES5* suppress expression of the proneural genes on a transcriptional level^{90,91} to inhibit neuronal differentiation such that loss of *HES1* and *HES5* results in reduced Notch signalling, and premature differentiation⁹⁵. Furthermore, the *HES* transcription factors can bind and sequester E proteins to also inhibit proneural TF activity, in a manner similar to the ID proteins⁹⁶. Also, as mentioned before, *HES1* and *HES5* repress their own promoters, resulting in their own expression oscillating in 2-3hr periods. Through these oscillations and transcriptional repression of proneural genes, the oscillatory expression patterns of the proneural TFs that promote proliferation is set up while Notch signalling occurs^{90,91,93}. Additionally, Notch signalling also inhibits the differentiation of aRGs into basal progenitors⁹⁷. The result is that Notch signalling inhibits differentiation and promotes self-renewal of neural progenitors.

However, as briefly mentioned, the proneural TFs themselves can trigger expression of the Delta ligands *DLL1* and *DLL3* and thus initiate Notch signalling in neighbouring cells. *DLL3* is unique from the other Delta ligands in that it does not trigger Notch signalling, but rather serves as an inhibitor by occupying Notch receptors in *cis*, preventing neighbouring cells from triggering Notch signalling in the sender cell⁹⁸. Accordingly, an interplay between Notch signalling and proneural TF levels occurs between neighbouring cells, known as “lateral inhibition” (Figure 3). Cells expressing more proneural TFs than their neighbours will have more *DLL1* and *DLL3* and thus become more resistant to Notch signalling (via *DLL3*) while triggering Notch signalling in their neighbours. These neighbours thus become receiver cells with higher levels of *HES* proteins that in turn further suppress their levels of proneural TFs. As the neighbours have lower levels of proneural TFs, they are also less able to express Delta ligands and trigger Notch signalling in the original sender cell, allowing it to differentiate non-cell-autonomously. Through lateral inhibition,

proneural TFs thus restrict their activity to single progenitor cells and inhibit their own expression in, and the differentiation of, neighbouring cells – giving the salt and pepper expression pattern described previously. Because of this, differentiation and proliferation decisions in neural progenitors are also partly non-cell-autonomous and not made in isolation.

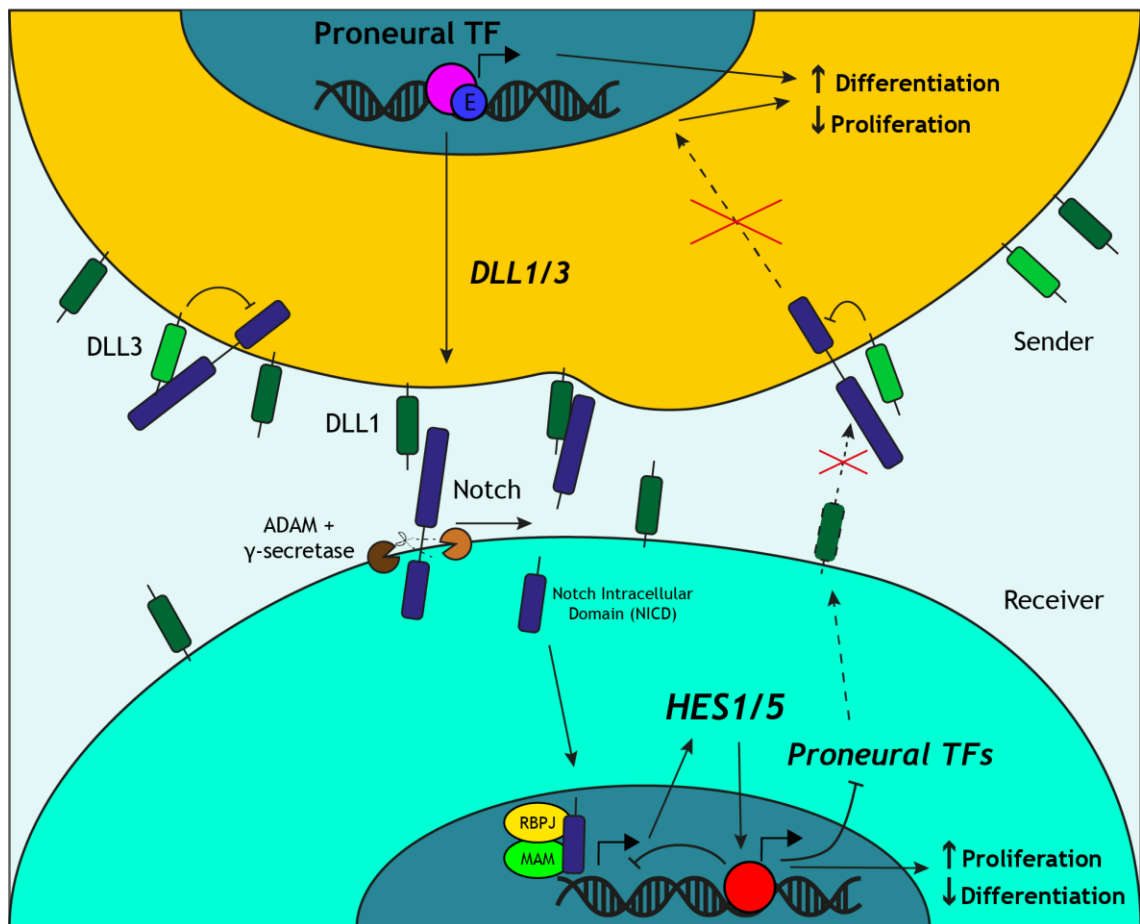


Figure 3. Lateral inhibition of Notch pathway intersects with proneural activity. Sustained expression of proneural transcription factors upregulates genes involved in differentiation, as well as the Notch signalling ligands *DLL1* and *DLL3*. *DLL1* binds to Notch receptors on neighbouring cells (Receiver), leading to their cleavage and release of NICD. NICD interacts with RBPJ and MAM to act as a transcription factor, promoting expression of *HES1* and *HES5* that in turn act as transcription factors repressing their own expression and that of proneural genes while upregulating genes involved in proliferation. Meanwhile, *DLL3* in the sender cell inhibits Notch receptors on the sender. Consequently, receiver cells, which also have less *DLL1*, cannot trigger Notch signalling in sender cells with higher proneural TF levels. Illustration: Yu Xuan Tan.

Other than via the proneural TFs, Notch signalling also has cross-talk with the morphogen pathways⁹⁹. Briefly, FGF signalling can also activate Notch signalling to promote radial glia proliferation, though the exact mechanism was not investigated⁴⁹.

WNT signalling similarly leads to increased Notch signalling and HES1 levels, as GSK3 (inhibited by WNT signalling) also phosphorylates and inhibits NICD.¹⁰⁰ SHH, via GLI1/2 also activates transcription of *HES1*, *NOTCH1*, and *JAG1*, increasing Notch activity¹⁰¹. Similarly, BMP signalling, via the SMADs, can activate transcription of *HES1* and *JAG1*¹⁰².

Besides promoting proliferation, Notch signalling also contributes to progenitor identity by promoting expression of *HER2* (*ERBB2*) in radial glia to suppress gliogenesis¹⁰³ and maintain radial glia¹⁰⁴. Later, however, Notch signalling is a gliogenic signal, with Hes1 promoting STAT3–JAK2 binding to facilitate STAT3 phosphorylation and gliogenesis¹⁰⁵. Another of its target genes, *NFIA*, also encodes a transcription factor that demethylates promoters of gliogenic genes such as *GFAP*¹⁰⁶.

1.2 Modelling cortical neurogenesis

1.2.1 2D and 3D *in vitro* models

Simultaneous to researchers gaining an understanding of the mechanisms underlying cortical development *in vivo* (as covered in the previous sections), work was being done in: establishing human embryonic stem cells (hESCs)¹⁰⁷; generating human induced-pluripotent stem cells (hiPSCs)¹⁰⁸; and isolating foetal neural progenitor cells (hNPCs)^{109,110}. Together, these efforts culminated in the first *in vitro* differentiation protocols to model human cortical neurogenesis^{111–117}, and presented opportunities to overcome the limitations inherent in accessing and studying neurodevelopment in humans. As hESCs and hiPSCs (collectively hPSCs for human pluripotent stem cells) are easier to obtain and maintain long-term than hNPCs (which also lose neurogenic potential over passages¹¹⁸), differentiation protocols largely use hPSCs, and hNPCs will not be discussed further.

The first types of models I will cover are directed differentiation protocols, inspired by our understanding of the morphogens' actions during neurogenesis. The guiding principle of the earliest attempts at modelling cortical neurogenesis *in vitro* has been

the identification of the right combination and temporal application of growth factors and small molecules to specify, pattern, and differentiate, stem cells towards the neurogenic lineage^{119,120}. To that end, differentiation protocols often begin by inhibiting BMP and TGF β signalling, in what is termed dual-SMAD inhibition, to pattern hPSCs into neuroectoderm¹²¹, similar to how BMP pathway inhibition is necessary for neural plate induction *in vivo*^{41,122} by preventing the adoption of mesodermal fates¹²³. Following neural induction, the cultured cells are then exposed to morphogens to confer positional identity based on anterior-posterior and dorso-ventral axes and generate the appropriate neural progenitor. For instance, FGF8 promotes a forebrain identity in opposition to RA, while FGFs and WNTs promote a dorsal cortical identity in opposition to SHH¹²¹. Prolonged or excess WNT signalling however leads to neural crest identities and so requires tight temporal and dosage control¹²⁴. Finally, after neural progenitors with the desired positional identity are obtained, cell-cycle exit and terminal differentiation into neurons can be promoted by inhibiting the Notch and FGF pathways¹²⁴.

“Dorsal cortical” neurons generated in this way exhibit a strikingly similar development profile to *in vivo* neurons. Firstly, they lose pluripotency markers (*POU5F1/OCT4*, *NANOG*) and acquire early markers for cortical neural identity such as *NES*, *PAX6* and *FOXP1*. They also spontaneously organize into neural rosettes – radial arrangements of columnar neuroepithelial cells with apico-basal polarity¹²⁵ – reminiscent of the neural tube. These progenitors then produce early neurons expressing generic early neuronal markers (*TUBB3/TUJ1*, *MAP2*) and deep-layer markers (*BCL11B/CTIP2*, *TBR1*) before producing neurons expressing upper-layer markers (*CUX1*, *SATB2*), and lastly astrocytes (*GFAP*, *S100 β*), following the temporal order seen in the cortex^{121,126}. These neurons also exhibit the expected electrophysiological properties and form synapses with each other¹²⁶ and can survive and integrate into mouse cortices¹²⁷. These similarities suggest we are now capable of a high degree of fidelity in modelling cortical neurogenesis – something which has also been demonstrated in transcriptomic comparisons between fetal brain tissue and cultured neurons^{128,129}.

These earlier efforts to model cortical neurogenesis subsequently led to protocols for neuronal differentiation in 3-dimensional cultures, in an effort to better recapitulate

cell-cell interactions, and model neuronal maturation and later developmental stages^{10,130}. Such 3D cultures can be broadly categorised into organoids and spheroids, with organoids arising from undirected differentiation and thus having wider cell-type diversity¹¹⁵, and spheroids arising from directed differentiations using morphogens^{10,116,117,131,132}. Both methods allow for longer-term culture than 2D methods, allowing the modelling and study of gliogenesis, neuronal maturation, and other postnatal events¹⁰. More recently, sliced cultures have been developed to overcome the growth-limits imposed by diffusion, and allow for even longer-term maintenance and maturation of spheroids¹³³. Other advances such as assembloids – fusions of domain-specified spheroids with other spheroids or even spinal cord and muscle organoids – represent our first steps into modelling the wider human bodily systems^{134,135}.

Another type of model, made possible by earlier work on proneural TFs, is the induced neuron. By overexpressing *ASCL1* and *NEUROG2* in cells^{136–140}, hNPCs, hPSCs, and even human fibroblasts and other cell types such as hepatocytes, can be converted into neurons within days (reviewed in Dennis et al. 2019⁶⁷). This also forms the basis of the doxycycline-induced neurons generated from hPSCs by the OPTi-OX platform¹⁴¹.

By opening the doors to modelling human cortical development, both 2D and 3D differentiation protocols have also ushered in a new era of studies looking into the aetiology of neurodevelopmental disorders. By converting easily-accessible patient fibroblasts into iPSCs then differentiating them, many insights have been gained (reviewed in Tamburini and Li 2017¹⁴²). For example: organoids derived from microcephalic patients with *CDK5RAP2* mutations showed premature differentiation¹¹⁵; spheroids from lissencephalic Miller-Dieker Syndrome patients (chr17p13.3 deletion) showed increased apoptosis and decreased self-renewal of NEs¹⁴³; 2D differentiations¹⁴⁴ and organoids¹⁴⁵ from Autism Spectrum Disorder patients showed changes in the dynamics of GABAergic neuron generation and its proportions.

1.2.2 CRISPR-Cas9 and differentiation models

These efforts to study neurodevelopmental processes and dysfunction were given a further boost with the widespread introduction of CRISPR-Cas9 as a gene-editing tool^{146,147}. Briefly, Cas9 is an RNA-guided nuclease that makes double-stranded breaks in DNA based on where its associated guide RNA (gRNA) forms base-pairs with. This double-stranded break can then be repaired by nonhomologous end-joining (NHEJ) which introduces seemingly-random¹⁴⁸ insertions and deletions (useful for disrupting a gene's function) or by homology-directed repair (HDR) in which repair involves integration of an exogenously-provided DNA template bearing ends homologous to the cut locus. The guide RNA must however lie upstream of a PAM sequence (5'-NGG-3'), which restricts the range of genomic sites that can be edited, though efforts are constantly being made to expand this range^{149,150}. HDR is also inefficient and rare, though again, efforts are continuously being made to understand and improve this¹⁵¹, such that newer methods like Prime Editing¹⁵² and PASTE¹⁵³ can introduce edits in excess of 30kb.

With CRISPR technology rapidly advancing, researchers have also adopted variants of the technology in studying neurodevelopment. For instance, by using CRISPR interference (CRISPRi) screens – a variant in which nuclease-dead Cas9 guides a transcriptional repressor to multiple target sites based on a library of gRNAs – in 2D-differentiated hiPSCs, genes necessary for neuronal differentiation, survival and subtype-specification were found¹⁵⁴. The opposite, CRISPR activation (CRISPRa), where a transcriptional enhancer is fused instead, was similarly used to find genes that protected neurons under oxidative stress conditions, several of which were associated with neurodegenerative diseases¹⁵⁵. CRISPRa was also used to identify genes that improved neuronal differentiation efficiency and maturation, finding 17 candidate neurogenic TFs that included known ones such as NEUROG2¹⁵⁶.

The more conventional CRISPR-Cas9 genome-editing techniques can and have also been used in neurodevelopmental disorder modelling, presenting an advantage especially in instances when patients samples and matching controls are rare and hard to obtain, or a well-characterised genetic background is desired¹⁴². It also allows for specific mutations, such as a complete deletion to study a protein's function, to

be introduced. For example, heterozygous knockout iPSCs of the autism-associated *CHD8* gene have been neuronally-differentiated to find genes that CHD8 regulates the accessibility of¹⁵⁷. iPSC mutants for the intellectual-disability (ID) associated *GRIN2B* (an NMDA receptor subunit) gene were more proliferative and showed delayed differentiation¹⁵⁸. Similarly, iPSC knockouts for the ID-associated *ACTL6B* showed delayed differentiation with no MAP2-positive cells at the timepoint investigated¹⁵⁹. Evidently, CRISPR-Cas9 technology is a powerful tool that can complement neuronal differentiation techniques in the investigation of the mechanisms governing human neurogenesis.

1.3 HUWE1 and neurogenesis

1.3.1 HUWE1-related genetic conditions

One group of neurodevelopmental disorders for which no mechanism was proposed at the start of my project are the HUWE1-related genetic disorders (OMIM #309590). These are characterised by X-linked syndromic and non-syndromic intellectual disability (ID) at birth, with a high (>50%) penetrance of: absent/limited speech, growth and developmental milestone delay, microcephaly, hypotonia, and less-commonly epilepsy (~30%)¹⁶⁰. Originally described in the literature as “Juberg-Marsidi Syndrome”^{161,162} (JMS), “X-linked Intellectual Disability”¹⁶⁰ (XLID), or “Mental Retardation, X-linked, Syndromic, Turner-type”¹⁶³ (MRXST), the patient-family community (<https://www.huwe1.org/>) would prefer to term it “HUWE1-related genetic conditions” (private correspondence with Ursula and Alex Green) and so the condition shall be referred to as HUWE1-related genetic disorders or conditions throughout my thesis. To date (Mar 2023), 48 patients with missense mutations, 1 with a splice-site mutation, and 36 with microduplications of the X chromosome encompassing the entirety of *HUWE1* have been reported^{160,164–166}. *In vitro* assays for HUWE1 activity have indicated that missense mutations can be either hypomorphic^{162,167,168} or hypermorphic¹⁶², though none led to complete loss of activity, suggesting loss of HUWE1 is not tolerated and that it has an essential role in general embryogenesis¹⁶⁹. Patients with *HUWE1* microduplications were also demonstrated to have ~2-fold increased *HUWE1* mRNA levels¹⁶⁶, consistent with a gain-of-function mechanism.

Clearly, *HUWE1* regulates some aspect(s) of human developmental neurogenesis, but the protein has thus far not been studied in human models of neurogenesis, save for two studies (Bosshard et al. 2017¹⁷⁰, and Aprigliano et al. 2021¹⁶⁸). That microduplications and hypomorphic mutations both lead to ID also indicates *HUWE1*'s function in neurodevelopment is dose-sensitive. Consequently, any model for its functions needs to account for both gain and loss-of-function leading to similar phenotypes.

1.3.2 *HUWE1* in developmental neurogenesis

HUWE1 (HECT, UBA and WWE domain containing 1; Mule, LASU1, HECTH9, UREB1 or ARFBP1 in older literature) is an X-linked gene encoding a 4374aa, 482kDa HECT E3 ubiquitin ligase¹⁷¹, expressed throughout the body at moderate levels¹⁷². First identified in 2005 by multiple groups through independent partner-interaction screens, it was found to ubiquitinate MCL1¹⁷³, ARF¹⁷⁴, histones¹⁷⁵, and c-MYC¹⁷⁶. Like canonical E3 ligases, *HUWE1* can target substrates for proteasomal degradation via K48-polyubiquitination¹⁷¹ – however it can also perform K63-polyubiquitination to modify its substrates' activity, as in the cases of c-MYC¹⁷⁶ and DVL¹⁷⁷. *Huwe1*-knockouts are embryonically-lethal in mice between E9-14^{178–181}, perhaps due to its ubiquitous expression and wide repertoire of targets. Notably, increased p53 levels and the apoptotic marker cleaved-Casp3 were detected prior to lethality¹⁸¹. However, due to its ability to tag proteins of interest in oncology such as p53, ARF, c-MYC, MCL1, and BRCA1 for degradation, much of the literature on *HUWE1* focuses on its unresolved, conflicting, roles as both a tumour-suppressor and a proto-oncogene¹⁷¹, rather than its developmental role.

Huwe1 was first implicated in neurogenesis through a screen for binding partners of the bHLH transcription factor Mycn (N-Myc), then known to undergo proteasomal degradation¹⁷⁸. In that study, it was demonstrated that *Huwe1* targeted Mycn for degradation, and that *Huwe1* gene-trapped mESCs and neural progenitors of brain slices electroporated with *Huwe1*-siRNA were hyper-proliferative and either unable to neurally differentiate or were greatly delayed in differentiation. These effects were

found to be mediated by an accumulation of *Mycn*, the knockdown of which completely masked the phenotypic effects of *Huwe1* knockdown¹⁷⁸. Also notable was that p53 levels remained unchanged in neural progenitors, despite loss of *Huwe1*, suggesting regulation by HUWE1 activity is dependent on cell-type¹⁷⁸. These results were subsequently confirmed *in vivo* through *Nestin-Cre* driven *Huwe1* KO mice which have an expansion of the proliferating neural stem cell population and a concomitant reduction in their differentiation to neurons in the dorsal forebrain, leading to a disorganized laminar structure of the cortex¹⁸². The same study also discovered that *Mycn* stabilization in *Huwe1*'s absence led to an overexpression of *Dll3* and an upregulation of Notch signalling that opposed the terminal differentiation of neural progenitors¹⁸².

Similar observations of *Huwe1* ablation in other mouse neural progenitor populations followed, mediated however by the stabilization of other bHLH transcription factors. Loss of *Huwe1* in cerebellar granule neuron precursors (cGNPs) results also in hyperproliferation and impairment of differentiation and failure of the cerebellum to develop¹⁸³ – although mechanistically this has been separately linked to the stabilization of *Mycn*¹⁸³ and *Atoh1*¹⁸⁴. These studies also showed an increase in *Huwe1* expression as neural progenitors differentiated, with expression highest in neurons^{178,183}. Similarly, deletion of *Huwe1* in adult mice hippocampal neural stem cells promotes the proliferation and impairs differentiation of their already-cycling population, through stabilization of *Ascl1* which prevents re-entry into quiescence¹⁸⁵. In inner ear progenitors, loss of *Huwe1* also stabilizes *Atoh1*, but this instead promotes cell-cycle exit and differentiation, resulting in extra inner hair cells¹⁸⁶.

Taken together, these results suggest *Huwe1* is a key regulator of the transition from the proliferative to differentiation phase of neurogenesis, at least in a murine context. In the only studies providing information on HUWE1 function in human neurogenesis, an immunofluorescence staining for HUWE1 in wildtype cerebral organoids showed its localization in the progenitorial zones, away from TUBB3-positive neurons¹⁷⁰, at odds with the results from mouse studies. I should also note that the TUBB3-positive regions of the organoids shown had a dense DAPI staining and curvature more resembling a ventricular zone. In the other study, published midway through my project, patient-derived iPSCs were differentiated into cerebral organoids which

failed to survive and suffered a block in differentiation and expression of neuronal markers due to a stabilization of p53 that could be rescued by constitutively knocking-down p53¹⁶⁸. The regulation of p53 levels, however, cannot satisfactorily explain HUWE1-related genetic conditions, for reasons I will discuss in my Results and Discussion chapters, and thus cannot be HUWE1's main role in human neurogenesis. I will discuss these two studies and their issues in-depth, and in relation to my results, where relevant throughout my thesis.

1.3.3 Molecular regulation of HUWE1 activity

HUWE1 structures and domains have been characterised via truncated c-terminus constructs^{187,188}, full-length nematode orthologues¹⁸⁹, and full-length protein¹⁶⁷, giving some insight into its function. From these (primarily Hunkeler et al. 2021¹⁶⁷), it was found that HUWE1 has four Armadillo-like domains (ARLDs 1-4) that form a ring-shaped solenoid (a “donut-like” structure) together, atop which sit accessory modules (UB module 1, WWE, HWA, Tower, and UB module 2), and the flexibly-linked c-terminal catalytic HECT domain that contains a catalytic Cys4341 and possesses the ubiquitin ligase activity. Under the ring is a BH3 domain (Figure 4, including summary of some HUWE1 substrates and the consequence of regulation). The solenoid has been proposed to provide multiple low-affinity degenerate binding sites on its inner surface to jointly allow for its promiscuous substrate binding, with the out-of-plane modules providing specificity for certain key substrates^{167,189}. For example, the UB modules are critical for loading upstream E2 ligases and ubiquitin to HUWE1¹⁶⁷, the BH3 domain for binding to MCL1¹⁷³, and the WWE and HWA domains for binding to PARylated substrates¹⁹⁰. The flexibly-tethered HECT domain then scans the docked substrate for ubiquitination sites¹⁸⁹.

Patient mutations have been found distributed throughout all domains, though many are also clustered in the catalytic HECT domain (reviewed in Giles and Grill 2020¹⁶⁴). It has been suggested that HUWE1's “closed-loop” solenoid structure – whereby multiple linearly-distant domains contribute structurally to the same inner surface (and hence substrate-binding) – is why these mutations can occur across multiple domains yet lead to the same disease, and why HUWE1 appears intolerant to edits

such as tagging (idea presented by Lilian Fennel during a lab retreat, from her discussions with Tim Clausen's group¹⁸⁹).

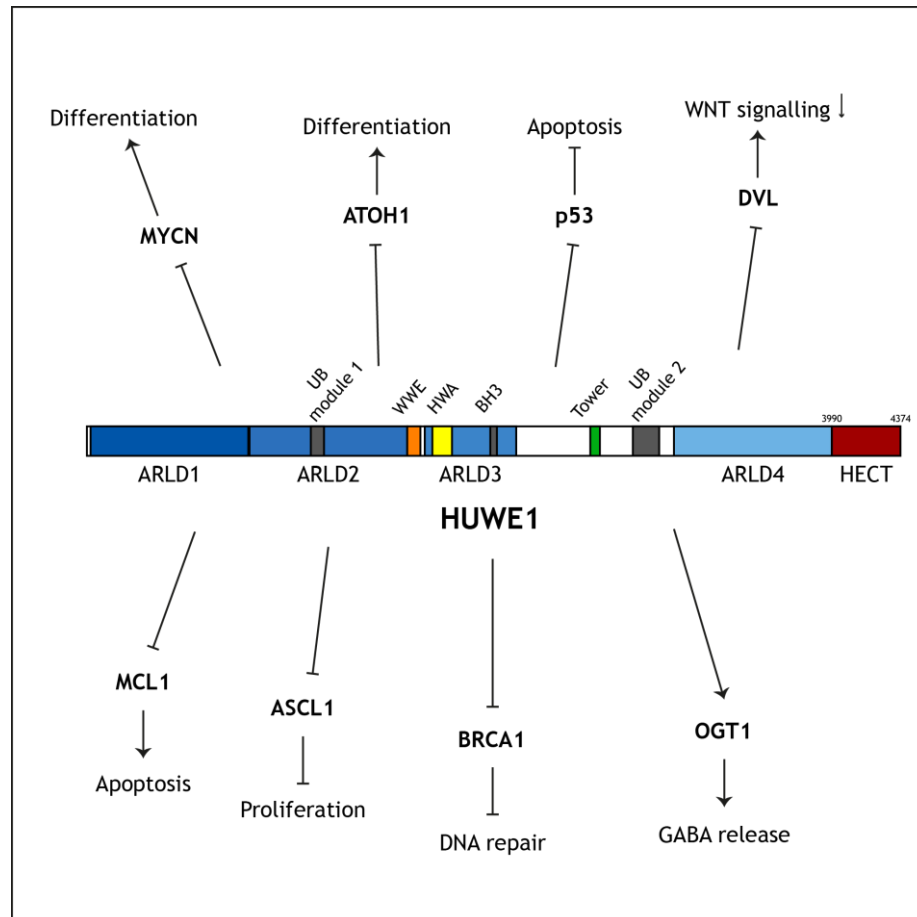


Figure 4. Summary of HUWE1 domains and some of its targets. HUWE1 adopts a ring-shaped structure, with the ARLD domains forming a ring with surfaces to which substrates bind loosely. Above and below the plane of the ring are loops containing accessory modules (UB modules 1/2, WWE, HWA, BH3) that recognise specific substrates, as well as the flexibly-linked HECT domain that ubiquitinates bound substrates. HUWE1 ubiquitinates most of its substrates for proteasomal degradation, resulting in the outcomes shown. In the case of DVL, HUWE1 ubiquitination prevents its multimerization, but does not induce its degradation. For OGT1, HUWE1 physically associates with OGT1 but does not ubiquitinate it. ARLD: Armadillo-Like Domain. Adapted from Hunkeler et al. 2021¹⁶⁷ and Kao et al. 2018¹⁷¹.

As mentioned, HUWE1's activity on its substrates appears to be differentially regulated both temporally and across cell types. Currently, it has been shown that HUWE1 can be prevented from degrading ATOH1 in cGNPs through the dephosphorylation of ATOH1, triggered by SHH signalling, suggesting it preferentially recognizes phosphorylated-ATOH1¹⁸⁴. HUWE1's activity may also be restricted subcellularly to cytosolic targets as nuclear-localised Ascl1 is not

ubiquitinated by Huwe1 whereas cytosolic Ascl1 is poly-ubiquitinated by Huwe1 for proteasomal degradation¹⁹¹.

HUWE1 can also be targeted for proteasomal degradation by the E3 ligases MDM2¹⁹², CUL4B¹⁹³, and itself¹⁹⁴, at least in several cancer cell-lines, in response to DNA damage^{193,194} or EGFR inhibition¹⁹². Conversely, HUWE1 can also be stabilized through deubiquitination, by the deubiquitinases USP4¹⁹⁵ and USP7¹⁹⁴.

HUWE1 may also be inhibited when homodimerized at its catalytic c-terminus – an inhibition that is relieved during binding of an upstream activation domain to the dimerization domain *in cis* to prevent dimerization¹⁸⁷. This property is thought to be exploited by p14ARF (an endogenous HUWE1 inhibitor¹⁷⁴), which binds and blocks the activation domain, to trap HUWE1 in an inactive homodimer¹⁸⁷. It should be noted though that a more recent study using full-length HUWE1 found that it does not actually dimerize, though the activation domain was still found to enhance activity¹⁶⁷.

Finally, considering its large size and multiple domains, one might speculate that HUWE1 could also have other ubiquitination-independent functions, although this is an unexplored area. This would be inspired by how the similarly large HECT E3 ligase HERC1 is a multifunctional protein that possesses guanine nucleotide exchange factor activity¹⁹⁶. To date, there is only one reported example of HUWE1 potentially having a ubiquitination-independent role via its formation of a complex with the glycosyltransferase OGT1 to promote GABAergic transmission in *c. elegans* (discussed in the next section)¹⁹⁷. In those experiments, OGT1 levels were not increased in a pulldown fraction of catalytically-dead EEL-1 (HUWE1 orthologue) relative to WT EEL-1, suggesting that it bound to EEL-1 without being ubiquitinated/degraded. Additionally, loss of OGT1 or EEL-1 both led to reductions in GABAergic activity which is unlikely if deletion of EEL-1 would increase OGT1 levels¹⁹⁷. It is thus possible that HUWE1 is performing a ubiquitination-independent function with OGT1. More likely, however, OGT1 is serving as an adaptor protein for HUWE1 similar to how other HECT E3 ligases such as E6AP and WWP2 are recruited to ubiquitination substrates by means of adaptor proteins¹⁹⁸, since ubiquitin ligase activity but not glycosyltransferase activity was required for GABAergic

transmission¹⁹⁷. It remains to be seen if HUWE1 truly does possess ubiquitination-independent functions.

1.3.4 HUWE1 dysregulation may affect mature neuronal function

Missense mutations and microduplications in *HUWE1* both result in intellectual disability (ID)¹⁶⁰, suggesting a role in human brain development. This, however, could stem from either a defect in neuralization of progenitor populations, or from a defect in neuronal maturation and function. Unlike in mouse models, knockouts of the *HUWE1* orthologue *EEL-1* in *C. elegans* are viable and develop grossly functional nervous systems^{197,199}. Pharmacological challenge with an acetylcholinesterase inhibitor and patch clamp experiments revealed however that *eel-1* knockouts had reduced GABAergic signalling and axonal branching defects – phenotypes that could only be rescued by wild-type *EEL-1*, but not mutants with substitutions corresponding to mutations associated with ID¹⁹⁹. These phenotypes were subsequently found to involve *EEL-1* binding to OGT1 to promote GABA transmission, with human HUWE1 also found to bind OGT1¹⁹⁷. Likewise, constitutive overexpression of human HUWE1 in *Drosophila* neurons to levels similar to those in human patients with microduplications results in axonal branching defects via excess WNT signalling – with the caveat that no behavioural defect was found and overexpression was driven only in adult-stage²⁰⁰. The possibility therefore is open that human *HUWE1* is involved primarily in neuronal maturation rather than neurogenesis.

1.4 Project Aims

With the refinement of several key protocols (hPSC culture, CRISPR-Cas9 genome editing, and *in vitro* neural differentiation) in the years since HUWE1's link to neurogenesis was first uncovered, we are now in a better position to understand HUWE1's role in neurogenesis. Furthermore, this can now be done directly in a human context, which could inform on its associated pathologies.

Given that Huwe1-knockout mice regularly show a block in differentiation in favour of proliferation, I started with the hypothesis that the same might be true in human neurogenesis. However, because Huwe1 was also shown to degrade Ascl1 (at least in the adult mouse context), I hypothesised that this might also occur in human embryonic neurogenesis, though this link has never been shown yet. In such a case, I would expect premature differentiation of HUWE1-mutants instead, given ASCL1's prominent proneural activity. This project thus aims to clarify *HUWE1*'s role, if any, in promoting or inhibiting human neurogenesis – specifically in the cortex, and with ASCL1 as a possible candidate.

To test these hypotheses, I decided to generate and differentiate HUWE1-knockout hPSCs. A complete loss-of-function approach was chosen (rather than overexpression of HUWE1) partially because the characterised ID-associated missense mutations are primarily hypomorphic – the recurrent R2981H mutation impairs HUWE1's ubiquitin-binding and polyubiquitination activity, for example²⁰¹ – suggesting this approach would be sufficient to obtain a phenotype to study in human cells undergoing neural differentiation. It would also be more practicable, given HUWE1's large coding region.

- In Results Chapter 1, I shall characterise the expression profile of HUWE1 during a 2D model for human cortical differentiation. I then generate and validate a constitutive HUWE1 HECT domain mutant, before differentiating it, finding early-differentiation defects that agree with a patient-derived iPSC model reported during my project (Aprigliano et al. 2021¹⁶⁸).

- In Results Chapter 2, I generate and characterise a 4OHT-inducible HUWE1 knockout to study HUWE1's role in neural progenitors, finding mosaicism of the knockout occurring during differentiation. ASCL1 levels were shown to be regulated by HUWE1, but with no discernible impact on differentiation.
- In Results Chapter 3, I will describe single-cell RNA sequencing experiments that attempted to tease apart the mosaicism, but ultimately led instead to discoveries regarding ASCL1 function in our model.
- In Results Chapter 4, I will describe flow cytometry-based experiments that revealed phenotypes specific to HUWE1-knockouts within my mosaic differentiations that relate to ASCL1 stabilization.
- In Results Chapter 5, I describe attempts to address the issues of cell-autonomy that might arise from working on a mosaic system, and attempts to rescue the HUWE1-knockout phenotypes.

Materials & Methods

2.1 Cell Culture

2.1.1 Routine maintenance of hPSCs

Two lines were used to generate the lines studied in this thesis: KOLF2 (HPSI0114ikolf2, clone C1), generated and kindly gifted by the Wellcome Sanger Institute's Human Induced Pluripotent Stem Cell Initiative (HipSci) project (www.hipsci.org); and Human Embryonic Stem Cell Line, H1 (WAe001-A, WiCell Research Institute, USA). All lines were maintained feeder-free on Geltrex (Gibco #A1413201), with KOLF2-based lines maintained in TeSR-E8 (Stemcell Technologies #05990), and H1-based lines maintained in mTeSR1 (Stemcell Technologies #85850). Media was supplemented with 100U/ml Penicillin/Streptomycin (Gibco #15140), and media was changed daily. Cells were kept at 37°C, 5% CO₂, until approximately 80% confluent, after which they were passaged or frozen down.

For passaging, cells were washed once with DPBS (Gibco #14190144), then incubated for 7min in 0.5mM EDTA (Lonza #51201) diluted in DPBS. EDTA was then removed, and cells detached in culture media and replated, typically at a 1:6 ratio.

For freezing, cells were detached as above, in DMEM F-12 (Gibco #11320033), and pelleted at 300g, 3min. The pellet was then resuspended in 10% DMSO (Sigma-Aldrich D2650) + 90% Knockout Serum Replacement (KSR, Gibco #10828010) and transferred into cryogenic vials (ThermoFisher Scientific #377224). Vials were chilled to -80°C in cell-freezing containers and transferred into liquid nitrogen for long-term storage. For thawing, frozen cell suspensions were thawed quickly in a 37°C water bath and diluted into DMEM F-12. Cells were then pelleted at 300g, 3min, and the pellet resuspended and plated in culture media supplemented with 10µM ROCK inhibitor Y-27632 (Tocris #1254). Media was changed to remove ROCK inhibitor the next day.

2.1.2 Gene-editing of hPSCs

At least 30min prior to nucleofection, media was replaced with media supplemented with ROCK inhibitor Y-27632 (ROCKi). 200 μ M crRNA and 200 μ M tracrRNA (IDT #1072533) were mixed in IDT duplex buffer (IDT #11-05-01-12), in a 1:1:2.5 ratio, and hybridized by heating to 95°C for 2min before returning to room temperature. 5 μ l of this solution is then mixed with 20 μ g of Alt-R® S.p. HiFi Cas9 Nuclease V3 (IDT #1081060) and incubated at RT for at least 20min.

Cells were detached using accutase + ROCKi and washed twice with media + ROCKi. Cells were then passed through a 40 μ m cell strainer to achieve a single-cell suspension before counting. 10⁶ cells were then resuspended in 100 μ l P3 buffer (Lonza #V4XP-3024), mixed with the Cas9-gRNA complexes, and 5 μ l EP enhancer (IDT #1075916) and 5 μ l 100 μ M ssODN repair template added (2 μ g if using plasmids). Suspension was then transferred into a cuvette and nucleofected on an Amaxa 4D Nucleofector using program CA137 (Lonza #AAF-1002X). Cells were then transferred into a Geltrex-coated 6-well, in media + ROCKi. Media was changed the next day to remove ROCKi. All centrifugation steps were carried out at 300g for 3min. Protocol was adapted from Skarnes et al. 2019²⁰².

Nucleofected cells were then expanded, and frozen stocks of the population made. Cells were then plated at low density, at 500 cells per 10cm dish, to obtain colonies of single clones. Recovery was done in CloneR (Stemcell Technologies #05888)-supplemented media, following manufacturer's instructions. Clones were then manually picked and transferred into 96-well plates for subsequent expansion, genotyping, and freezing.

Custom Alt-R® crRNAs were designed and ordered using IDT's Alt-R Custom Cas9 crRNA Design Tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM) to target the introns of *HUWE1*. To generate constitutive *HUWE1* mutants, KOLF2 iPSCs were nucleofected with crRNAs against introns 80 and 82 to delete the intervening region containing exons 81 and 82. To flox exon 81, ssODNs with a single loxP site flanked by 40bp homology arms were used as repair templates, and two sequential rounds of editing and clone-derivation (See 2.1.2.2

Screening for gene-edits), in a H1 hESC background, performed to introduce the loxP sites individually in *cis* – henceforth termed HUWE1E81-fl hESCs. Sequences given in Table 1.

2.1.2.1 Knock-in of CreERT2 into AAVS1 locus

2 x 10⁶ H1, and HUWE1E81-fl hESCs were nucleofected and plated onto a 10cm dish each as described in 2.1.2, with crRNA targeting the AAVS1 locus and pAAVS1-Nst-MCS²⁰³ with a CAG-driven CreERT2 cloned in (gift from W. Skarnes) as a repair template. 48hrs after nucleofection, 175µg/ml G418 was added into culture media, and selection proceeded for 8 days. Surviving colonies were then picked and transferred into a 96-well plate for subsequent expansion, genotyping, and freezing. These lines are henceforth referred to as H1Cre and HUWE1cKO (*HUWE1* conditional knockout) respectively.

Name	Sequence	Comments
<i>HUWE1</i> Intron 80 Alt-R crRNA	5'-GTAACGGAATGTAATCTCTT-3'	Used simultaneously with Intron 82 crRNA to generate constitutive knockouts. Used with <i>HUWE1</i> Intron 80 + loxP ssODN to insert a loxP into Intron 80.
<i>HUWE1</i> Intron 81 Alt-R crRNA	5'-ATACTTAACTTTGGGCACGG-3'	Used with <i>HUWE1</i> Intron 81 + loxP ssODN to insert a loxP into Intron 81.
<i>HUWE1</i> Intron 82 Alt-R crRNA	5'-ACTTCCTCTAAGAGTACTAC-3'	
<i>HUWE1</i> Intron 80 + loxP ssODN	5'- GGCAGTTAAGGTTACTCTTCTCCTAAATTCTTTTCCT AAGATAACTTCGTATAATGTATGCTATACGAAGTTAT AGATTACATTCCGTTACATATGTTAGCCATTCCCTTT TCC-3'	Homology-Directed Repair template 1. loxP site highlighted in yellow.
<i>HUWE1</i> Intron 81 + loxP ssODN	5'- AAAGACAAAACACTTGATCAAAAATACTTAACTTTGG GCAATAACTTCGTATAATGTATGCTATACGAAGTTAT CGGTGGCTCACACCTATAATCCCAGCACTTTGGGAG GCCA-3'	Homology-Directed Repair template 2. loxP site highlighted in yellow.
AAVS1 Alt-R crRNA	5'-GGGGCCACTAGGGACAGGAT-3'	Sequence from Ocegüera-Yanez et al. 2016 ²⁰³ . Used to introduce CAG-CreERT2 into AAVS1 locus.

Table 1. Oligo sequences for gene-editing of hPSCs.

2.1.2.2 Screening for gene-edits

After isolating clones in 96-well plates, a genotyping screen for possibly successful edits was carried out as soon as practicable. During the next split, 1/3 of the cell suspension was mixed with an equal volume of DPBS + Proteinase K (Roche #3115801001) for a final concentration of 250µg/ml, then incubated at 56°C for 1hr, followed by 95°C for 10min to obtain a crude lysate. To screen clones for desired edits, PCR was performed using Thermoprime Plus Taq (ThermoFisher Scientific AB0301) on crude lysates followed by gel electrophoresis (2.2.1 PCR, genotyping, and gel electrophoresis). For PCRs with multiple bands, gel-extraction (Qiagen #28704) was carried out on the major band of the expected size prior to Sanger sequencing. Sanger sequencing was then carried out on promising clones to ensure the desired edits were made. PCR and Sanger sequencing for the top 4 off-target genomic loci for each crRNA, as predicted by Off-Spotter²⁰⁴, was then performed on these clones. Sanger-sequencing was done by the Crick's Genomics Equipment Park STP or outsourced to GeneWiz. Primer sequences are given in Table 2.

Name	Sequence	Annealing Temp.	Comments
<i>HUWE1</i> F1	5'-AGAACTAGCACTGCGTGACC-3'	58°C	~2431bp (2499bp in <i>HUWE1</i> cKO due to LoxPs) amplicon containing <i>HUWE1</i> exons 81-83. A ~1053bp amplicon results if exons 81-82 are deleted successfully. A 1886bp amplicon results if exon 81 is deleted by recombination of its flanking loxP sites.
<i>HUWE1</i> R1	5'-TCGCCCATAGGCTAAAGTGC-3'		
<i>HUWE1</i> Intron 80 loxP R1	5'-TAACGGAATGTAATCTATAACTTCG-3'	58°C	3' end sits on loxP, highlighted in yellow. When used with <i>HUWE1</i> F1, a 325bp amplicon is produced if loxP is present in <i>HUWE1</i> Intron 80.
<i>HUWE1</i> Intron 81 loxP F1	5'-CTTAACCTTTGGGCAATAACTTCG-3'	58°C	3' end sits on loxP, highlighted in yellow. When used with <i>HUWE1</i> R1, a 1625bp amplicon is produced if loxP is present in <i>HUWE1</i> Intron 81.

<i>HUWE1</i> F2	5'-CCCAAGGGTAGGTCTTTGTAGA-3'	N/A	For Sanger sequencing of Intron 80
<i>HUWE1</i> R2	5'-GCAATGCTCTCCAGAACCAC-3'		For Sanger sequencing of Intron 82
<i>HUWE1</i> int81seq	5'- AGTTCGTGACCTCAAACCCA-3'		For Sanger sequencing of Intron 81
<i>AAVS1</i> F1	5'-TCGACTTCCCCTCTTCCGATG-3'	60°C	2083bp amplicon in WT cells. No product if <i>AAVS1</i> locus is disrupted by insertion of pAAVS1-Nst-CAG-CreERT2.
<i>AAVS1</i> R1	5'-CACCCACAGTTGGAGGAGAATCCA-3'		Used with <i>AAVS1</i> F1. Forms a 1213bp amplicon only if pAAVS1-Nst-CAG-CreERT2 is integrated.
NeoR_R1	5'- GAGCCTAGGGCCGGGATTCTC-3'		Used with <i>AAVS1</i> R1. Forms a 2195bp amplicon only if pAAVS1-Nst-CAG-CreERT2 is integrated.
ERT2_F1	5'- TCTGCTGGAGACATGAGAGC-3'		
Intron 80 Off-target 1 F1	5'-GCGAATACAGAGCCCTTTGG-3'	56°C	570bp amplicon to check chr11:56294932-56294954
Intron 80 Off-target 1 R1	5'-GCTGTGTGAGGGAGAACAAG-3'		
Intron 80 Off-target 2 F1	5'-GCTCTGCTTGTCTCCCTAA-3'		344bp amplicon to check chr2:219873635-219873657 (<i>CCDC108</i>)
Intron 80 Off-target 2 R1	5'-AGTGGAAGGACGAGAAGGTG-3'		
Intron 80 Off-target 3 F1	5'-ACCTTCCTGTGTTGTGCCTA-3'		568bp amplicon to check chr5:163088007-163088029
Intron 80 Off-target 3 R1	5'-GGTTGCCTGGTGAATAGCTG-3'		
Intron 80 Off-target 4 F1	5'-ACAGGTCCAGGATTCAAGGG-3'		479bp amplicon to check chr14:21040464-21040486
Intron 80 Off-target 4 R1	5'-GCAGAGTGACCCAAACCATC-3'		
Intron 81 Off-target 1 F1	5'-TTTCAGGCCACCATAGAGCA-3'	56°C	608bp amplicon to check chr2:32340302-32340324 (<i>SPAST</i>)
Intron 81 Off-target 1 R1	5'-ACAGACTGGGTAAGCAAACT-3'		
Intron 81 Off-target 2 F1	5'-GTCCAACAGTCCCATCTCCA-3'		306bp amplicon to check chr1:12737112-12737134
Intron 81 Off-target 2 R1	5'-CCCAAGTAGCTGAGACCACA-3'		
Intron 81 Off-target 3 F1	5'-TATCCCCTTGCCAACCTCAG-3'		441bp amplicon to check chr12:101445123-101445145 (<i>ANO4</i>)
Intron 81 Off-target 3 R1	5'-ATGGGCAATGCTTAGGACTG-3'		

Intron 81 Off-target 4 F1	5'-ACGGCAAAGACCCATACAGA-3'	533bp amplicon to check chr2:69642818-69642840 (<i>NFU1</i>)
Intron 81 Off-target 4 R1	5'-AGGCATGAACTACCACACCT-3'	

Table 2. Primer sequences for clone-screening and CRISPR off-target verification. Off-target loci occurring within genes have their gene names specified in brackets.

2.1.3 Quality Control

Generated cell lines containing the desired edits were subjected to qRT-PCR (2.2.3 RNA extraction, cDNA synthesis and qRT-PCR) for *NANOG*, *SOX2*, and *OCT4* to verify pluripotency was not lost. qRT-PCR for *HUWE1* was similarly performed to verify edits did not affect its steady-state transcription. Karyotyping was also done through low-pass whole genome sequencing (performed by the Crick's Advanced Sequencing Facility STP) on purified genomic DNA (Qiagen #69504) to avoid inadvertent selection for clones with aneuploidy.

2.1.4 2D neuronal differentiation and 4OHT treatment

Cell lines were differentiated into adherent cortical neurons using a dual-SMAD inhibition protocol adapted from Shi et al. 2012¹¹⁴ by Clementina Cobolli-Gigli, Cristina Dias, and Oana Păun, summarised in Figure 5. hPSCs were dissociated in EDTA as described and replated in a 2:1 ratio on Geltrex, in 3x the usual volume of media, supplemented with ROCK inhibitor, at least 8 hours before neural induction (we typically split the day before). A ~100% confluent layer of attached cells is achieved, and the media was then replaced with N2B27 (a 1:1 mixture of N2 and B27, compositions listed in Table 3) supplemented with 10 μ M SB431542 (Abcam ab120163) and 100nM LDN193189 (Stemcell Technologies #72147) to begin neural induction (Day 0). Media was replaced daily, and cells were split 1:1 on Days 7 and 12. For splits, cells were washed once with DPBS then detached, using Accutase (Sigma-Aldrich A6964) or a 1:3 dilution of Accumax (Sigma-Aldrich A7089, used from Mar 2022 due to global accutase shortage) in DPBS, 7mins at 37°C. Cell suspensions were then washed twice in DMEM F-12, with centrifugations done at 132g for 2min, before replating on Geltrex in media supplemented with ROCK inhibitor. From Days 12 – 23, cells were cultured in N2B27 without inhibitors, and passaged 1:1-1.5 on Days 16 and 20, with centrifugations at 255g. Cultures should

aim to remain confluent until Day 16. On Day 23, cells were terminally plated onto Poly-O-Laminin (Poly-O-laminin: 1:100 10mg/ml poly-L-ornithine (Sigma #P3655) in water, 37°C overnight, then 1:100 laminin (Sigma #L2020) in DMEM/F-12, 37°C overnight the following day), in B27 supplemented with 10μM DAPT (Tocris #2634) and ROCK inhibitor, with centrifugations at 255g. Half the media was replaced on Days 26, 29 and 33, then weekly after, with DAPT removed from the media changes from Day 29.

To acutely delete exon 81 of *HUWE1* in the HUWE1cKO line, 4-hydroxytamoxifen (4OHT, Sigma-Aldrich H6278, dissolved in absolute ethanol, Fisher Chemical E/0650DF/17) was supplemented into the media at 100nM concentration on Days 12 and 16, during the splits, while cells are in suspension. Media supplemented with 4OHT was renewed daily until after the split on Day 16. Vehicle-treated controls were performed concurrently on half the cells during the Day 12 split.

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
N2 (DMEM + N2supp) : B27 (Neurobasal + B27 supp)																							B27 (Neurobasal + B27 supp)											
GTX							GTX					GTX				GTX				GTX				Poly-O-laminin										
100 nM LDN193189 + 10 μM SB431542												10 μM DAPT																						
MC +	MC +	MC +	MC +	MC +	MC +	MC +	MC +	MC +	MC +	MC +	MC +	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC +										
																											1/2 MC +							
																													1/2 MC					

Figure 5. Summary of neural differentiation protocol. Confluent hPSCs were differentiated using the protocol above for all experiments described. GTX: Geltrex; MC: Media change; MC+: Media change with inhibitors; Days 7, 12, 16, 20, 23: Passaging days. Media compositions listed in Table 3.

	Component	Catalogue Number	Volume
N2 (50ml)	DMEM F-12	Gibco #11320033	48.5ml
	N2 supplement (100X)	Gibco #17502001	0.5ml
	GlutaMAX (100X)	Gibco #35050061	0.5ml
	Penicillin/Streptomycin (100X)	Gibco #15140	0.5ml
B27 (50ml)	Neurobasal	Gibco #21103049	48ml
	B27 Supplement (50X)	Gibco #11530536	1ml
	GlutaMAX (100X)	Gibco #35050061	0.5ml
	Penicillin/Streptomycin (100X)	Gibco #15140	0.5ml

Table 3. N2 and B27 media composition.

2.1.5 Cre-dependent reporter generation

Both H1Cre and HUWE1cKO lines were transduced with a lentivirus encoding a Cre-dependent reporter (pEF1a-EGFP-P2A-FLEX-tagRFP). Briefly, the Cre-dependent reporter is an EF1a promoter-driven EGFP linked by the self-cleaving P2A peptide to a 'FLExed' tagRFP. The tagRFP is originally in an antisense orientation and flanked by lox2272 and loxP pairs such that in the presence of Cre activity, tagRFP is permanently restored into the correct reading frame for transcription and expressed. As such, transduced cells are EGFP⁺, but only additionally become tagRFP⁺ in response to Cre activity. Plasmid cloning, lentivirus generation and titration, were done by Molly Strom of the Viral Vector Core STP.

On the day of transduction, both lines were split in the morning to achieve small colonies. 7 hours later, the culture media (mTeSR1) was replaced to include 10 μ g/ml polybrene (Sigma-Aldrich TR1003-G) and 5 x 10⁵ ml⁻¹ lentiviral pEF1a-EGFP-P2A-FLEX-tagRFP. Media was replaced the next day to remove polybrene and unused virus, and replaced daily as per usual culture conditions (2.1.1 Routine maintenance of hPSCs).

After 2 passages, both lines were enriched for EGFP⁺, tagRFP⁻, cells by Fluorescence-Activated Cell-Sorting (FACS) with the aid of Emma Russell of the Flow STP, to derive mixed-population Cre-reporting lines. On the day of sorting, cells were washed twice with DPBS, then detached and dissociated into single cells using Accutase for 5mins at 37°C using a P1000 pipette. Cells were transferred into 3 volumes DMEM F12 and centrifuged at 300g for 3mins, then resuspended in 3% w/v Bovine Serum Albumin (Sigma-Aldrich A9647) in DPBS and strained into FACS tubes (Corning #3522345) before sorting. Cells were then sorted into 10% CloneR2 (Stemcell Technologies #100-0691) in mTeSR1, and centrifuged, before replating in 10% CloneR2 in mTeSR1.

2.1.6 Lentiviral transduction

On Day 13 of differentiation, one 6-well each of cells was transduced with lentivirus carrying pLKO.1 zsGreen (modified from Addgene plasmid #8543) with short hairpin

RNA (shRNA) targeting Renilla luciferase or ASCL1 (Table 4). To do so, media was replaced with N2B27 supplemented with 10 μ g/ml polybrene (Sigma-Aldrich TR1003-G), 100nM 4OHT, and 109,375 TU/cm² of concentrated virus (PEG-precipitated then resuspended in DPBS). This was repeated 3 hours later for a second round of infection. Media was then replaced with N2B27 + 4OHT after another 3 hours to remove polybrene and virus. Differentiation was then carried out as per usual. Cells were harvested and fixed for flow cytometry on Day 20.

Cloning of shRNA plasmids, lentiviral production and titration were done by the Viral Vector Core STP. Briefly, zsGreen expression is driven by a PGK promoter, with shRNA expression driven by a U6 promoter.

Name	Sequence		Comments
shRenilla	Sense	5'- CCGGATGTTTACTACACTCGGATATCTCGAGATATCCGAG TGTAGTAAACATTTTTTG-3'	Control non- targeting shRNA
	Antisense	5'- AATTCAAAAATGTTTACTACACTCGGATATCTCGAGATAT CCGAGTGTAGTAAACAT-3'	
shASCL1-1	Sense	5'- CCGGCAACTACTCCAACGACTTGAACGAGTTCAAGTCG TTGGAGTAGTTGTTTTTG-3'	Sequence obtained from Wang et al. 2019 ²⁰⁵ , also TRCN0000013550
	Antisense	5'- AATTCAAAAACAACACTACTCCAACGACTTGAACGAGTTC AAGTCGTTGGAGTAGTTG-3'	
shASCL1-2	Sense	5'- CCGGCCAACTACTCCAACGACTTGACTCGAGTCAAGTCG TTGGAGTAGTTGGTTTTTG-3'	Sequence obtained from Wang et al. 2019 ²⁰⁵ , also TRCN0000235657
	Antisense	5'- AATTCAAAAACCAACTACTCCAACGACTTGACTCGAGTCA AGTCGTTGGAGTAGTTGG-3'	

Table 4. shRNA sequences used in pLKO.1 zsGreen.

2.1.7 Nucleofection during differentiation

On the morning of Day 16, and at least 2 hours before nucleofection, cells were refed with 10 μ M ROCKi-supplemented N2B27 without 4OHT or ethanol. One Geltrex-coated 6-well containing 4ml of N2B27 + ROCKi for every two 6-wells of cells was prepared and pre-warmed to 37°C.

Cells were washed once with DPBS, then detached and dissociated into single cells with Accumax as per usual Day 16 splits. Cells will be more adherent due to prior exposure to ROCKi. After two washes with DMEM F12, cells were resuspended in 100µl of resuspension buffer + supplement (Lonza VPG1004) per 6-well. 6µg of endotoxin-free (Sigma-Aldrich NA0400) pLKO.1 zsGreen shRenilla/shASCL1 plasmid DNA (above) was then added. Cell suspensions were then transferred into electroporation cuvettes (Lonza VPG1004) and nucleofected using program A-033 on a Nucleofector 2b (Lonza AAB-1001). Two cuvettes of electroporated cells were then immediately combined into one pre-warmed well and incubated as usual.

This protocol was first established in our lab by Oana Păun.

2.2 Molecular Biology

2.2.1 PCR, genotyping, and gel electrophoresis

After establishing HUWE1cKO, genotyping was performed using the *HUWE1* F1 and *HUWE1* R1 primer pair (Table 2) for every 4OHT-treatment experiment, to qualitatively assess response to 4OHT, and initially to also assess 4OHT-induced recombination efficiency quantitatively (until this was subsequently proven to be unreliable). Purified genomic DNA (DNeasy Blood and Tissue Kit, Qiagen #69504) or crude lysate containing genomic DNA (2.1.2.2 Screening for gene-edits) was amplified for *HUWE1* exons 81-83 with Thermoprime Plus Taq (ThermoFisher Scientific AB0301). PCR products were then mixed with loading dye (BlueJuice Gel Loading Buffer, Invitrogen #10816015) and loaded onto 1% agarose (Invitrogen #16500-500) gels supplemented with StainIN RED nucleic acid stain (highQU NAS0101) and run. Gels were then imaged digitally on a BioDoc-It Transilluminator (UVP) or a ChemiDoc MP (Bio-Rad, from 2022 onwards). Quantification of bands for recombination efficiency calculation was performed in FIJI using the Gel Analyzer tool.

2.2.2 Western Blotting

Cell pellets were lysed in Pierce IP lysis buffer (Thermo Scientific #87787) with Halt Protease Inhibitor Cocktail w EDTA (Thermo Scientific #87786), and Phosphatase Inhibitor Cocktail (Thermo Scientific #78420), on ice for 40mins. Lysates were centrifuged at 12000g, for 10mins at 4°C, and supernatants quantified by BCA assay (Thermo Scientific #23227). 12 - 20µg protein per sample was subjected to SDS-PAGE electrophoresis on pre-cast gels (Bio-Rad #4561085, #4561025) and transferred onto a 0.2µm PVDF membrane (Bio-Rad #1704156) using a Trans-Blot Turbo (Bio-Rad #1704150), at 1.3A, 25V, for 15mins for blots that include HUWE1, and 1.3A, 25V, for 7mins for all others. When blotting for HUWE1, HiMark high molecular weight protein ladder (Invitrogen LC5699) was used initially – though it became unnecessary after switching HUWE1 antibodies from Bethyl A300-486A to either Invitrogen PA5-20374, CST#5695, or HPA002548 as these had less unspecific staining (Table 5). Consequently, all blots with these antibodies were run with PageRuler Plus prestained protein ladder (ThermoScientific #26619).

Membranes were blocked in 5% milk (Marvel) in Tris-Buffered saline (Bio-Rad, #1706435) + 0.1%Tween-20 (Sigma-Aldrich P1379) (TBST) for 60 minutes. After blocking, membranes were incubated in primary antibodies (Table 5) diluted in 5% milk in TBST overnight at 4°C with rocking. The next day, membranes were washed in TBST, then incubated in secondary antibodies (Table 6) diluted in 5% milk in TBST at room temperature for 60 minutes. Membranes were then washed again in TBST, then signal was generated using enhanced chemiluminescence (ECL) substrate (Cytiva, RPN2236), or SuperSignal West Dura (ThermoFisher Scientific #34075) for less abundant proteins, and visualized on an Amersham Imager 600 (Cytiva #29083461). Images were then quantified in FIJI using the Gel Analyzer tool.

Target	Catalogue Number	Application	Host species	Dilution	Comments
α-tubulin (TUBA4A)	Sigma T9026	WB	Mouse	1:5000	
Actin (ACTB)	Sigma A2066	WB	Rabbit	1:1000	
ASCL1	Abcam #211327	F/WB	Rabbit	1:500	
CHCHD2	PTG 19424-1-AP	WB	Mouse	1:1000	
Cleaved- CASP3	CST #9664	F	Rabbit	1:500	

Cre	Synaptic Systems #257004	IF	Guinea Pig	1:500	
DCX	Santa Cruz #8066	F	Goat	1:250	
GAPDH	CST #5174	WB	Rabbit	1:2000	
HuC/D (ELAVL3/4)	Invitrogen A21271	IF	Mouse	1:100	
HUWE1	Bethyl A300-486A	WB	Rabbit	1:1000	
HUWE1	Invitrogen PA5-20374	WB	Rabbit	1:1000	Targets C-terminus. ONLY use for WB
HUWE1	Atlas HPA002548	WB/IF	Rabbit	1:200/1:1000/1:200	Resolution in flow cytometry weaker than CST #5695
HUWE1	CST #5695	F/WB/IF	Mouse	1:200/1:1000/1:500	
Lamin B1 (LMNB1)	Abcam #16048	WB	Rabbit	1:5000	
OLIG2	R&D Systems AF2418	IF	Goat	1:800	
PAX6	Biolegend #901301	IF	Rabbit	1:500	
p53	CST #18032	WB	Mouse	1:1000	
p53	Novus #200-171	F	Rabbit	1:1000	
SOX2	eBioscience 14-9811-82	F	Rat	1:500	
TUJ1-AF647 (TUBB3)	BD #560394	F	Mouse	1:200	

Table 5. All primary antibodies used, and applications tested. F: Flow cytometry; WB: Western Blot; IF: Immunofluorescence

Target	Catalogue Number	Host species	Dilution
Anti-Goat-Dylight 350	Invitrogen SA5-10085	Donkey	1:1000
Anti-Rabbit-AF350	Invitrogen A10039	Donkey	1:1000
Anti-Rat-405	Abcam ab175670	Donkey	1:1000
Anti-Mouse-AF488	Invitrogen A21202	Donkey	1:1000
Anti-Rabbit-AF488	Invitrogen A21206	Donkey	1:1000
Anti-Rat-AF555	Invitrogen A21434	Goat	1:1000
Anti-Rat-Cy3	Jackson #712-166-153	Donkey	1:1000
Anti-Rabbit-Cy3	Jackson #711-166-152	Donkey	1:1000
Anti-Rabbit-AF568	Invitrogen A10042	Donkey	1:1000
Anti-Mouse-AF647	Invitrogen A31571	Donkey	1:1000
Anti-Goat-AF647	Jackson #705-606-147	Donkey	1:1000
Anti-Guinea Pig-AF647	Jackson #706-606-148	Donkey	1:1000
Anti-Mouse Immunoglobulins-HRP	Dako P0161	Rabbit	1:1000
Anti-Rabbit Immunoglobulins HRP	Dako P0448	Goat	1:1000

Table 6. All secondary antibodies used.

2.2.3 RNA extraction, cDNA synthesis and qRT-PCR

RNA was collected directly from adherent cells after one wash with DPBS by lysing in RLT buffer (Qiagen #74106). Lysates were then stored in -80°C for at least 24hrs before RNA extraction. RNA was then extracted using the RNeasy Mini Kit (Qiagen #74106) according to manufacturer's instructions, with a 15min on-column DNase I digestion (Qiagen #79254). The extracted RNA was then quantified on a NanoDrop Spectrophotometer (ThermoFisher Scientific ND-1000), and 1-2 µg of RNA reverse-transcribed using a Maxima First Strand cDNA Synthesis kit (ThermoFisher Scientific K1641).

For qRT-PCR, transcripts were quantified from 10ng of cDNA in individual wells on a 384-well plate, in 10µl reactions, using Taqman Universal qRT-PCR Master Mix (Applied Biosystems #4304437) and commercially-designed primer probes (Table 7). To measure Cre and HUWE1 exon 81 transcript levels, primers were designed (Table 8) and validated by PCR + gel electrophoresis to check for single bands of intended amplicon sizes; and by qRT-PCR on serially-diluted cDNA to check for primer efficiencies of approximately 1 less cycle per doubling of template concentration. Transcripts were then quantified as above, but using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems A25742) instead.

Triplicates were set up and plates were run on a Quantstudio 12K Flex Real-Time PCR System (Applied Biosystems) and data analysed using the 2^{(-Delta Delta C(T))} method²⁰⁶, normalizing the genes' expressions to the combined average of 2 housekeeping genes (UBC and GAPDH; only GAPDH in the cases of Cre and HUWE1 exon 81) before normalization to a reference sample or timepoint.

Target	Catalogue Number	Assay ID
<i>GAPDH</i>	Applied Biosystems #4331182	Hs02758991_g1
<i>UBC</i>	Applied Biosystems #4331182	Hs00824723_m1
<i>SOX2</i>	Applied Biosystems #4331182	Hs01053049_s1
<i>NANOG</i>	Applied Biosystems #4331182	Hs04399610_g1
<i>OCT4</i>	Applied Biosystems #4331182	Hs00999632_g1
<i>p21</i>	Applied Biosystems #4331182	Hs00355782_m1
<i>HUWE1</i> (exons 67-68)	Applied Biosystems #4331182	Hs00948075_m1
<i>ASCL1</i>	Applied Biosystems #4331182	Hs00269932_m1

<i>ACTL6B</i>	Applied Biosystems #4331182	Hs00211827_m1
<i>BCL11B</i>	Applied Biosystems #4331182	Hs01102259_m1
<i>ELAVL4</i>	Applied Biosystems #4331182	Hs00956610_mH
<i>TUBB3</i>	Applied Biosystems #4331182	Hs00801390_s1
<i>HES5</i>	Applied Biosystems #4331182	Hs01387463_g1
<i>PAX6</i>	Applied Biosystems #4331182	Hs01088114_m1
<i>MKI67</i>	Applied Biosystems #4331182	Hs00606991_m1
<i>DLL3</i>	Applied Biosystems #4331182	Hs01085096_m1
<i>RGS16</i>	Applied Biosystems #4331182	Hs00892674_m1
<i>IGFBP5</i>	Applied Biosystems #4331182	Hs00181213_m1
<i>DLL3</i>	Applied Biosystems #4331182	Hs01085096_m1

Table 7. Taqman probes used in this study

Target	Sequence	Comments
<i>GAPDH</i>	5'-TGCACCACTGCTTAGC-3'	87bp amplicon. Sequences from Cicinnati et al. 2008 ²⁰⁷
	5'-GGCATGGACTGTGGTCATGAG-3'	
<i>HUWE1</i> exon 81	5'-AGTTCGTGACCTCAAACCCA-3'	84bp amplicon
	5'-ATCTGGCATACCAGGTGTACATA-3'	
<i>Cre</i>	5'-CTGACGGTGGGAGAATGTTAAT-3'	109bp amplicon
	5'-CATCGCTCGACCAGTTTAGTT-3'	

Table 8. Primers for SYBRGreen-based qRT-PCR

2.2.4 Single-Cell RNA Sequencing and analysis

Cells were differentiated as per normal (2.1.4 2D neuronal differentiation) until the timepoints collected. For Day 20 samples, this was done in two batches: H1Cre + 4OHT, and HUWE1cKO + 4OHT (batch 1); H1Cre + Veh, H1Cre + 4OHT, HUWE1cKO + Veh, HUWE1cKO + 4OHT (batch 2). Informed by the batch effects in the day 20 analysis, for Day 24 samples three KOLF2 passages were differentiated concurrently with three ASCL1KO lines and harvested on the same day to minimize batch effects.

On the day of harvest, cells were washed twice with DPBS then detached using Accutase at 37°C for 6mins, and manually dissociated by pipetting with a P1000. 2 volumes of DMEM F-12 were added and cells passed through a 40µm cell strainer to achieve a single-celled suspension before counting on a NucleoCounter NC-200. 1.5×10^6 cells were then centrifuged at $255 \times g$ for 2mins. The pellet was resuspended in 750µl of 0.22µm-filtered 1% BSA in DPBS for a theoretical concentration of 2000 cells/ml. This suspension was then counted again in the Advanced Sequencing Facility (ASF) STP (typically resulting in ~1000 cells/µl).

Library preparation and sequencing was then performed by the ASF. Briefly, 10,000 cells were then loaded onto the Chromium X (10x Genomics) to generate single-celled Gel Beads-in-Emulsion (GEMs) for library preparation. Dual-index Chromium Single Cell 3' v3.1 Chemistry was used according to manufacturer's instructions, using 11 cycles for the cDNA amplification and 14 cycles for the library PCR. Libraries were then sequenced on a NovaSeq 6000 (Illumina). Single-cell collection and sequencing was performed on the following samples:

Sample ID	Cell line	Genotype	Differentiation day	Treatment
TAN2457A1	H1 hESCs	HUWE1cKO	20	4OHT
TAN2457A2	H1 hESCs	H1Cre	20	4OHT
TAN2457A5	H1 hESCs	H1Cre	20	Veh
TAN2457A6	H1 hESCs	H1Cre	20	4OHT
TAN2457A7	H1 hESCs	HUWE1cKO	20	Veh
TAN2457A8	H1 hESCs	HUWE1cKO	20	4OHT
TAN2457A19	KOLF2 iPSCs	WT	24	No DAPT
TAN2457A20	KOLF2 iPSCs	WT	24	No DAPT
TAN2457A21	KOLF2 iPSCs	WT	24	24hr DAPT
TAN2457A22	KOLF2 iPSCs	WT	24	24hr DAPT
TAN2457A23	KOLF2 iPSCs	ASCL1KO	24	24hr DAPT
TAN2457A24	KOLF2 iPSCs	ASCL1KO	24	24hr DAPT
TAN2457A25	KOLF2 iPSCs	WT	24	No DAPT
TAN2457A26	KOLF2 iPSCs	WT	24	24hr DAPT
TAN2457A27	KOLF2 iPSCs	ASCL1KO	24	24hr DAPT

ASCL1KO cells were generated by Siew-Lan Ang (Guillemot lab), and single-cell collection and sequencing of all day 24 cells was done together with Oana Păun (Guillemot lab), using the method I optimised above.

Bioinformatic analysis was done with the aid of Stephanie Strohbuecker and Avinash Ghanate of the Bioinformatics and Biostatistics (BABS) STP (Custom genome construction; Pre-processing; Integration of datasets; label transfer; and RNA velocity). I performed all visualization and other analyses on Seurat²⁰⁸ objects provided by the BABS team, in R-4.1.3²⁰⁹.

2.2.4.1 Pre-processing

Spliced and unspliced reads of whitelisted cell barcodes identified from Cell Ranger (10x Genomics) were quantified using alevin²¹⁰ and mapped to the human reference

genome GRCh37 release 75, or to a modified version when attempting to identify definite HUWE1-knock outs. Downstream analyses were carried out using the Seurat v4.1.1 package²⁰⁸. Sample-specific thresholds were established using median absolute deviation (MAD) measures for cells expressing > 3 MAD of mitochondrial gene expression along with < 3 MAD of total number of detected features/genes. Potential doublets were identified and removed using scDbfFinder²¹¹ v1.4.0. Day 24 samples were then normalized and integrated across three replicates using SCTransform²¹² with glmGamPoi²¹³ method. Day 20 samples (HUWE1cKO and H1Cre +/- 4OHT) however, were directly integrated together (6 samples total: 3 samples per genotype, 2x +4OHT, 1x Veh, see above) across conditions, skipping this step due to the lack of sufficient replicates.

2.2.4.2 Integration across conditions

After filtering of cells to discard potential doublets and poor-quality cells, Day 24 samples were then integrated across conditions using Seurat: (a) WT cells with and without DAPT treatment and b) WT cells and *ASCL1* KO cells with DAPT treatment.

For both Day 20 and 24 samples, after data normalization and variable feature detection in the individual samples using SCTransform, anchors were identified using the 'FindIntegrationAnchors()' function and datasets were integrated with 'IntegrateData()' across 30 dimensions for 3000 integration features identified from the datasets. We then performed PCA on the integrated data and the first 20 principal components were used to create a Shared Nearest Neighbor (SNN) graph using the 'FindNeighbors()' function. This was used to find clusters of cells showing similar gene expressions using the 'FindClusters()' function across a range of clustering resolutions (0.2-1.4 in 0.2 increments). For data visualization, dimensional reduction of the integrated data was achieved using Uniform Manifold Approximation and Projection (UMAP²¹⁴) with 20 principal components and with cosine correlation metric. The raw RNA counts of the integrated data were then normalized with the "LogNormalize()" method for downstream analyses. Cell cycle phases were then inferred using the "CellCycleScoring()" function.

Cell cycle phases together with canonical marker genes were then used to visually aid cell type assignment to clusters in the Day 20 data at the lowest resolution (0.2) that best fit the markers. Once the types of cells present in our model were determined, the same sets of genes found to broadly define cell types in the Day 20 data were applied to the Day 24 data as gene modules using the “AddModuleScore()” function to aid cell type assignment there. Markers used were obtained from PanglaoDB, for radial glia, neuroblasts, and neurons²¹⁵.

2.2.4.3 Label Transfer

Using the Day 24 WT + DAPT integrated data as a reference, cell identities for the ASCL1 KO dataset were identified with the help of transfer anchors (FindTransferAnchors() + MapQuery()) obtained using the first 20 principal components of SCTransform²¹² normalized features.

2.2.4.4 RNA Velocity

Spliced and unspliced read counts from the integrated Seurat objects where RNA velocities are displayed was used as input into scVelo²¹⁶ to calculate RNA velocity values for each gene of each cell. scVelo was used in the “dynamical” mode with default settings. The resulting RNA velocity vector was embedded into the PCA and UMAP space by translating the RNA velocities into likely cell transitions using cosine correlation to compute the probabilities of one cell transitioning into another cell. We identified driver genes, i.e., those genes that show dynamic behavior, as those genes with a fit likelihood in the dynamical model > 0.3.

2.3 Microscopy

2.3.1 Live imaging in culture

During culture maintenance or differentiations, whenever there was a need to take images of the live cells without terminating the culture, images of the cells would be taken using a Leica DMI1 inverted phase contrast microscope. When fluorescence was involved, both brightfield and fluorescence images were taken using an EVOS FL widefield microscope.

2.3.2 Immunofluorescence microscopy

During differentiation, cells were seeded at Day 16, during the scheduled split, onto 8-well chamber slides (ThermoFisher Scientific #177402) or black 96-well plates (greiner Bio-One #655090) at the same seeding ratio as usual (2.1.4 2D neuronal differentiation and 4OHT treatment), based on the relative surface areas of the original and the target vessels. On the day of analysis, cells were washed once with DPBS, then fixed in 4% paraformaldehyde in DPBS (Alfa-Aesar J61899) for 10mins at room temperature. The fixed cells were then washed once with DPBS, and stored at 4°C in DPBS until staining.

For staining, cells were first permeabilized and blocked in 3% Donkey serum (Jackson #017-000-121) + 0.2% Triton X-100 (Sigma-Aldrich T8787) in DPBS for 30mins at room temperature. Cells were then probed overnight at 4°C with primary antibodies diluted in 3% Donkey serum + 0.2% Triton X-100 in DPBS (Table 5. All primary antibodies used, and applications tested.). The next day, the cells were washed with DPBS 3 times over 30mins, then probed with fluorescence-conjugated secondary antibodies (Table 6. All secondary antibodies used.) + 1µg/ml DAPI (Sigma-Aldrich D9542) for 1hr at room temperature, in the dark. Cells were then washed with DPBS 3 times over 30mins, and either imaged directly (96-well plates), or mounted with a coverslip and mounting media (Polysciences #18606) before imaging the next day. Images were acquired in 1µm stacks using either a Leica SP5 II confocal microscope or an Olympus IX83 inverted widefield fluorescence microscope.

2.3.3 Image analysis and quantification

Analysis of images was performed in FIJI²¹⁷. Maximum-intensity projections were obtained and nuclei segmented by Stardist²¹⁸ to obtain nuclear regions of interest (ROIs). The average intensity of each fluorophore within individual nuclear ROIs was then measured. When reporting the proportion of positively-stained cells, a batch-specific threshold was applied.

2.4 Flow cytometry

On the day of harvest, cells were washed in PBS and dissociated with Accutase (Sigma A6964) using a P1000 pipette. Once detached, cells were collected into 15 ml tubes with 4 volumes of DMEM-F12 (Gibco 11320033) and pelleted at 300g, 3min. Cells were resuspended in PBS, pelleted, then resuspended in Live/Dead™ Fixable Near-IR Dead Cell Stain (Invitrogen L34976) as per manufacturer's instructions, and left to incubate for 30mins, protected from light. Cells were subsequently pelleted, and washed once in PBS, before fixation in 4% PFA/DPBS. Following 10mins incubation, cells were washed with PBS by dilution, then pelleted and resuspended in DPBS before storage at 4 °C for future analysis. When applicable, cells were incubated in media supplemented with 2.5µg/ml 5-Ethynyl-2'-deoxyuridine (EdU, Santa Cruz sc-284628) for 2hrs prior to harvest. As a negative control, cells not incubated in EdU were used, to control for the staining process.

On the day of analysis, cells were transferred for staining into V-bottomed 96-well plates (Corning #3894). Samples were pelleted (1000g, 4mins, 4 °C) and resuspended in 100µl primary antibodies (Table 5. All primary antibodies used, and applications tested.) diluted in DPBS + 0.2% Triton X-100 + 3% Donkey serum, and incubated overnight at 4 °C. The next day, samples were washed by dilution with 100µl DPBS, pelleted and resuspended in 100µl DPBS to complete the wash (1 wash step). After washing, cells were pelleted and resuspended and incubated in 100µl secondary antibodies (Table 6. All secondary antibodies used.) + 1µg/ml DAPI for 1hr at room temperature, in the dark. Typically, Alexa-Fluor 350 or DAPI; Alexa-Fluor 488; Cy3; and Alexa-Fluor 647 were used, making spill over of spectra negligible.

If EdU incorporation was to be analysed, cells were then washed once with DPBS, before pelleting and resuspending in Click-iT Plus reaction mix (Invitrogen C10640) made to manufacturer's instructions with a 1:1 mixture of copper protectant and copper sulfate. As a negative control, a well of cells not incubated in EdU was also processed, to account for the increased AF647 background from the procedure.

One additional wash was then performed before transferring into cell strainer-capped tubes (Falcon #352235) for acquisition on a Fortessa flow cytometer (BD

Biosciences) using FACSDiva software. Analysis was subsequently performed in FlowJo.

Results 1: Constitutive *HUWE1* mutant iPSCs fail to survive neural differentiation

To study *HUWE1*'s functions during human neurogenesis, access to readily available and easily manipulable human material was required. This precluded the use of scarce foetal tissue as a starting point for exploration, particularly since little was known about human *HUWE1*. Fortunately, the pioneering work of our predecessors – in establishing human embryonic stem cells (hESCs)¹⁰⁷, generating human induced-pluripotent stem cells (hiPSCs)¹⁰⁸, isolating foetal neural progenitor cells (hNPCs)^{109,110}, and differentiating these cell-types to model human cortical development *in vitro*^{111–117} – present an opportunity to bypass this limitation and provide sufficient, tractable, material to study *HUWE1* *in vitro*. As hESCs and hiPSCs are easier to obtain and maintain compared to hNPCs, thanks to their long-term self-renewal in culture, and do not lose neurogenic potential over passages¹¹⁸, I used both hESCs and hiPSCs (collectively human pluripotent stem cells, hPSCs) throughout my project. This was particularly important due to the need to generate stable gene-edited cell lines to study *HUWE1*.

Several protocols to model human cortical neurogenesis from hPSCs exist in both 2D and 3D formats (reviewed in Kelava and Lancaster 2016¹³⁰). Within 2D models, variants of the dual-SMAD inhibition protocol are further available, involving additional small molecules¹²⁴. In this project, a variant of the dual-SMAD inhibition protocol is used, established by prior members of the Guillemot and Srivastava labs, and adapted from Shi et al. 2012¹¹⁴. Briefly, it involves 12 days of dual-SMAD inhibition followed by Notch inhibition on day 23 using DAPT.

To study gene function, a typical approach involves perturbing the gene's expression in relevant cell-types or contexts. Similarly, I make use of CRISPR-Cas9 gene-editing¹⁴⁶, combined with hPSCs, and 2D neural differentiation extensively in this project to study *HUWE1*'s functions in human neurogenesis.

In this chapter, I first explore *HUWE1*'s expression profile in our differentiation model. After confirming its presence, I use CRISPR-Cas9 to disrupt *HUWE1*'s catalytic domain in a hiPSC line. I then model cortical neurogenesis in these mutants to infer the function of *HUWE1* in the process.

3.1 *HUWE1* is expressed throughout *in vitro* cortical neurogenesis

In order to study *HUWE1*, it first needs to be expressed in our model. Given the dearth of information regarding *HUWE1* in human neurogenesis contexts, there was no guarantee it would be. I therefore started by investigating its expression profile in our system, with the added benefit of potentially identifying relevant timepoints or cell-types to focus on subsequently.

For this, I differentiated wild-type (WT) KOLF2 hiPSCs and H1 hESCs using the protocol established in our labs (Figure 6A). These hPSCs were plated at confluent densities prior to neural induction to avoid a neural crest fate²¹⁹, and dual-SMAD inhibition performed using the small molecules LDN193189 and SB431542. Notch inhibition was performed using the small molecule DAPT at Day 23 to synchronize cell-cycle exit and promote a more homogeneous neural differentiation²²⁰.

Using this protocol, I collected mRNA at multiple timepoints (Days 13, 18, 23, 29, 33, 37) to track the change in *HUWE1* expression from predominantly neural progenitor cultures into predominantly neuronal cultures. Timepoints prior to day 13 were not checked as bulk-RNAseq data from 3 hiPSC lines performed by Lucia Dutan-Polit (Srivastava lab) indicated that *HUWE1* was expressed and its expression stable throughout days 0 – 8 (data not shown).

Similar to observations in the developing mouse cortex¹⁸² and cerebellum¹⁸³, *HUWE1* increases slightly in expression over differentiation time (i.e. as cultures shift from progenitors to neurons) (Figure 6B). In support of this, the increase in *HUWE1* expression is accompanied by a decrease in the expression of the progenitor markers *SOX2* and *PAX6*, and the increase in the early neuronal marker *TUBB3* (also known as *TUJ1*) (Figure 6B). This was subsequently supported in later chapters

by single-cell RNAseq and flow cytometry. I should however note that this is in direct opposition to the observations of Bosshard et al. 2017¹⁷⁰ where the authors showed by immunofluorescence that HUWE1 expression is specific only to the progenitor zones of cerebral organoids, away from TUBB3-expressing neurons where it is absent. This discrepancy is probably due to their reliance on a single antibody (Bethyl A300-486A) which I quickly disfavoured due to its unspecific staining in Western Blots (Figure 8), opting instead for a c-terminus-targeting antibody (Invitrogen PA5-20374) for Western Blots (Figure 9B), or subsequently HPA002548 and CST#5695 for Western Blots, flow cytometry, and immunofluorescence. Furthermore, Human Protein Atlas (proteinatlas.org) data shows by both immunocytochemistry¹⁷² (using the antibody HPA002548) and single-cell RNAseq²²¹ that HUWE1 is expressed even in adult neurons, in line with my observations.

Another transcript of interest was *ASCL1*, which the Guillemot lab¹⁸⁵ and others¹⁹¹ previously showed in mouse neural progenitors is ubiquitinated for degradation by HUWE1. *ASCL1* encodes a proneural transcription factor and is considered a master regulator of neurogenesis²²², both necessary and sufficient for neuronal differentiation. Its overexpression drives neuronal differentiation in hNPCs¹³⁶, hESCs and even human fibroblasts¹³⁷, though whether endogenous *ASCL1* can also promote neurogenesis was unknown at the start of my project. Given *ASCL1*'s importance in regulating neurogenesis, and the possibility of its interaction with HUWE1, I also checked for its expression. *ASCL1* was detectable from Day 18, and peaked in expression following Notch inhibition, before decreasing as neurons matured. Together, these results give a time window starting around Day 18 for when HUWE1 might interact (if at all) with *ASCL1* to have an effect on neurogenesis.

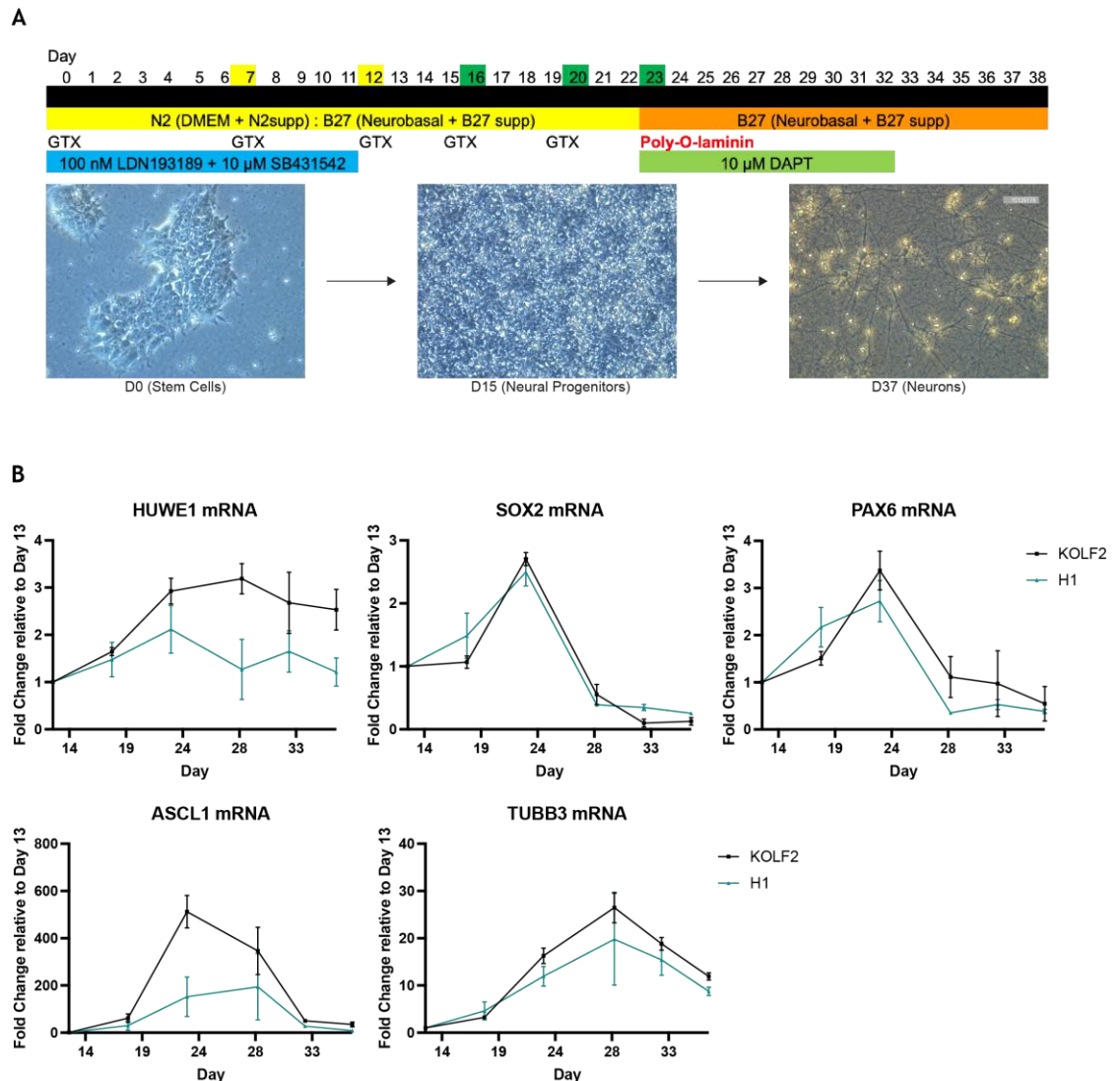


Figure 6. Characterisation of *HUWE1* mRNA expression during *in vitro* cortical neurogenesis.

- A)** Summary of the 2D *in vitro* cortical neurogenesis model used in this project. Dual SMAD-inhibition (100nM LDN + 10μM SB) followed by Notch inhibition (10μM DAPT) is used to induce neuronal differentiation of human pluripotent stem cells over the course of weeks. The protocol used here is adapted from Shi et al. 2012¹¹⁴. Heterogeneous cultures are present at all timepoints, with most cells being neural progenitors at Day 15, and most cells exiting the cell-cycle and becoming neurons by Day 37. GTX: Geltrex; Coloured days indicate days when passaging is performed.
- B)** qRT-PCR for indicated transcripts from differentiating cells harvested at various timepoints (Days 13, 18, 23, 29, 33, 37). Data represent mean ± SEM from 4 KOLF2 and 2 H1 differentiations.

3.2 *HUWE1* is necessary for survival during early neurogenesis

Having determined that *HUWE1* is expressed in our model, I next set out to design a *HUWE1* mutant to study how loss of HUWE1 activity might affect neural differentiation. HUWE1's catalytic HECT domain responsible for its ubiquitin ligase activity is encoded in its 3' end, and contains the catalytic Cys4341, without which it loses ability to ubiquitinate its substrates like N-Myc or itself^{174,178}. Huwe1 mutant mouse models used in prior studies^{182–185} also targeted the c-terminus (Figure 7A) to result in loss of Huwe1 function and their studied phenotypes. As such, I similarly targeted the HECT domain.

Originally, I had intended to delete either exon 81 or exon 82 (as both encode a Gly 4217 across their boundary) to create a frameshift mutation, loss of the Cys 4341, and a truncated HECT domain. However, due to forgetfulness, after testing guide RNAs (gRNAs) against introns 80, 81, and 82, I ended up proceeding with the gRNAs against introns 80 and 82, resulting in deletion of HUWE1 exons 81 **and** 82 (HUWE1 E81-82Del). Unfortunately, this is predicted to result in an in-frame deletion (Figure 7A) and retention of Cys 4341. Fortuitously, as the deletion is large (~1/4 of the HECT domain), and contains Arg 4187, Arg 4237 and Glu 4241 which have all been mutated in patients¹⁶⁰ – suggesting they are critical for activity – I continued with the generated HUWE1 E81-82Del mutants as they likely also represented the effects of impaired HUWE1 activity.

To generate HUWE1 E81-82Dels, I electroporated KOLF2 hiPSCs with CRISPR reagents and established several clonal lines (2.1.1 Gene-editing of hPSCs). The male line KOLF2 was selected as HUWE1 is an X-chromosome gene, and thus required fewer successful edits. Simultaneous cuts at both intronic gRNA target sites followed by non-homologous end-joining would result in excision of a 1378bp genomic region containing exons 81 and 82, whereas sequential cuts and repairs would result in small intronic insertions or deletions (indels) around the target sites (Figure 7B), imperceptible on gel electrophoresis.

I then performed a genotype screen for the deleted exons using a PCR strategy I employ throughout the rest of the project (Figure 7B), where I amplify a 2431bp

region of genomic DNA (gDNA) containing exons 81-83 and run the products on agarose (Figure 7C) to look for the expected size changes. In total, 72 clones were picked, of which 50 were genotyped and 6 were found to harbour HUWE1 E81-82Del (Figure 7C). 6 clones carrying WT-sized bands were also banked. Sanger sequencing was then performed to pinpoint the deletions and clonality of each line, during which it was discovered that Clone 1H which had a WT-sized band actually had an inversion of the 1378bp genomic region (Figure 7D, E). By sequencing around both the intron 80 (Figure 7E) and 82 (not shown) cut sites, I was able to confirm the 5 clones with intronic indels (henceforth EP controls) and most mutants were unique, independent, lines. EP controls are not expected to have impaired HUWE1 activity, and were made to control for potential off-target activity of the gRNAs and Cas9.

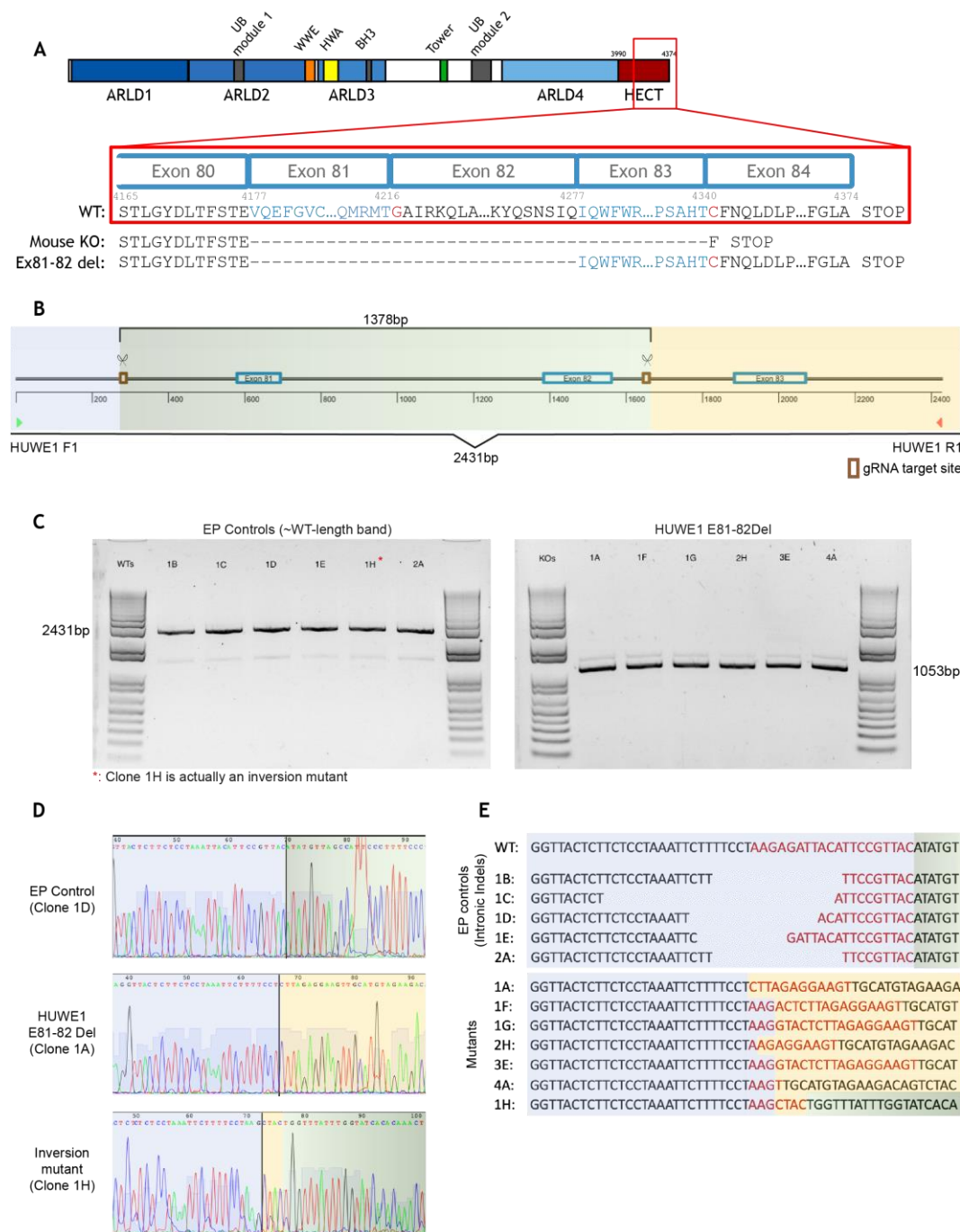


Figure 7. Generation and verification of *HUWE1* E81-82Del mutants.

- A)** Schematic of *HUWE1*, with particular focus on the exons coding for part of the catalytic HECT domain (c.4165 – c.4374). The amino acid sequences for different models are shown, with alternating black-blue colours to demarcate exon boundaries. Residues coloured red are encoded across an exon junction. The human wild-type (WT) HECT domain is shown, and for comparison: the human-equivalent modification for the *Huwe1* conditional knockout mice (from Zhao et al. 2009¹⁸²), and the *HUWE1* exon 81-82 deletion KOLF2 mutant (*HUWE1* E81-82Del) generated in this study are shown.
- B)** Genotyping strategy to screen for KOLF2 clones with the desired *HUWE1* E81-82Del mutation. Using *HUWE1* F1 and *HUWE1* R1 primers, a ~2431bp amplicon is obtained from WT genomic DNA (gDNA) or cells with short intronic insertions and deletions (indels), whereas a 1053bp amplicon lacking a 1378bp fragment between the 2 intronic cut sites is

obtained upon successful CRISPR editing. Regions are colour-coded, with a gradient for the 1378bp to indicate direction.

- C)** Gel electrophoresis of PCR products, described in **B)**, from crude lysates of 6 clones with a WT-sized band (Electroporated controls; EP controls), and 6 clones with the expected 1378bp deletion (HUWE1 E81-82Del). Subsequent Sanger sequencing of Clone 1H revealed it to harbour an inversion of the 1378bp fragment.
 - D)** Example chromatograms of intron 80 from Sanger sequencing of the PCR products in **C)**, using *HUWE1* F2 primers (Table 2. Primer sequences for clone-screening and CRISPR off-target verification.), without need for gel-extraction. The same colour code is used as in **B)**, showing the sequences around the intron 80 gRNA target site in each of the indicated clones.
 - E)** Sequences for intron 80, around the gRNA target site, for each of the clones in **C)**. The same colour code is used as in **B)**, and both the intron 80 and intron 82 gRNA target sequences are additionally written in red. EP controls are unique clones when also considering their intron 82 indels, while mutants 1G and 3E have identical mutations.
-

Having generated HUWE1 mutants, I then selected and performed basic validations on 2 EP controls and 2 HUWE1 E81-82Dels. The selected lines retained iPSC morphology, growing in compact colonies with defined boundaries (Figure 8A), and expressed the pluripotency markers *SOX2*, *OCT4*, and *NANOG* at levels equal to or higher than unedited KOLF2 (Figure 8B). Critically, *HUWE1*'s transcript expression, using a probe targeting exons 67-68, was unaltered in EP controls, although it is also unaltered in HUWE1 E81-82Dels (Figure 8B). When investigating the effect of the edits by Western Blot however, I found a reduction in HUWE1 protein in only HUWE1 E81-82Dels, suggesting loss of protein stability, while EP controls retained normal expression (Figure 8C). Levels of a potential HUWE1 substrate, C-MYC¹⁷⁸, were unaffected by the edits (Figure 8C). C-MYC was chosen as it was shown to be destabilized somewhat in mouse ESCs¹⁷⁸ and no other HUWE1 substrates in hPSCs or closely-related cell types had been reported then. That it did not increase in HUWE1 E81-82Dels was disappointing, but within expectations given the small effect in mESCs¹⁷⁸ and a report that C-MYC ubiquitination by HUWE1 enhances C-MYC activity rather than degrades it¹⁷⁶, and is not an indication that HUWE1 E81-82Dels retain HUWE1 activity.

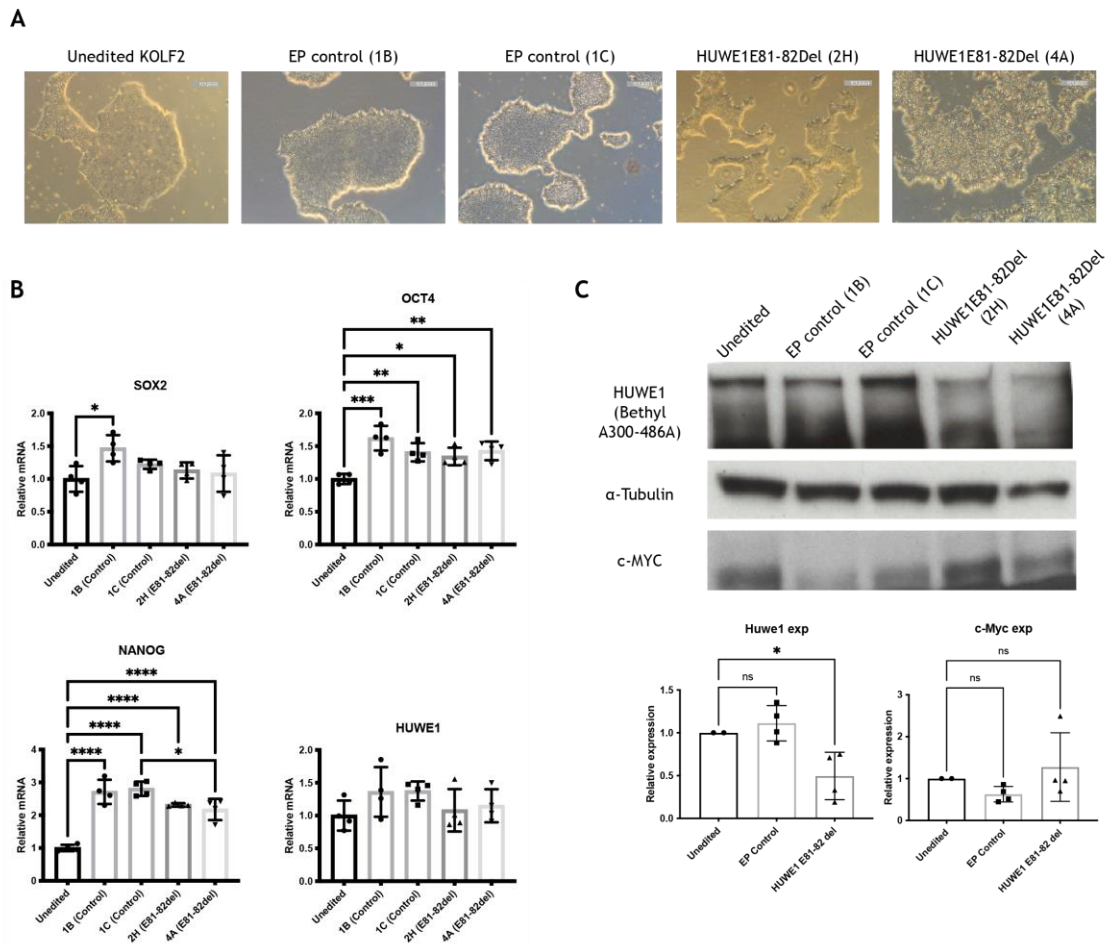


Figure 8. HUWE1 E81-82Dels are pluripotent and have reduced HUWE1 stability.

A) Brightfield microscopy images of the indicated clones showing their morphology.

B) qRT-PCR for pluripotency markers *SOX2*, *OCT4*, and *NANOG*, and for *HUWE1*, in the indicated clones/genotypes. Clones retain pluripotency, showing similar or higher expression for each of the markers compared to unedited KOLF2. *HUWE1* mRNA expression (probe targets exons 67-68) is unaffected by the intronic indels or the loss of exons 81-82. Data represent mean $\pm$ SD from 4 passages each. One-way ANOVA with Tukey's HSD test.

C) Western Blot for HUWE1 shows reduced levels in HUWE1 E81-82Dels. The top band ($\sim$ 480kDa) was measured. HUWE1's previously reported substrate C-MYC was not stabilized in KOLF2 iPSCs. Data represent mean $\pm$ SD from 2 passages of each clone in **B)**, grouped by genotype. One-way ANOVA with Tukey's t-test.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Undeterred, and instead encouraged by the reduced HUWE1 protein stability, I proceeded to subject HUWE1 E81-82Dels to our neuronal differentiation model. While both EP controls and HUWE1 E81-82Dels were able to initiate neural induction similar to WT KOLF2, HUWE1 E81-82Dels consistently detached en masse between

Day 9-10 (5 differentiations from 3 clones, Figure 9A), leaving few attached survivors, through unknown mechanisms.

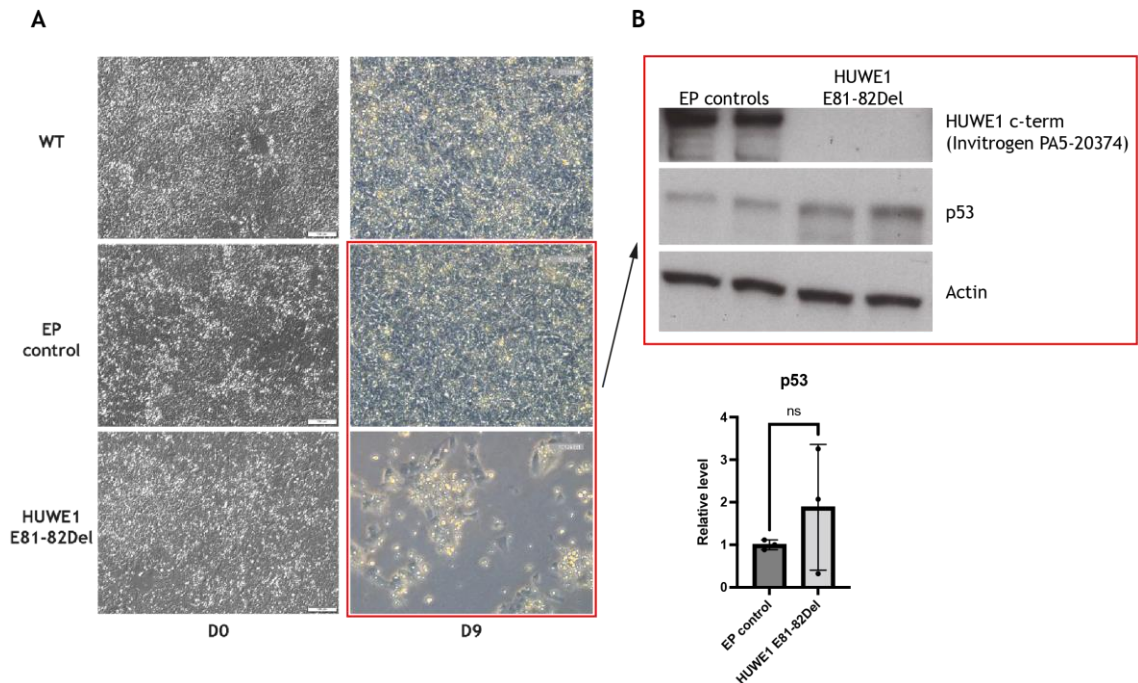


Figure 9. HUWE1 E81-82Dels fail to survive early neural differentiation, with potential involvement of p53.

- A)** Representative brightfield microscopy images from 2D neural differentiations of the indicated genotypes at Day 0 and Day 9. All genotypes undergo differentiation similarly, until around Day 9, when HUWE1 E81-82Dels progressively experience severe detachment and are lost. N = 5 total differentiations of 3 clones per genotype (EP controls: 1B, 1C, 1E; HUWE1 E81-82Del: 1G, 2H, 4A).
- B)** Western Blot from cells collected at Day 9 show potential increase in p53, in line with Aprigliano et al. 2021¹⁶⁸. The HUWE1 antibody used here targets an epitope within the deleted region of HUWE1 E81-82Dels. Data represent mean $\pm$ SD, 3 clones from each genotype.

Given the need in our model to maintain confluent cultures, particularly early during induction to avoid neural crest fates²¹⁹, I was not confident the identities of continued cultures of HUWE1 E81-82Dels would be neuronal nor relevant to neurogenesis. There would also always be a severe shortage of material for downstream analyses, and controls for HUWE1 E81-82Dels would be less relevant over time. Essentially, HUWE1 E81-82Del can only model neural induction (i.e., neuroepithelial and radial glia-like cells) at best and not neuronal differentiation. As such, I started exploring the generation of an inducible *HUWE1* knockout to circumvent this differentiation block.

It was also around this time (early 2020) a preprint was uploaded (Aprigliano et al. 2021¹⁶⁸, eventually published) in which the authors described the same phenotype in *HUWE1* patient-derived iPSC lines (p.G4310R, 2 clones from 1 patient) differentiated into cerebral organoids. Only 1-2 of 96 differentiations survived past day 35 with the rest arresting at day 14, never forming rosettes, despite starting differentiations similar to WT. They further demonstrated that this was due to an accumulation of HUWE1's substrate, p53, in patient cells starting from the iPSCs till day 14, as constitutive lentiviral-mediated knockdown of p53 was able to restore neural differentiation and rosette formation. Given this report, I proceeded to investigate p53 levels in my differentiations at day 9, finding that p53 may indeed similarly be increased and involved (Figure 9B) in the observed phenotype, though statistical significance was not reached. Despite the independent mutual confirmation between our results, the limitations mentioned above persist. Crucially, both our phenotypes appear too severe to be compatible with life, whereas patients are born intact and alive, casting doubts on the biological relevance of our results.

Because the phenotype – which may be limited only to *in vitro* systems – has been better-characterised by Aprigliano et al. 2021, and the possibility exists that HUWE1 still has an undiscovered function in neural progenitors past this early block, I refocussed my efforts on generating a conditional HUWE1 knockout instead.

Results 2: Acute *HUWE1* knockouts undergo neural differentiation with stabilization of ASCL1

The failure to survive differentiation, with increased p53, observed in HUWE1E81-82Del and Aprigliano et al. 2021¹⁶⁸ in the previous chapter mirror perfectly prior observations in two distinct constitutive *Huwe1* knockout mouse models^{179,181} which both saw embryonic lethality post-gastrulation. Kon et al. 2012¹⁸¹ even showed increased p53 and cleaved Caspase-3 in neural tissue at E13.5, prior to lethality around E14.5. However, as mentioned, these observations are incongruent with human *HUWE1* patients being alive – a contradiction Cox et al. 2010¹⁷⁹ pointed out and attempted to reconcile as likely due to “tissue-specific loss of gene expression caused by mutations in regulatory regions” in patients. In other words, generalised null mutations could be much more severe than milder, or more regionalised, patient mutations. To see a coding mutation (p.G4310R) also cause null-mutation phenotypes is thus surprising, furthermore so given HUWE1^{G4310R} appeared to retain ubiquitination activity against p53 and was only mildly decreased in stability¹⁶⁸. Ultimately, because *HUWE1* patients are alive – and *HUWE1*’s severe p53-related phenotypes are incompatible, and cannot be explained by mutation type (point, null, or truncated) – it is likely that human-specific compensation occurs *in vivo* to reduce or even nullify the impact of p53-driven development arrest.

Furthermore, *HUWE1*’s role in intellectual disability (and by extension, neurogenesis) likely goes beyond p53 degradation altogether, since two HUWE1^{R4063Q} (also in the HECT domain) siblings’ lymphoblast lines were shown to have decreased p53 levels¹⁶², along with increased HUWE1 levels. Microduplications encompassing *HUWE1* also cause intellectual disability¹⁶⁶ and would also be expected to cause reduced p53 levels. While elevated p53 levels have been linked to microcephaly in humans²²³ and mice²²⁴, reduced p53 activity instead causes female-specific neural tube closure defects in mice^{225–227} (reviewed in Xiong et al. 2020²²⁸) – decidedly not a *HUWE1* patient phenotype. Other roles and targets for *HUWE1* during human neurogenesis therefore likely exist.

In this chapter, continuing from the lines of reasoning above, I sought to circumvent the physiologically questionable p53-related differentiation block, to uncover other *HUWE1*-relevant roles during human neurogenesis. To that end, I first generated and characterised inducible *HUWE1* knockouts. After verifying the line functions as intended, I successfully bypassed the early differentiation failure through postponing deletion of *HUWE1*, albeit as a mosaic culture, to uncover new population-level phenotypes. Additionally, I also used the line to validate and compare currently available HUWE1 antibodies.

4.1 HUWE1cKO: a 4OHT-inducible HUWE1 knockout hESC line

Having previously generated an unintended mutant (HUWE1E81-82Del) that retained reduced levels of HUWE1, I now had the opportunity to generate the intended mutation in an inducible form, using the gRNAs that I had already tested. To do this, I aimed to first flox (flank-by-loxPs) exon 81 of *HUWE1* (HUWE1E81-fl) through successive rounds of CRISPR-Cas9 gene-editing and clone isolation, this time in the male H1 hESC line. The KOLF2-C1 hiPSC line previously used was not chosen as by this point, a 19bp heterozygous deletion in the SWI/SNF chromatin remodeling complex subunit *ARID2* (linked to Coffin-Siris Syndrome – which includes intellectual disability) had been discovered in it²⁰² and the lab collectively moved away from the line to avoid potential complications. Again, a male line was chosen to reduce the number of copies of *HUWE1* for editing. Successful floxing of *HUWE1* exon 81 was predicted to result in frameshift, premature stop, and a truncated HECT domain missing the catalytic Cys4341 (Figure 10A). As the premature stop codon would occur prior to the final exon, nonsense-mediated decay of *HUWE1* transcripts could also occur²²⁹, to produce a model with complete loss-of-function.

To generate HUWE1E81-fl, I electroporated CRISPR reagents similar to before, including a guide against intron 80, and a 114bp single-stranded oligodeoxynucleotide (ssODN) repair template containing a 34bp loxP site interrupting the gRNA site and two 40bp homology arms. Clones were obtained as before and screened for insertion of loxP with the strategy shown in Figure 10B (loxP1). 13/85 clones screened successfully incorporated loxP1.

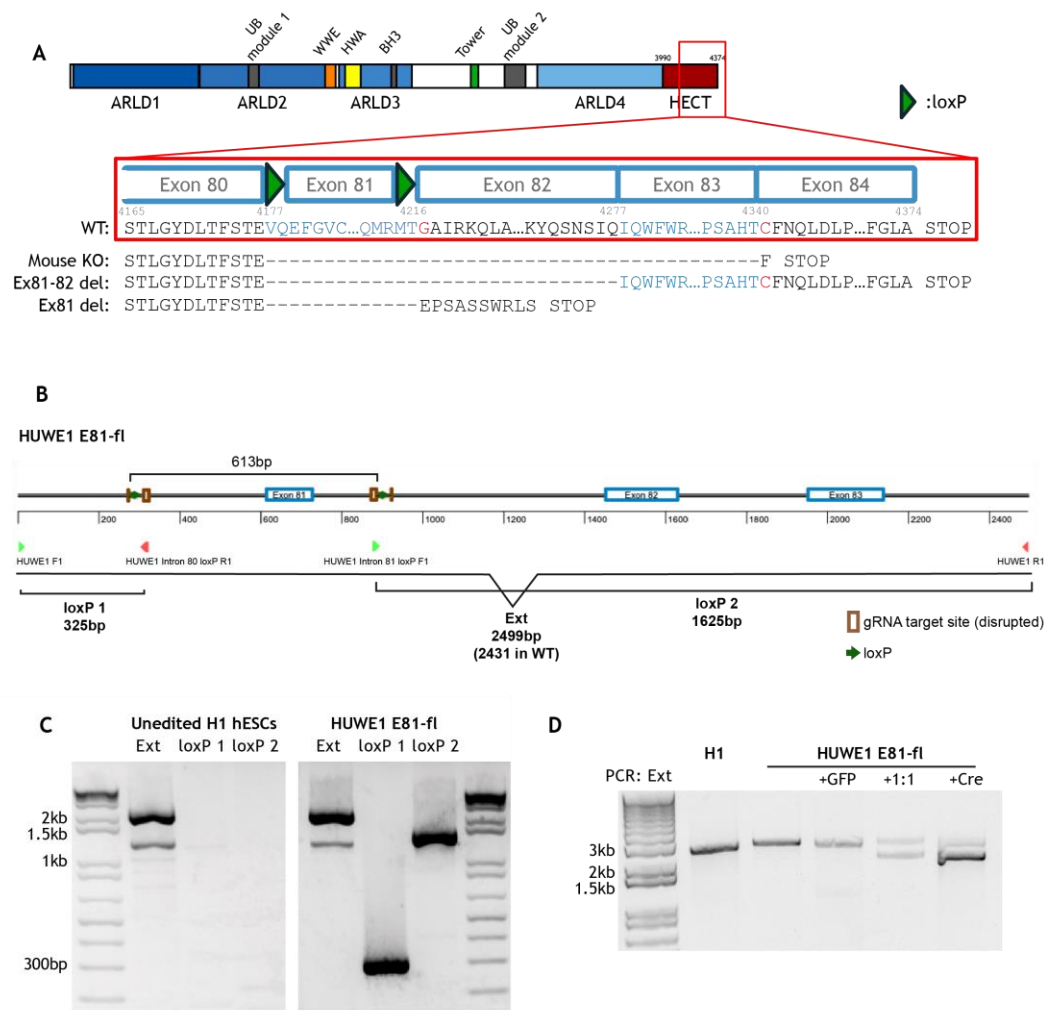


Figure 10. Generation, verification, and validation of a floxed *HUWE1* exon 81 in H1 hESCs.

- A)** Schematic of *HUWE1*, with particular focus on the exons coding for part of the catalytic HECT domain (c.4165 – c.4374). The amino acid sequences for different models are shown, with alternating black-blue colours to demarcate exon boundaries. Residues coloured red are encoded across an exon junction. The human wild-type (WT) HECT domain is shown, and for comparison: the human-equivalent modification for the *Huwe1* conditional knockout mice (from Zhao et al. 2009¹⁸²); the *HUWE1* E81-82Del KOLF2 mutants from the previous chapter; and the predicted outcome of an exon 81 deletion in the planned *HUWE1* exon 81 floxed mutant are shown. Exon 81 deletion is predicted to result in frameshift and a premature stop codon before the final exon, potentially resulting in nonsense-mediated decay.
- B)** Genotyping strategy to screen for H1 clones with the desired loxP insertions. Using *HUWE1* F1 and *HUWE1* R1 primers (the Ext combination), a 2431/2499bp amplicon is obtained from gDNA, serving as a positive control for presence of gDNA. *HUWE1* intron 80 loxP R1 and *HUWE1* intron 81 loxP F1 have their 3' ends complementary to the loxP sequences, and only produce 325bp (the loxP1 combination with *HUWE1* F1) or 1625bp (the loxP2 combination with *HUWE1* R1) amplicons respectively when a loxP has been successfully integrated into their respective introns. Deletion of the floxed 613bp region results in a 1886bp amplicon when using the Ext combination.

- C) Gel electrophoresis of PCR products from WT cells and the eventual clone with floxed *HUWE1* exon 81 (HUWE1 E81-fl) made with PCR primers in the combinations described in **B**), demonstrating the efficacy of the screening method.
 - D) Gel electrophoresis of PCR products (Ext combination) from HUWE1 E81-fl after electroporation with plasmids coding for GFP, Cre, or a 1:1 mixture by mass of both, to demonstrate functional loxPs. Deletion of the floxed 613bp region occurs only in the presence of Cre, and is dose-dependent. A WT-sized band persists due to the imperfect efficiency of electroporation. As controls, the same PCR was also performed on unedited H1, and HUWE1 E81-fl at steady-state.
-

One clone was then selected and edited again similar to before to introduce a second loxP in *cis* into intron 81 (loxP2). Clones were obtained and screened for loxP2 as shown in Figure 10B. 24/88 clones successfully incorporated loxP2, and one HUWE1E81-fl clone was selected for future experiments. Figure 10C demonstrates the efficiency of the screening method and the presence of both loxPs in this clone.

To demonstrate functional loxPs, I then electroporated 5 μ g pCAG-Cre (Addgene #13775), pmax-GFP (Lonza), or a 1:1 mixture by mass of the two plasmids, into HUWE1E81-fl to delete *HUWE1* exon 81. After recovery, cells were harvested for genomic DNA (gDNA) and genotyped using the Ext combination (Figure 10B). Two bands were seen only in the presence of Cre, with the smaller KO band corresponding to HUWE1E81-Del being higher in proportion with a higher Cre dose (Figure 10D), demonstrating successful Cre-dependent deletion of *HUWE1* exon 81. The ability of the Ext combination genotyping strategy to resolve both bands and track *HUWE1* deletion in a seemingly quantitative manner here will be relied upon heavily and revisited in subsequent figures.

Having generated HUWE1E81-fl, I now needed a way to reliably delete *HUWE1* exon 81 efficiently. The electroporation method above, while successful, was inefficient, resulting in WT cells remaining – as evidenced by the larger WT band (Figure 10D). The method also results in high cell-death which could skew cultures to a neural crest fate²¹⁹, and its efficacy was untested for use during neural differentiation. As such, an alternative method to deliver Cre activity on demand was required. For this, I decided to permanently integrate a CAG-promoter-driven CreERT2 into the *AAVS1* safe-harbour locus of HUWE1E81-fl, to allow for Cre activity upon 4-hydroxytamoxifen (4OHT) addition. While lentiviral-delivered Cre might have been

suitable, it would also have required more work in the long-run producing lentiviruses for experiments, with potential batch-to-batch variation in titres and thus recombination efficiencies.

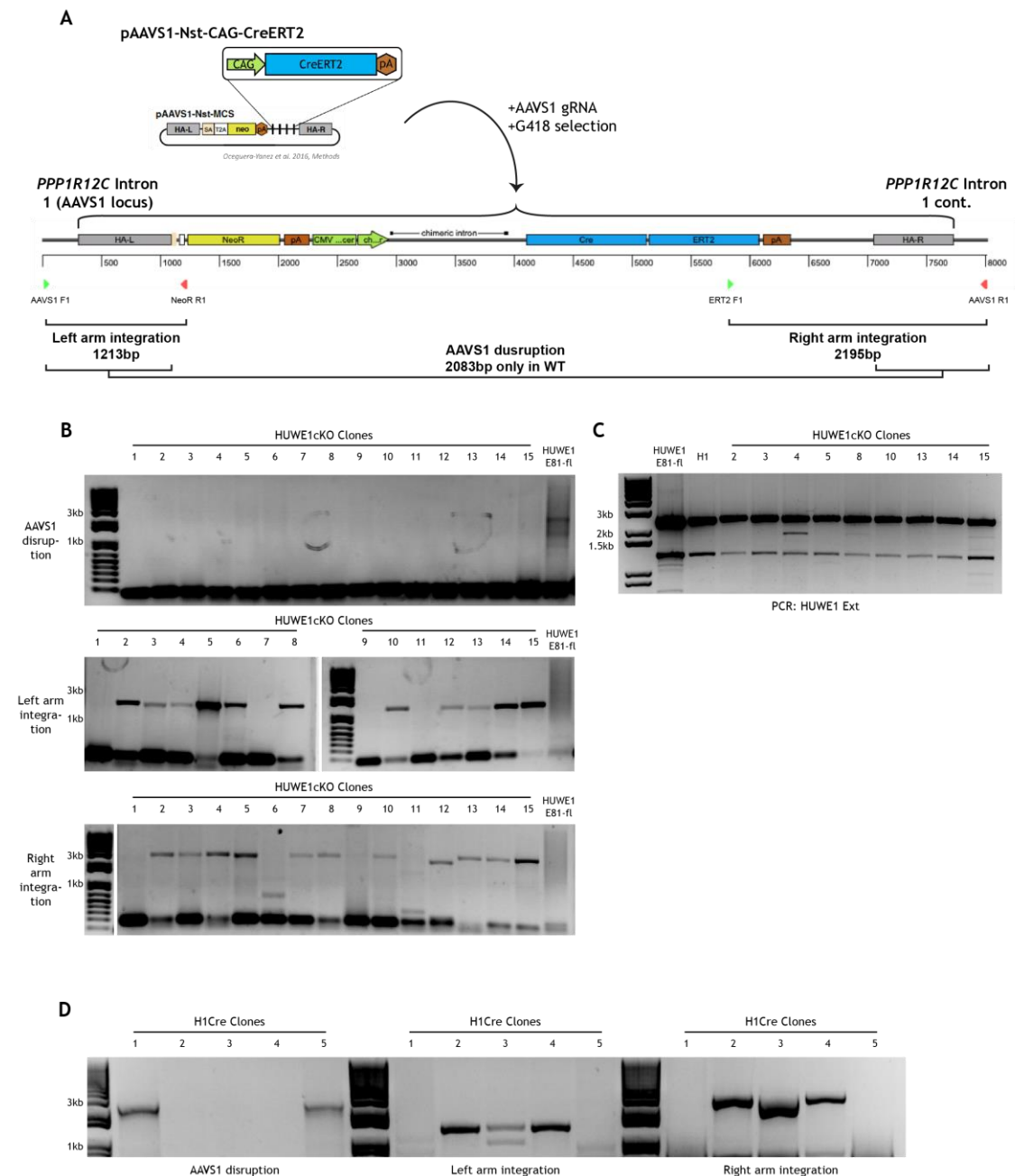


Figure 11. Integration of CreERT2 into the AAVS1 locus of HUWE1 E81-fl.

A) Using CRISPR-Cas9, a 7438bp region of the pAAVS1-Nst-CAG-CreERT2 plasmid shown was integrated into intron 1 of the *PPP1R12C* gene (AAVS1 locus) of HUWE1 E81-fl. Desired clones were then found through antibiotic selection with G418, and verified through 3 combinations of PCR primers. HA-L: left homology arm; HA-R: right homology arm; SA: splice acceptor; T2A: self-cleaving polypeptide sequence; NeoR: neomycin/G418 resistance gene; pA: poly-adenylation site; CAG: CAG promoter. pAAVS1-Nst-CAG-CreERT2 was a gift from

W. Skarnes, made by E. Pellegrino, and based off the original protocol for editing the human *AAVS1* locus from Ocegüera-Yanez et al. 2016²⁰³.

- B)** Gel electrophoresis of PCR products from a screen of 15 clones after G418 selection using the primer combinations described in **A**). In the last lane of each gel is the same PCR performed on the parental HUWE1 E81-fl line to serve as a control. Clones without either left or right arm bands or that had an abnormal band size (Clone 12) were discontinued. Clones 2, 3, 4, 5, 8, 10, 13, 14, and 15 were found to have homozygous insertions of both the left and right arms of the repair template and thus likely to be HUWE1 conditional knockouts (HUWE1cKO).
 - C)** Gel electrophoresis of PCR products (Ext combination) from the potential HUWE1cKO clones to screen for leaky Cre activity. Clones 4, 8, and 15 were found to have inadvertently excised *HUWE1* exon 81 in some fraction of their population through the course of the editing. Subsequent experiments were performed only on Clones 10 and 13. As controls, the same PCR was performed on the parental HUWE1 E81-fl line and unedited H1.
 - D)** Gel electrophoresis of PCR products similar to **B**), from 5 H1 hESC clones also subjected to CRISPR-Cas9 to introduce CreERT2 as in **A**), and following G418 selection. Clones 2 and 4 both had left and right arm integration products of the expected sizes, while Clones 1 and 5 had neither, and likely retained WT *AAVS1* loci as they also had products when checking for *AAVS1* disruption. Subsequent experiments were thus performed only on Clones 2 and 4.
-

In practice, this was achieved by electroporating CRISPR reagents including a gRNA targeting intron 1 of *PPP1R12C* (*AAVS1* locus), and pAAVS1-Nst-CAG-CreERT2 (gift from W. Skarnes) as a repair template – a strategy adopted from Ocegüera-Yanez et al. 2016²⁰³. pAAVS1-Nst-CAG-CreERT2 uses two ~800bp homology arms (HA-L and HA-R) complementary to the *AAVS1* locus to deliver a CAG-promoter-driven CreERT2 (Figure 11A). Additionally, it carries a splice-acceptor (SA) to co-opt the promoter of the *PPP1R12C* gene to drive expression of a neomycin resistance (neo/NeoR) selectable marker after self-cleaving from *PPP1R12C* exon 1's polypeptide using T2A²³⁰. Disruption of the *PPP1R12C* gene product occurs, though this is not associated with any known disease²⁰³.

After 8 days of G418 selection, surviving clones were expanded and genotyped. Successful integration in clones was screened for with 3 pairs of primers (Figure 11A). Left arm integration was verified through PCR using *AAVS1* F1 and NeoR R1, giving a 1213bp product only if the 5' region of the neomycin resistance gene was integrated at minimum. Right arm integration was verified through PCR using ERT2 F1 and *AAVS1* R1, giving a 2195bp product only if at least the 3' end of the CreERT2 construct was integrated. *AAVS1* F1 and *AAVS1* R1, which both target endogenous sequences, produce a 2083bp together only if at least 1 copy of the *AAVS1* locus remains undisrupted by the 7438bp repair template, due to the limited amplification

time in each PCR cycle. 15 clones were obtained and screened (Figure 11B), of which 9 appeared to have homozygous insertions of CAG-CreERT2. The Ext combination PCR was then performed to genotype *HUWE1* exon 81, and discontinue clones that had leaky Cre activity at some point during the electroporation (Figure 11C). Two clones (10 and 13) were then selected for all future validation experiments, and henceforth termed HUWE1cKOs (*HUWE1* conditional knockouts).

With HUWE1cKO, an isogenic culture differing only in *HUWE1* exon 81 from the parental culture is obtained after 4OHT addition. However, the effects of 4OHT and Cre activity might need to be controlled for. As such, I repeated the previous integration of CAG-CreERT2, this time into H1 hESCs, obtaining 5 clones (Figure 11D), of which 2 had homozygous integrations of the repair template (henceforth H1Cre).

Basic quality-control checks were then performed on H1Cre and HUWE1cKO lines. Both lines possessed the expected colony morphologies of hESCs (Figure 12A), and expressed pluripotency markers *SOX2*, *OCT4*, and *NANOG* similar to each other and unedited H1s (Figure 12B). Like in HUWE1E81-82Del, the intronic edits did not affect *HUWE1* expression from exons 67-68. The top 4 genomic off-targets for each of the 3 gRNAs used, as predicted by Off-Spotter²³¹, were also PCR-amplified and Sanger-sequenced. No off-target edits were found (not shown). Low-pass whole genome sequencing was also done to karyotype 1 clone each of H1Cre and HUWE1cKO, with no abnormalities found (not shown). Arguably, given the isogenic HUWE1cKO model I intended to use, these additional genomic checks were unimportant, since off-targets would be shared between 4OHT and Vehicle-treated (ethanol) conditions.

As the genotyping strategy in Figure 11 only checks for integration of the repair template's ends, and not integrity of the entire construct, mistakes could still occur that remove CreERT2 activity in the lines. To demonstrate functional CreERT2 activity, particularly in the H1Cre line, I electroporated a Cre-reporting plasmid pEF1a-Flex-GT (Figure 12C) (Gift from T. Branco lab) into the lines. pEF1a-Flex-GT encodes for EGFP and the transmembrane tumour virus subgroup A receptor TVA950 (not relevant to this project) in an inverted reading frame, under the control

of an EF1a promoter. If Cre activity is present, specific pairwise lox-site recombinations occur sequentially to restore the reading frame and EGFP expression. EGFP⁺ cells were found only in H1Cre and HUWE1cKO, and not H1, after 1 μ M 4OHT was added to the culture (Figure 12D).

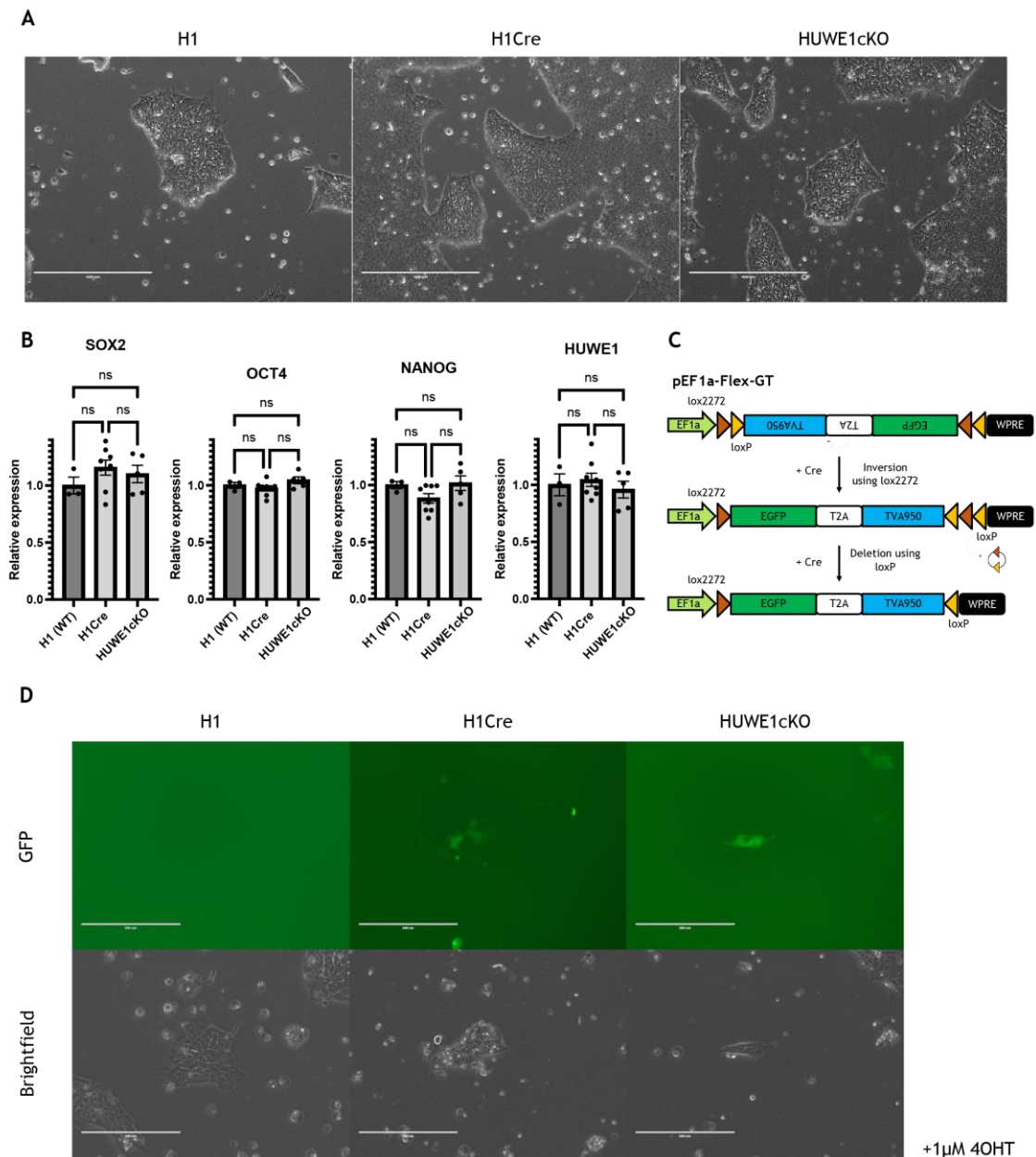


Figure 12. HUWE1cKOs are pluripotent and possess Cre activity.

A) Brightfield microscopy images of unedited H1, H1Cre, and HUWE1cKO showing their colony morphology. Scalebar: 400 μ m.

B) qRT-PCR for pluripotency markers *SOX2*, *OCT4*, and *NANOG*, and for *HUWE1*, in unedited H1, H1Cre, and HUWE1cKO at steady-state. *HUWE1* mRNA expression was unaltered by the edits. Data represent mean $\pm$ SEM from separate passages of 2 clones each for H1Cre and HUWE1cKO. One-way ANOVA with Tukey's HSD test conducted.

- C)** A Cre-reporting plasmid – pEF1a-Flex-GT – was electroporated into unedited H1, H1Cre, and HUWE1cKO. In the presence of Cre activity, inversion followed by excision (the Flex system) of specific pairs of loxP sites (i.e., recombination of loxP with loxP, and lox2272 only with lox2272) occurs, resulting in permanent restoration of the reading frame, and expression of EGFP. pEF1a-Flex-GT was a gift from T. Branco, made by P. Iordanidou.
- D)** Brightfield and GFP-channel images from electroporated cells described in **C)**, cultured in 1 μ M 4OHT for 3 days post-electroporation. GFP-positive cells were only found in H1Cre and HUWE1cKO cultures. Scalebar: 200 μ m.

p>0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****)

I next had to show HUWE1cKO responds to 4OHT treatment as intended, and that HUWE1 expression is disrupted. Following 3 days of exposure to 100nM 4OHT, HUWE1cKO indeed underwent recombination of *HUWE1* exon 81 as assessed by the Ext combination PCR (Figure 13A). This deletion or band size shift was not due to 4OHT and/or off-target CreERT2 activity alone as H1Cre does not exhibit the same shift (as a demonstration of the utility of having H1Cre as an additional control). Additionally, the deletion appeared to be 100% efficient as no WT-sized band persisted, unlike following electroporation of Cre (Figure 10D) into HUWE1E81-fl, suggesting HUWE1cKO can indeed allow for higher recombination efficiency through gentler means.

To characterise the consequences of *HUWE1* exon 81 deletion, I next performed a qRT-PCR for both the deleted exon, and for exons 67-68. Not only was the deleted exon's expression completely abolished – again suggesting 100% efficient recombination – expression upstream from exons 67-68 was also greatly reduced (Figure 13B), in support of the intended nonsense-mediated decay during model-design (Figure 10A). Both observations were dependent on having both 4OHT and the HUWE1cKO genotype ($p^{\text{interaction(exon 81)}} = 0.0014$, $F(1, 12) = 16.89$) ($p^{\text{interaction(exon 67-68)}} < 0.0001$, $F(1, 12) = 41.64$), and specific to HUWE1cKO.

As a result, HUWE1 expression on the protein-level was also abolished, which was accompanied by an increase in p53 levels specific to HUWE1cKO + 4OHT ($p^{\text{interaction(genotype X 4OHT)}} = 0.0228$, $F(1, 8) = 7.900$) (Figure 13C). This increase in p53 is supported by an increase in expression of p53's transcriptional target *CDKN1A*(p21), though statistical significance was not obtained due to the large

variance (Figure 13C). These findings again echo those of Aprigliano et al. 2021¹⁶⁸ which found a mild increase in p53 levels in the *HUWE1* patient-derived iPSCs.

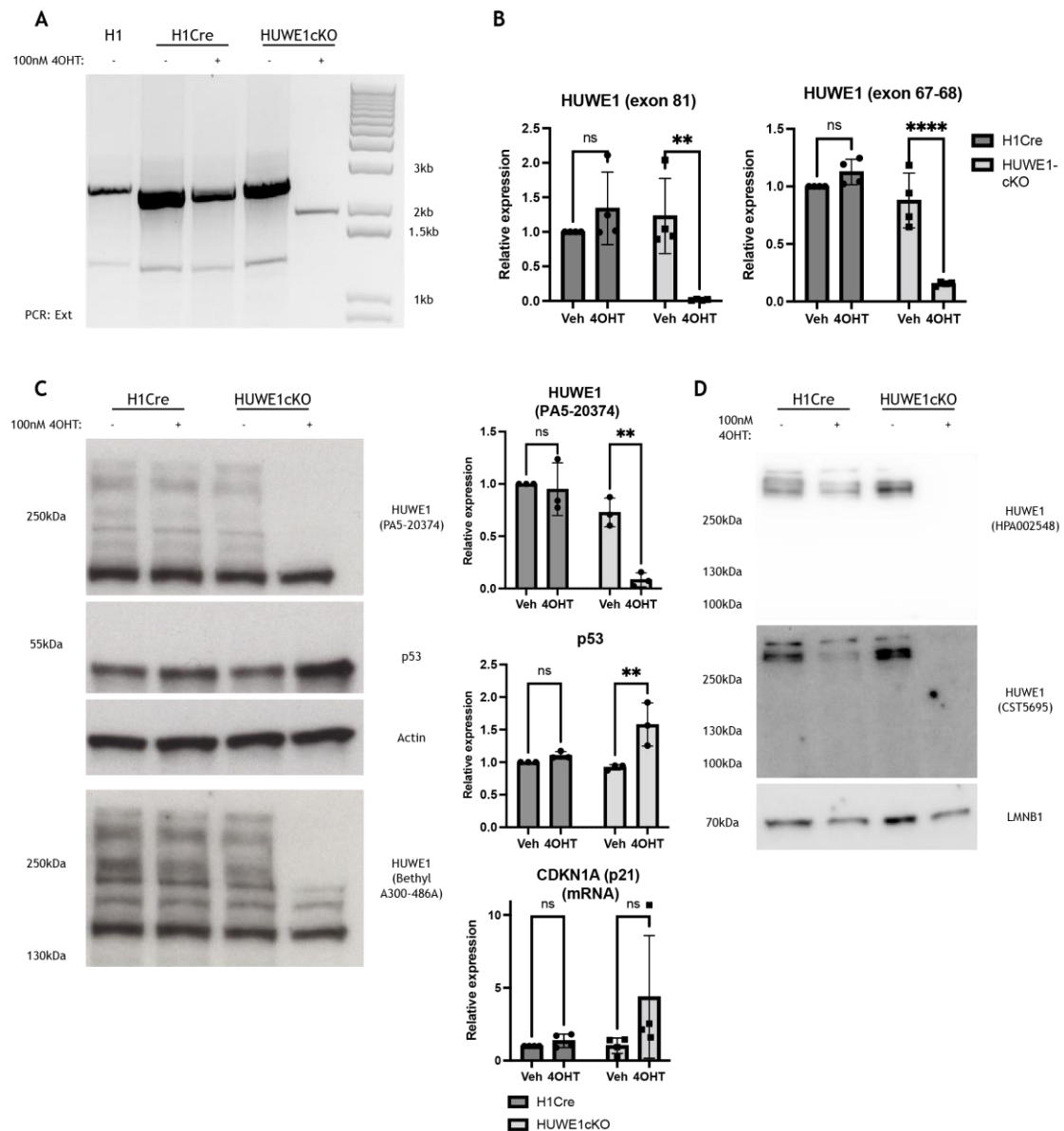


Figure 13. 4OHT-dependent deletion of *HUWE1* in HUWE1cKO hESCs results in loss of *HUWE1* on both protein and transcript levels, with increase in p53.

- A)** Gel electrophoresis of PCR products (Ext combination) from H1Cre and HUWE1cKO following 3 days of 100nM 4OHT or vehicle (ethanol) exposure. The first lane is obtained from unedited H1. HUWE1cKO undergoes a complete 4OHT-dependent deletion of *HUWE1* exon 81.
- B)** qRT-PCR for *HUWE1* exon 81, and the exon 67-68 junction in H1Cre and HUWE1cKO following 3 days exposure to 100nM 4OHT, showing 4OHT-dependent loss of *HUWE1* transcripts in HUWE1cKO beyond the deleted region. Data represent mean $\pm$ SD from 2 clones. Two-way ANOVA with Sidak's multiple comparisons test between the predefined pairs shown.

- C)** Western Blot for HUWE1 and p53 in HUWE1cKO following 4OHT treatment shows absence of HUWE1 protein and increase in p53, quantified on the right. qRT-PCR also potentially shows increase in p21 expression. Both PA5-20374 and A300-486A antibodies give unspecific bands between 130-250kDa despite loss of *HUWE1* transcripts demonstrated in **B)**. Data represent mean $\pm$ SD from 2 clones. Two-way ANOVA with Sidak's multiple comparisons test between the predefined pairs shown.
- D)** For comparison, Western Blots for HUWE1 in HUWE1cKO following 4OHT treatment, using the preferred HPA002548 and CST5695 antibodies give specific bands above 250kDa, with no other unspecific bands.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

As an aside, a strong >130kDa band was detected in HUWE1cKO when probed for HUWE1 using a c-terminus-targeting antibody (Invitrogen PA5-20374). The band had unchanged intensity between all conditions (Figure 13C) despite *HUWE1* being greatly diminished on a transcript level, and the epitope the antibody was raised against being deleted in HUWE1cKO upon 4OHT addition. For comparison, the HUWE1 (Bethyl A300-486A) antibody gives additional unspecific bands of constant intensities across conditions, despite the expected near-complete loss of HUWE1 protein. This prompted me to look for an alternative, more specific, HUWE1 antibody. CST#5695 and HPA002548 turned out to fulfil this role, with both detecting only a >250kDa band that is completely lost in HUWE1cKO after 4OHT treatment (Figure 13D), and no other bands.

As further demonstration of the different specificities of the antibodies, I performed immunofluorescent microscopy on fixed HUWE1cKO samples stained with PA5-20374, CST#5695, and HPA002548 for HUWE1. The samples were also stained for Cre (Synaptic Systems #257004), which was found to have a cytosolic localisation in vehicle-treated samples, but a nuclear localisation upon 4OHT addition (Figure 14A, B), again demonstrating functional CreERT2 activity. PA5-20374 stained all cells uniformly regardless of 4OHT addition (Figure 14A), whereas both CST#5695 and HPA002548 had greatly diminished staining in HUWE1cKO treated with 4OHT (Figure 14B quantifications, images not shown). CST#5695 was more specific, with intensities equal to a secondary-only control. Both antibodies, however, were specific enough to discriminate 2 populations of HUWE1 intensities in a co-culture of unedited H1s with HUWE1cKOs treated with 4OHT, with Cre identifying the HUWE1cKOs (Figure 14B). Unfortunately, because these antibodies were discovered last, not all experiments were performed with them.

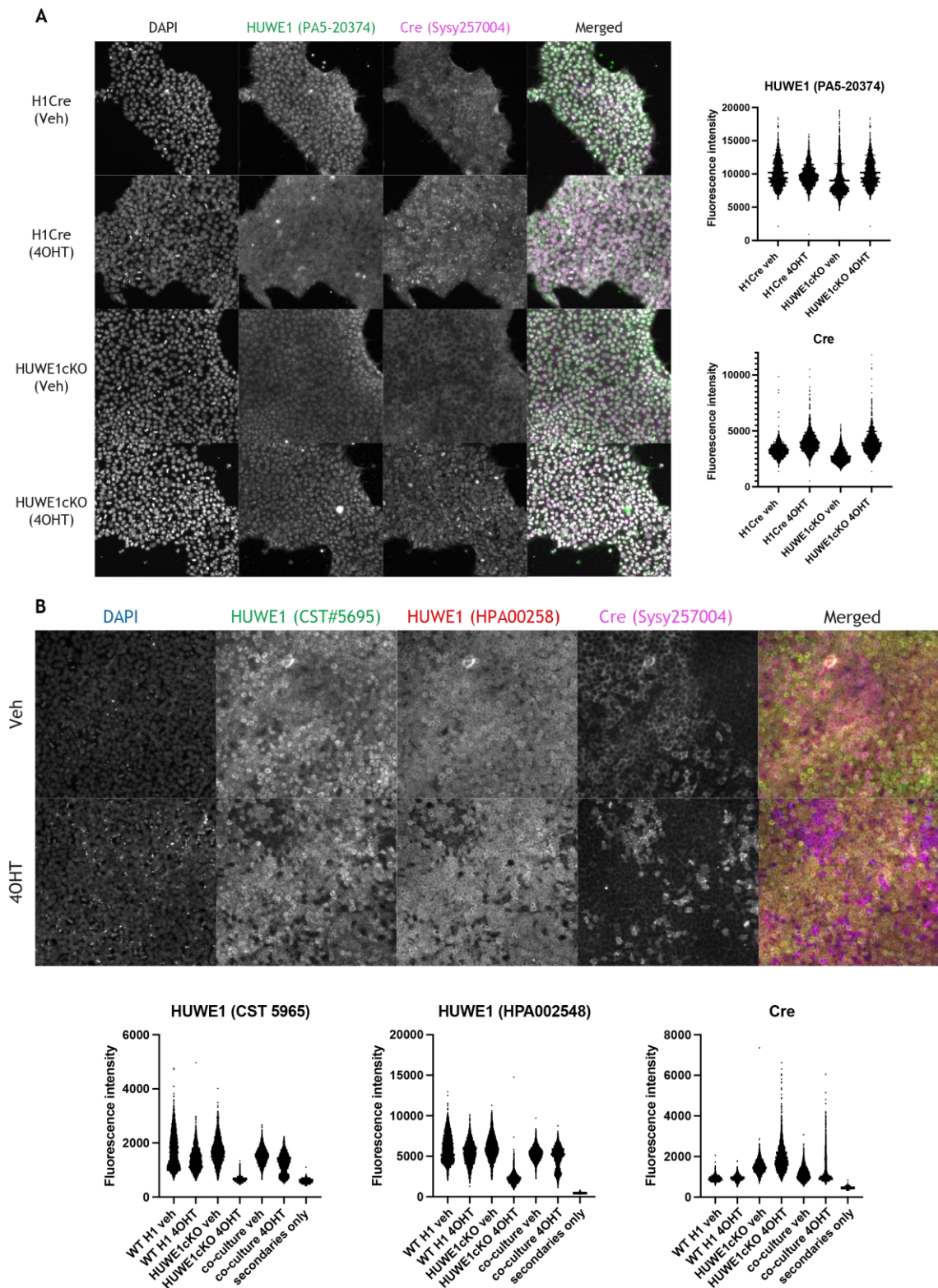


Figure 14. Validation of HUWE1 antibodies by immunofluorescence.

A) Immunofluorescence images of H1Cre and HUWE1cKO $\pm$ 4OHT, stained for HUWE1 (PA5-20374 antibody) and CreERT2, with nuclear intensity quantifications shown. CreERT2 changes from cytosolic to nuclear localization resulting in increased intensity following 4OHT treatment, but HUWE1 intensity is unchanged with PA5-20374.

B) Immunofluorescence images of a 1:1 co-culture of unedited H1 with HUWE1cKO $\pm$ 4OHT, stained for HUWE1 (CST5695 and HPA002548 antibodies) and CreERT2, with nuclear intensity quantifications shown, including from other conditions not shown. The Cre antibody marks only HUWE1cKO cells, which all lose HUWE1 expression in 4OHT, resulting in 2 populations of HUWE1 intensities in the co-culture + 4OHT condition. Both HUWE1 antibodies capture a decrease in fluorescence intensity following 4OHT treatment, with CST5695 showing levels equal to a secondary-only control.

Data from 3 fields of view of one coverslip per condition. Images captured on an Olympus IX83 widefield microscope.

As a final validation of HUWE1cKO, and a sanity-check of the p53-related phenotypes observed in HUWE1E81-82Del, the mouse mutants^{179,181}, and HUWE1 patient iPSC¹⁶⁸, I differentiated HUWE1cKO once, supplementing the media with 100nM 4OHT daily for 8 days from Days 0-7 (Figure 15A). Similar to before, HUWE1cKOs treated with 4OHT started differentiation identical to Vehicle-treated cultures, but also experienced widespread detachment on Day 10 (Figure 15A). Western Blotting of the remaining cells also revealed an increase in p53 in HUWE1cKO (4OHT) beyond that expected from 4OHT treatment alone (H1Cre 4OHT vs H1Cre Veh) (Figure 15B), again supporting the previously observed and reported results. Additionally, HUWE1 expression was not completely abolished this time (Figure 15B), suggesting incomplete recombination during neural differentiation – an observation that was seen again in subsequent differentiation dose regimens (Figure 16).

The replication of previous results in HUWE1cKO upon 4OHT treatment, along with all other validations in previous figures, suggests that HUWE1cKO functions as an inducible loss-of-function HUWE1 model as intended. All subsequent experiments were performed in a single clone each of H1Cre and HUWE1cKO, even though validations were performed in 2 clones each. Given the isogenic nature of the experiment setup however, I do not foresee this being a problem, since in knockout studies multiple clones are typically studied to average out off-target effects.

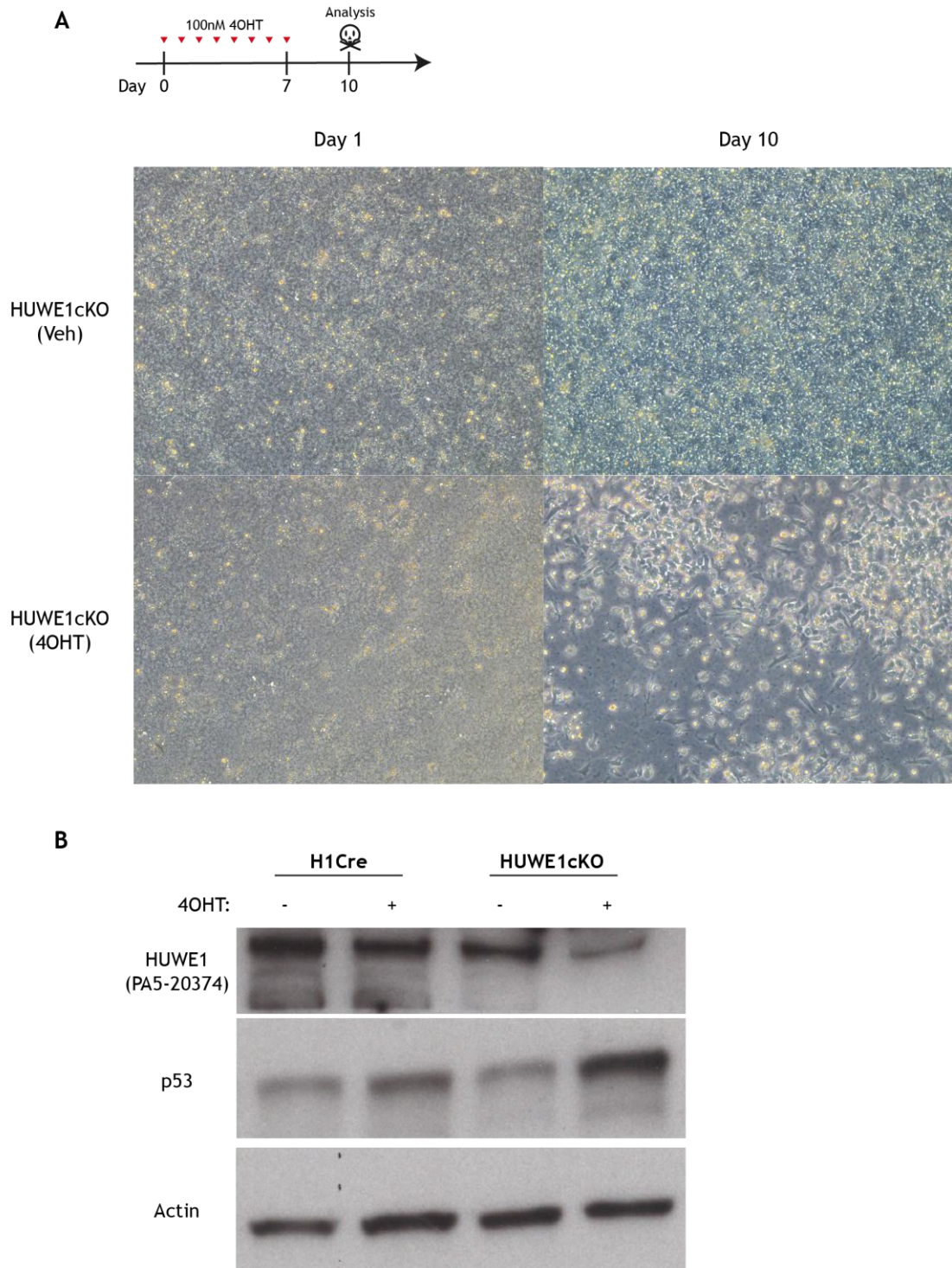


Figure 15. HUWE1cKO also fails to differentiate following early 4OHT treatment.

- A)** HUWE1cKO subjected to neural differentiation in the presence of 4OHT for 8 days starting from Day 0 also fail to survive early neural differentiation, albeit less severely than HUWE1E81-82Del.
- B)** Western Blot for HUWE1 and p53 at Day 10 of differentiation for the remaining cells shows p53 elevation similar to HUWE1E81-82Del beyond the effect of 4OHT alone (H1Cre + vs H1Cre -). Residual HUWE1 protein was detected, showing incomplete response to 4OHT and recombination during the protocol.

4.2 HUWE1cKOs show stabilized ASCL1, but not p53, despite mosaicism

Having established the new inducible model, I proceeded to test if knocking out HUWE1 after the early differentiation block (~Day 10) could allow me to obtain viable HUWE1-knockout neural cell types. For this, I supplemented the media daily with 4OHT for 5 days from Days 12-16, inclusive (Figure 16A). No visible differences were seen between the 4OHT and Vehicle cultures in both H1Cre and HUWE1cKO, with all able to form visible neurites by Day 20 (Figure 16A). By performing an Ext combination PCR on the Day 20 cultures, I was also able to confirm that deletion of *HUWE1* exon 81 had occurred in what appeared to be a majority of cells (Figure 16B), though it also meant a mosaic culture of KO and WT cells was present. No clear difference in the relative proportions of KO band to WT band was observed when a higher 4OHT concentration of 1 μ M was used between Days 12-16 versus 100nM, thus I settled on using the lower 100nM 4OHT concentration for all differentiation experiments. This was also driven by a desire to avoid potentially masking any differentiation phenotypes with 4OHT itself, as a study²³² found tamoxifen and 4OHT to promote premature neural differentiation in mice, including *in vitro*.

Given the heavy reliance on the Ext combination PCR as a means of genotyping HUWE1cKO up to this point, and now also to report on recombination efficiency, I performed a validation experiment to check the accuracy of the method. Theoretically, the Ext combination PCR is an internally-controlled one as both the WT and KO bands (which differ by 613bp) are produced using the same primer pairs from the same reaction, avoiding complications related to primer efficiencies. However, preferential amplification of the shorter KO band could also occur to inflate the proportion of KO bands in the final mixture. To verify that this was not occurring, I performed the Ext combination PCR on varying proportions by mass of 4OHT-treated HUWE1cKO to Veh-treated HUWE1cKO gDNA at the hESC stage (Figure 16C) – when recombination is 100% efficient as shown by qRT-PCR, Western Blot, and IF previously. This allowed me to simulate gDNA from mosaic cultures with known proportions of KO cells. By quantifying the proportion of area taken by the KO band

to the total area of the bands in a densitometric analysis, I was able to show that the measured KO proportion tracked well with the simulated KO proportion. The method was thus quantitative, and no preferential amplification was occurring (Figure 16C, right).

Using the Ext combination genotyping method, I then measured KO proportions over time in HUWE1cKO from Days 17-27 after 100nM 4OHT treatment as before. I found again that the majority of cells are deleted of HUWE1 exon 81 and thus HUWE1 knockouts, although this proportion decreases gradually over time, suggesting a selection bias in favour of the remaining WT cells in culture (Figure 16D).

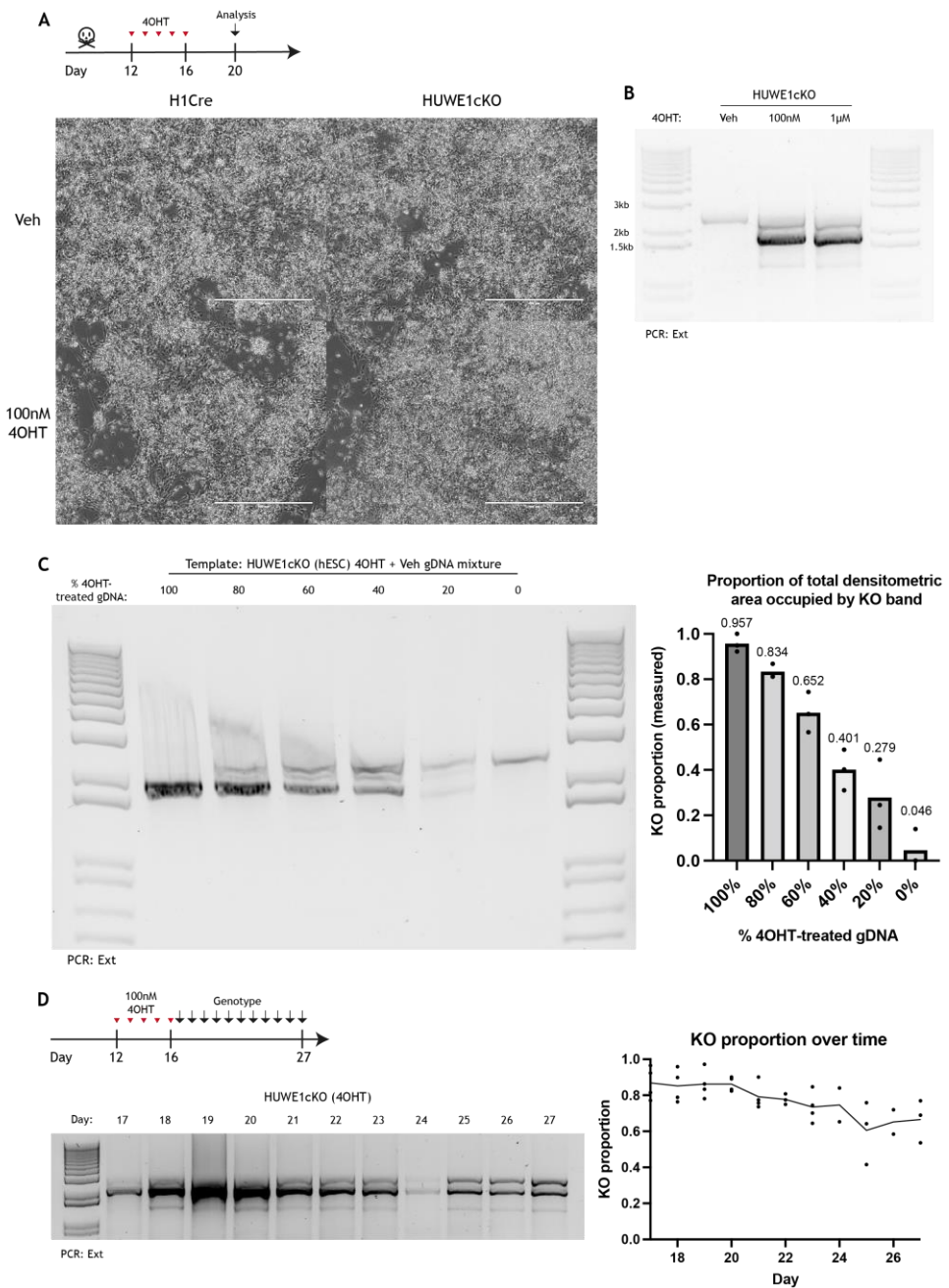


Figure 16. HUWE1cKO can bypass early differentiation failure but undergo mosaic recombination.

- A)** Treating HUWE1cKO with daily doses of 4OHT for 5 days from Day 12, after the critical period for survival, results in survival and outwardly indistinguishable cultures at Day 20 between HUWE1cKO (4OHT) and all other conditions, with neurites visible. Scalebar: 400µm.
- B)** Gel electrophoresis of Ext combination PCRs for *HUWE1*, from Day 20 HUWE1cKO cultures treated with 2 concentrations of 4OHT in the regimen shown in **A**). A larger ~2.5kb WT-sized band persists at both doses, along with the expected ~1.9kb band corresponding to *HUWE1ex81Del*, with no discernible difference in the relative proportions of the bands between doses.
- C)** Gel electrophoresis of Ext combination PCR performed on varying proportions by mass of 4OHT-treated HUWE1cKO to Veh-treated HUWE1cKO gDNA at the hESC stage – when recombination is 100% efficient – showing shifting relative proportions of the KO:WT bands

as the starting amount of *HUWE1*ex81Del DNA changes. Densitometric quantifications performed in FIJI show high correlation between the theoretical proportion of starting KO gDNA, and the measured KO band proportions.

- D)** Using the Ext combination PCR and quantification method from **C)**, the efficiency of recombination was assessed daily during neural differentiation of HUWE1cKO from Days 17-27, treated with 100nM 4OHT in the dosing regimen from **A)**. The majority of cells lost *HUWE1* exon81, with a decline in the proportion of KO cells over time.
-

To verify this result orthogonally, I collected mRNA at multiple timepoints from Days 14-27 and performed qRT-PCR for *HUWE1* exons 81 (the deleted region) and exons 67-68, quantifying the proportion of *HUWE1* transcripts left after 4OHT treatment versus the vehicle condition (Figure 17A). Unfortunately, the results disagreed with those from Figure 16D, with at most 50% of cells deleting exon 81 and becoming HUWE1 knockouts at Day 14 instead of a majority. However, similar to the previously-observed results, the proportion of knockout cells gradually decreased over time, starting from around Day 17, again suggestive of a selection bias. This continued until HUWE1cKO cultures appeared to have *HUWE1* expression levels similar to their vehicle-treated counterparts at around Day 20, suggesting that only WT cells remained by then. The error bars at Day 20 are large however, and later experiments show knockouts are still present then.

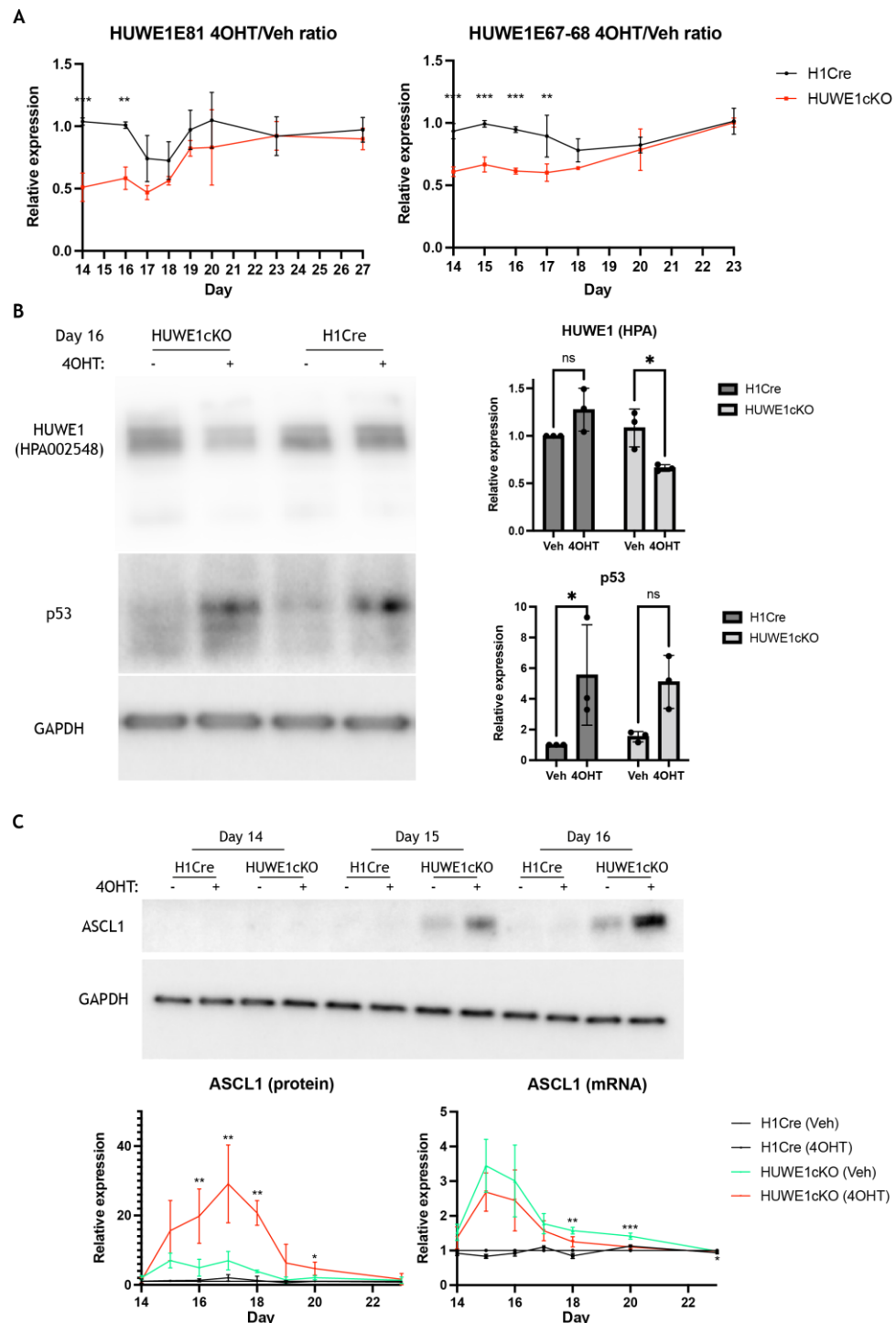


Figure 17. HUWE1cKO show stabilization of ASCL1 but not p53 despite mosaic recombination.

A) qRT-PCR for *HUWE1* transcripts at the deleted exon, and upstream show a reduction in *HUWE1* transcripts in *HUWE1cKO* from Day 14, when treated with 4OHT, consistent with mosaicism. This reduction is gradually lost after Day 17. Data represent mean $\pm$ SD.

B) Western Blot of *HUWE1cKO* at Day 16, with quantifications shown. *HUWE1* is reduced to levels consistent with qRT-PCR results in **A**, but not to that expected from PCR-based quantifications (Figure 16D). 4OHT treatment increased p53 levels in both *HUWE1cKO* and H1Cre, with no interaction dependent on genotype ($F(1, 8) = 0.209$, $p = 0.660$). Data

represent mean $\pm$ SD. Two-way ANOVA with Sidak's multiple comparisons test on pre-defined pairs shown.

- C)** Western Blot for ASCL1 from HUWE1cKO at multiple timepoints, quantified below. ASCL1 is first detected at Day 15, and strongly stabilized in HUWE1cKO + 4OHT, before declining to levels similar to that in H1Cre. qRT-PCR for *ASCL1* shows no increase in transcripts in HUWE1cKO (4OHT) vs HUWE1cKO (Veh). The HUWE1cKO line expressed *ASCL1* earlier than H1Cre. Data represent mean $\pm$ SD. One-way ANOVA was performed at each timepoint with Sidak's multiple comparisons test on both pre-defined Veh vs 4OHT pairs. All ns unless indicated.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Western Blotting for HUWE1 at Day 16, when KO cells are definitely present as assessed by both gDNA and qPCR methods, showed HUWE1 levels to be decreased to levels (~60%, suggesting a ~40% knockout proportion) more in-line with numbers from the qPCR method (Figure 17B). In a later chapter, HUWE1cKO is shown by flow cytometry to undergo recombination and knockout in ~40% of cells, in agreement with both the Western Blotting and qPCR results here. Why the gDNA-based method failed despite being shown to be quantitative remains unknown.

Previously, p53 was shown to be increased in the absence of HUWE1, even during early neural differentiation. However, at Day 16, I found that p53 no longer appeared to be a substrate of HUWE1 (Figure 17B), with no increase in p53 levels beyond the increase caused by 4OHT alone ($p^{\text{interaction}(\text{genotype} \times 4\text{OHT})} = 0.660$, $F(1, 8) = 0.209$) despite the ~40% knockout rate. This is reminiscent of observations from Zhao et al. 2008¹⁷⁸ which showed that p53 is not increased in IMR-32 (a human neuroblastoma line) subjected to siRNA against *HUWE1*, nor is it increased in *Huwe1* gene-trapped mouse ESCs subjected to neural differentiation. Together, these would suggest that p53 is not a substrate of HUWE1 in neural cell types, and thus that HUWE1's role in neurogenesis does not involve p53, at least at later stages. Additionally, this clearly demonstrates the advantage of including H1Cre as a control for 4OHT-specific effects to avoid false-positives.

However, it is also possible that no further increase in p53 was observed in my case as the knockout proportion was too low, or the 4OHT-induced p53 increase masked the effects of HUWE1. Regarding the latter point, as seen in Figure 15B, loss of HUWE1 should be able to cause an increase in p53 levels beyond that induced by

4OHT alone if p53 is a valid substrate in the cell-type. As for the former point, to address it would require single-cell analyses limited only to the HUWE1 knockouts – a theme that will recur in the following figures.

Given that I now appeared to have viable HUWE1-knockout neural progenitors at timepoints when *ASCL1* should be expressed (Figure 6), I proceeded to investigate if HUWE1 regulates *ASCL1*'s degradation in humans, similar to in mouse neural stem cells^{185,191}. By Western Blotting of protein samples from Days 14-23, I found that *ASCL1* was first detectable from Day 15 (Figure 17C), with a highly significant increase in HUWE1cKO (4OHT) up to Day 20. This increase was on a post-translational level since *ASCL1* mRNA levels are either similar or lower in HUWE1cKO (4OHT) compared to its isogenic vehicle-treated condition (Figure 17C, red vs green lines). 4OHT itself did not have an effect on *ASCL1* expression at all timepoints (black lines comparing H1Cre 4OHT vs H1Cre Veh). Together, this demonstrates for the first time that *ASCL1* protein stability is regulated by HUWE1 in human neural progenitors. Incidentally, the increase in *ASCL1* levels immediately precedes the gradual loss of HUWE1 knockout cells in culture after Day 17. The observation that *ASCL1* is still significantly stabilized at Day 20 also suggests there are still HUWE1-knockouts then, contrary to Figure 17A.

A line-specific difference in *ASCL1* expression was also found, with HUWE1cKO expressing *ASCL1* earlier than H1Cre, consequently resulting in earlier protein detection (Figure 17C). Fortunately, since H1Cre is only included to control for 4OHT's effects, and the experiment design is such that HUWE1cKO 4OHT and Veh conditions come from the exact same culture prior to splitting at Day 12, any differences observed are highly controlled for, and derive solely from a difference in HUWE1 status in the HUWE1cKO line – with these line-specific differences being less relevant. Another observation is that *ASCL1* expression in HUWE1cKO (4OHT, red line) eventually returns to levels similar to HUWE1cKO (Veh, green line), perhaps due to the HUWE1 knockout cells gradually being replaced by WT cells in culture and *ASCL1* transcript levels in H1Cre catching up with HUWE1cKO.

4.3 HUWE1cKOs show no population-level differentiation phenotypes

I next sought to look for other HUWE1-knockout phenotypes and verify some of the previous observations through qRT-PCR, focusing on Days 14-20 when HUWE1 knockout cells' presence is less debatable. Cre expression was found to be decreasing in both H1Cre and HUWE1cKO over time as cells become more neural (Figure 18A), suggesting a silencing of either the CAG promoter or the *AAVS1* locus, which could explain why incomplete and mosaic recombination occurs in response to 4OHT during neural differentiation. More critically, even though 4OHT treatment should not affect Cre expression (H1Cre 4OHT vs Veh, black lines), there is a gradual divergence leading to a greater-than-expected reduction in Cre expression in HUWE1cKO treated with 4OHT. This suggests a selection bias against Cre-expressing HUWE1cKO cells in the presence of 4OHT – cells which should tend to be HUWE1 knockout cells, since Cre expression is necessary for *HUWE1* exon 81 deletion. This again falls in line with my previous observations on both gDNA and *HUWE1* transcript levels showing a gradual loss of knockout cells in culture, although the mechanism is unknown for now.

I also showed previously a 4OHT-specific increase in p53 levels, with no involvement of HUWE1, in neural progenitors (Figure 17B). This was also supported by qRT-PCR for p53's transcription target *p21* (Figure 18B) which showed an increase in transcript levels with 4OHT treatment, in both H1Cre and HUWE1cKO. Crucially, *p21* levels in HUWE1cKO (4OHT) are consistently lower than H1Cre (4OHT). This would also argue against suppressing p53 signalling being the mechanism through which HUWE1 affects neurogenesis in neural progenitors, since loss of HUWE1 does not increase the expression of p53's target *p21*, and having more p53 signalling than HUWE1cKO (4OHT) has no effect on neural differentiation in H1Cre (4OHT) (Figure 18C, D, black lines). Together, these results would suggest that p53 is only a substrate of HUWE1 during early neurogenesis, irrelevant after a critical timepoint (~Day 10 in this protocol, and Day 14 in Aprigliano et al. 2021¹⁶⁸). An implication therefore is also that rescuing the p53-dependent differentiation block seen in HUWE1 patient-derived iPSCs during neural differentiation by constitutive knockdown of p53¹⁶⁸ only allows HUWE1-deficient cells to survive the early critical

timepoint, but other p53-independent phenotypes may still be present, such as the ASCL1 stabilization I observed and its effects.

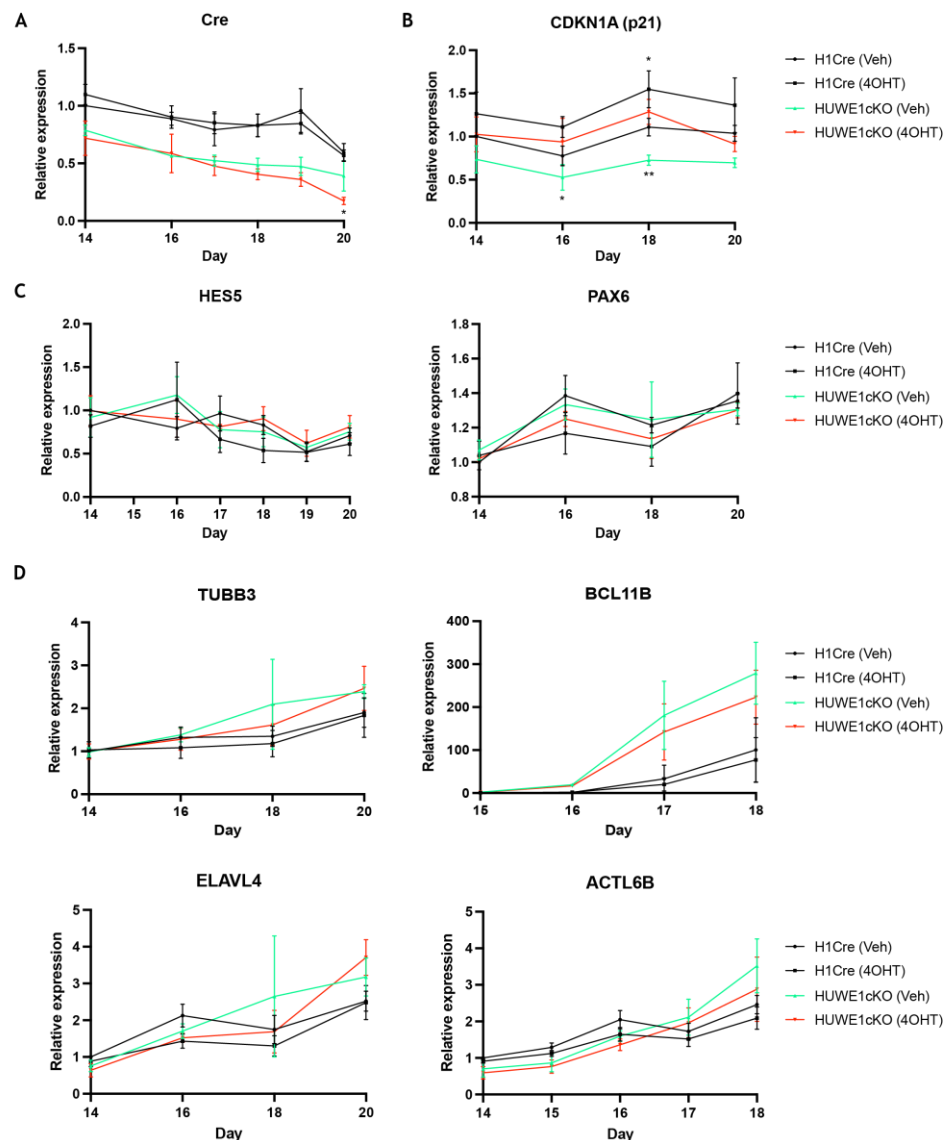


Figure 18. HUWE1cKO shows no difference in differentiation markers by qRT-PCR.

- A)** qRT-PCR for *Cre* transcripts in HUWE1cKO over Days 14-20. *Cre* expression, which should be unaffected by 4OHT (H1Cre), decreases over time in HUWE1cKO with 4OHT treatment supporting a hypothesis of selection pressure against *Cre*-expressing HUWE1cKO cells.
- B)** *CDKN1A* (p21) expression is increased with 4OHT treatment, in line with increased p53 following 4OHT exposure (Figure 17B), but also not higher than expected in HUWE1cKO.
- C)** Markers of neural progenitors, *HES5* and *PAX6* are not altered in HUWE1cKO with 4OHT exposure.
- D)** Neuronal markers *TUBB3* (TUJ1), *BCL11B* (CTIP2), *ELAVL4* (HuD), and *ACTL6B*, are also unchanged in HUWE1cKO (4OHT) vs HUWE1cKO (Veh).

All data represent mean $\pm$ SD. One-way ANOVA was performed at each timepoint with Sidak's multiple comparisons test on both pre-defined Veh vs 4OHT pairs. All ns unless indicated. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

While endogenous ASCL1's role in human cortical neural differentiation has not been studied (prior to Păun et al. 2023²²⁰), the expected consequences of an overexpression of ASCL1, such as what is induced in HUWE1cKO (4OHT), are well-studied. Overexpression of ASCL1 has been shown in several studies to force neural differentiation in a variety of cell types due to its pioneer activity^{136–139,233}, thus I next looked at expression of canonical markers of neural progenitors (*HES5*, *PAX6*) and neurons (*TUBB3*, *ELAVL4*, *BCL11B*, *ACTL6B*). Strangely, despite the strong stabilization of ASCL1 in HUWE1cKO (4OHT), there was no greater decrease in expression of progenitor markers (Figure 18C) nor greater increase in neuronal markers (Figure 18D) over the vehicle control at any timepoint after ASCL1 stabilization. This could have been due to the knockouts being in the minority, and thus any phenotype being diluted by wildtypes, especially at later timepoints when HUWE1 knockouts are gradually replaced – a limitation of the mosaic nature of HUWE1cKO (4OHT) cultures applicable to all negative results I obtained.

To address this drawback, I needed to individually identify HUWE1-knockouts to perform single-cell-resolution analyses. For this, I attempted to use the HUWE1 antibody (CST#5695) previously characterised (Figure 13D, Figure 14B) to discriminate between wildtype and HUWE1 knockout cells in HUWE1cKO (4OHT) cultures at Day 20 (Figure 19A), since it was able to do so in a mosaic co-culture of HUWE1cKO and unedited H1 hESCs (Figure 14B). Unfortunately, even though some cells appeared by eye to lack HUWE1 expression (DAPI+ HUWE1-, overall green cells) (Figure 19B), there was a high variability in HUWE1 intensities between fields of view and even within a single field of view that was probably due to the culture confluency and stacking of cells into multiple depths of view. As a result, in this context CST#5695 was unable to reliably discriminate between WT and KO cells when attempting to quantify images in an automated and unbiased manner (Figure 19C), failing to give 2 distinct populations of HUWE1 intensities.

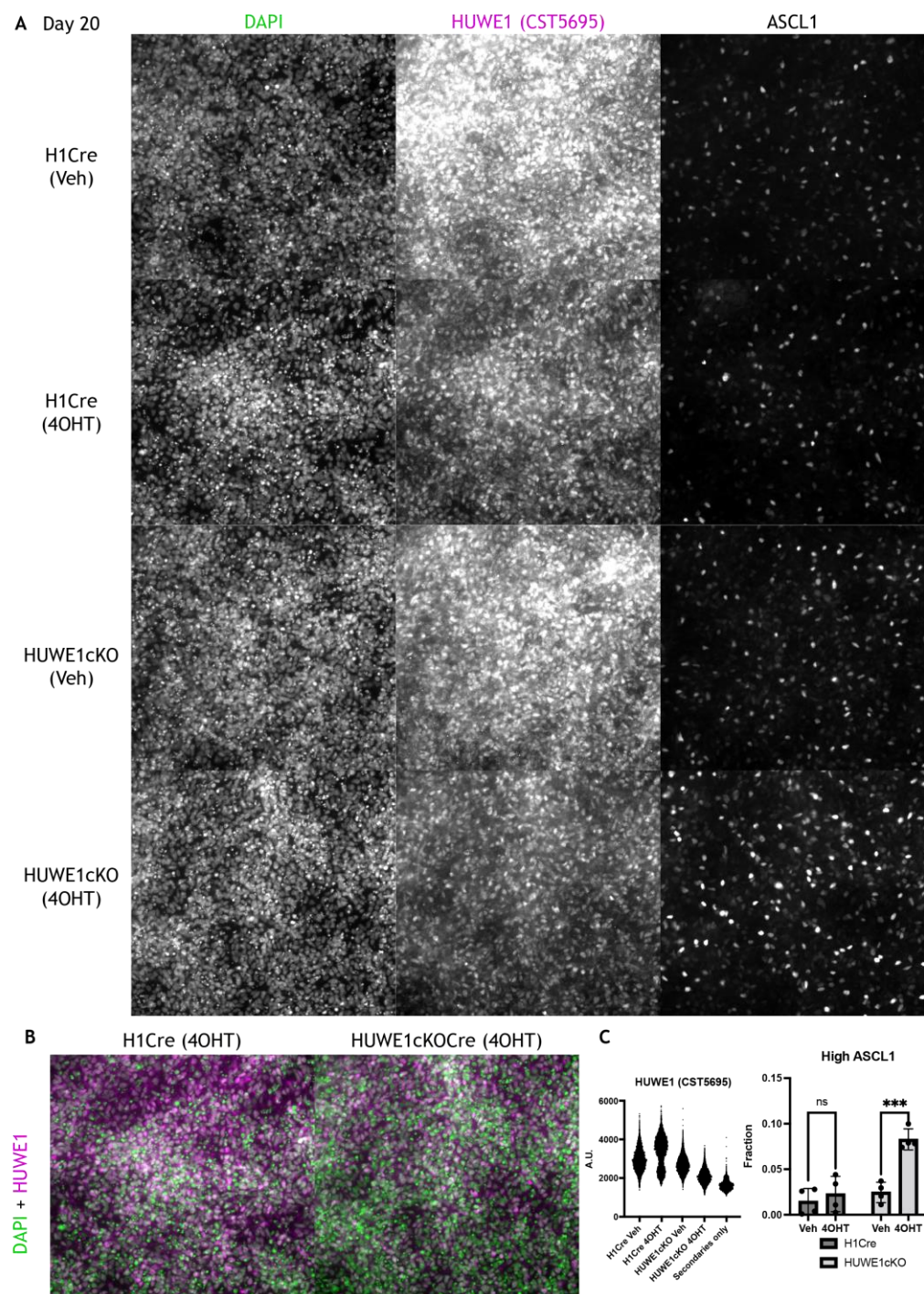


Figure 19. Immunofluorescence cannot reliably identify HUWE1 KO cells in mosaic neuronal cultures.

- A)** Immunofluorescence images of Day 20 cultures stained with the CST5695 antibody and for ASCL1 (ab211327). ASCL1 is expressed in a salt-and-pepper pattern, with more highly-expressing cells in HUWE1cKO (4OHT). Images captured on an SP5 II confocal microscope.
- B)** Merge of the DAPI and HUWE1 channels for both 4OHT-treated cultures.
- C)** Quantification of HUWE1 fluorescence intensities and fraction of ASCL1-high cells. CST5695 failed to resolve two populations of HUWE1 intensities in HUWE1cKO 4OHT. Data from 3 fields of view from one coverslip per condition. The fraction of cells highly-expressing ASCL1

is increased in HUWE1cKO (4OHT). Data represent mean $\pm$ SD from multiple differentiations. Two-way ANOVA with Sidak's multiple comparisons test between the predefined pairs shown.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Despite this setback, I was able to use the collected Day 20 cells to confirm previous observations by immunofluorescence. For example, ASCL1 was found to be expressed in a heterogeneous “salt-and-pepper” pattern with many more cells expressing the highest levels of ASCL1 in HUWE1cKO (4OHT) (Figure 19A, C), confirming the ASCL1 stabilization previously seen. Additionally, on a population-level there was again no difference in neural differentiation markers (PAX6, ELAVL3/4) (Figure 20A, B) despite the ASCL1 stabilization. Since ASCL1 is expressed in the ventral telencephalon in mouse where it confers a ventral identity in neural stem cells²³⁴, I also checked for OLIG2 (ventral neural progenitor and glial marker) expression, finding almost no expression and no change in HUWE1cKO (4OHT) (Figure 20A, B). This suggests that the increase in ASCL1 is also not causing a ventralization of the cultures, unlike what may have been expected based on observations from *Ascl1* upregulation in *Neurog1/2*^{-/-} mice⁷². Additionally, this again supports the idea that knockouts are still present at Day 20, which is subsequently confirmed by flow cytometry in a later chapter.

Just like before, these negative results are from population-level analyses, and thus unfortunately of less value in my mosaic system, as there could still be a phenotype that is diluted by the larger presence of wildtype cells. Additionally, the ASCL1 stabilization seen is not necessarily specific to HUWE1 knockout cells, and could be non-cell-autonomous for example. Clearly, a method to successfully discriminate between HUWE1 knockouts and unrecombined WT cells in mosaic HUWE1cKO cultures was sorely needed. I therefore focused my subsequent efforts on finding a single-cell analysis strategy capable of doing just that.

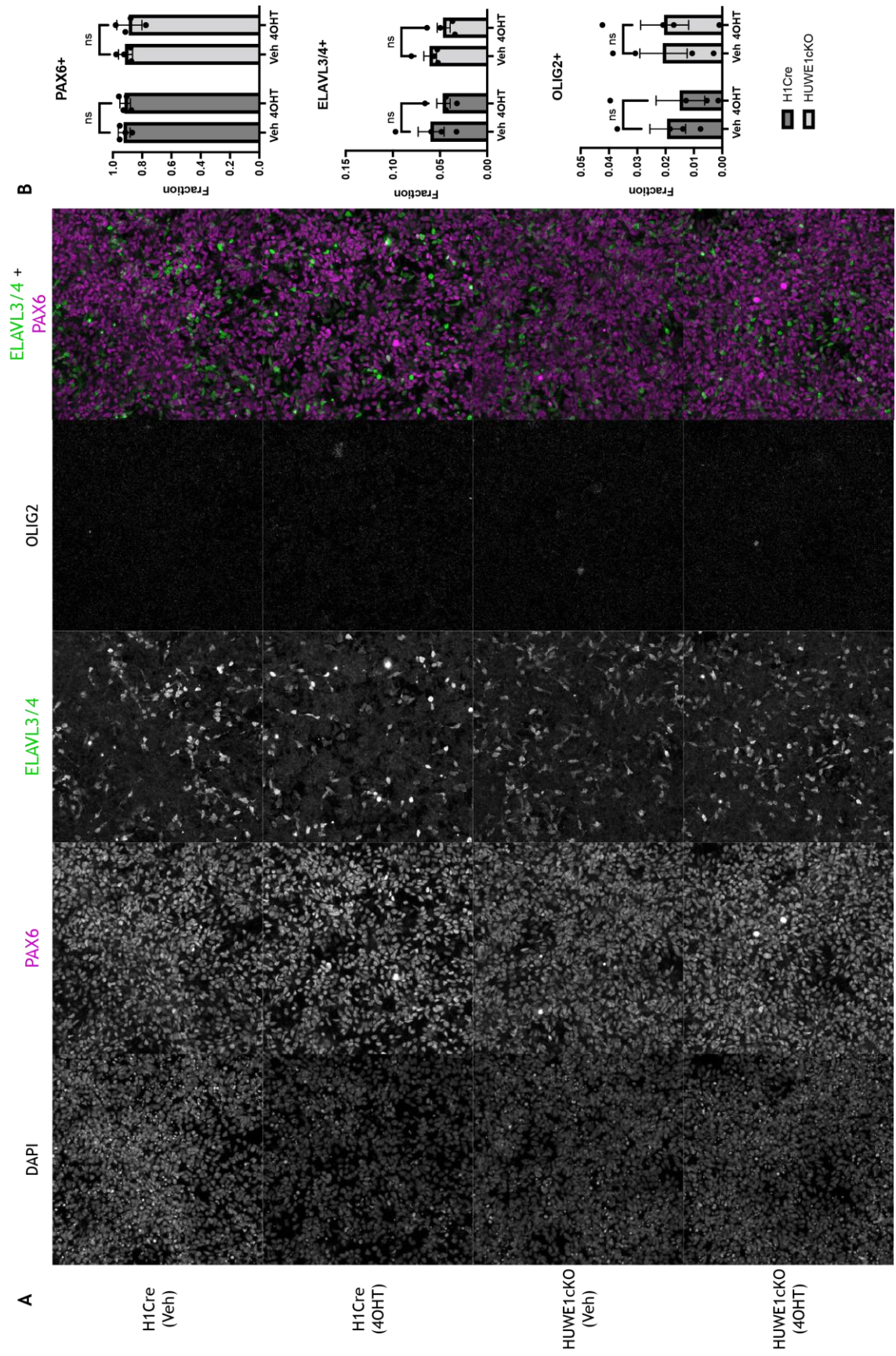


Figure 20. Immunofluorescence shows no change in differentiation markers in HUWE1cKO.

A) Immunofluorescence images of Day 20 cultures stained for PAX6 (neural progenitors), ELAVL3/4 (neurons), and OLIG2 (glia and ventral neural progenitors). Images taken on an SP5 II confocal microscope.

B) Quantification of fraction of cells positive for each marker in **A)**. None of the markers were altered in HUWE1cKO (4OHT). Data represent mean $\pm$ SD. Two-way ANOVA with Sidak's multiple comparisons test between the predefined pairs shown.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Results 3: Single-cell RNA sequencing identifies an *ASCL1*-dependent transitional progenitor

A method gaining in popularity in recent years, and an ideal candidate to both identify *HUWE1*-knockouts and detect phenotypic changes in them, is single-cell RNA sequencing (scRNAseq). Broadly-speaking, scRNAseq exploits the combination of single-cell isolation techniques, library preparation, and next-generation sequencing to obtain transcriptomes on a single-cell resolution. Each of these categories has seen rapid development in recent years, from manually-picking cells to flow-assisted cell-sorting (FACS) and microfluidics for single-cell isolation; and microarrays to Illumina short-read sequencing and Nanopore long-read sequencing (reviewed in Olsen and Baryawno 2018²³⁵, and Hwang et al. 2018²³⁶). Concurrently, we have seen an explosion in the number of bioinformatics tools available to process (such as read-quality assessment, read-alignment, normalisation) and analyse such data²³⁷ (such as batch-correction and integration, data visualisation, differential gene expression analysis, trajectory inference).

scRNAseq has successfully been used to study the effects of autism-associated risk genes on neural differentiation in organoids, finding shifts in neuronal subtype proportions over time in an unbiased manner²³⁸. Our lab was also able to use it to aid in distinguishing proliferative and quiescent hippocampal neural stem cells from tamoxifen-treated *Ki67CreERT2;lox-stop-lox-tdTomato;Nestin-GFP* mice by their *tdTomato* and *GFP* transcripts²³⁹. These examples gave hope that *HUWE1*cKO cells could similarly be genotype-segregated and have their phenotypes investigated in an unbiased manner via scRNAseq.

In this chapter, I first perform scRNAseq on our dual-SMAD inhibition differentiation model in an attempt to identify *HUWE1*-knockouts. While ultimately unsuccessful, I instead discovered an *ASCL1*-enriched population expressing markers of differentiating neural progenitors which we term “transitional progenitors”. I subsequently show in collaboration with Oana Păun and Siew-Lan Ang of the Guillemot Lab that this transitional progenitor population requires *ASCL1* to form,

and gives rise to neurons. The complete results of this collaboration can be found in Păun et al. 2023. I then show that the stabilization of ASCL1 in the HUWE1cKO line treated with 4OHT results in an increase in some of the unique markers for transitional progenitor. All scRNAseq in this chapter was performed using the 10X Genomics Chromium platform, with Illumina short-read sequencing, and analysed using Seurat^{208,240}.

5.1 scRNAseq reveals an ASCL1-defined transitional progenitor but cannot identify HUWE1-knockouts

H1Cre and HUWE1cKO cells at Day 20, following 4OHT treatment as before, were dissociated into single-cells and barcoded-libraries prepared from them using the 10X Chromium microfluidics-based platform. Day 20 was chosen for this pilot as a compromise between waiting too long (by which time HUWE1-knockouts may have been completely lost) and giving sufficient time for a phenotype to develop after the end of 4OHT exposure. There were also prior indications that HUWE1-knockouts were present at Day 20 (more ASCL1-high cells, Figure 19; increased ASCL1 levels, Figure 17), which flow cytometry analyses in the next chapter confirmed (two HUWE1-intensity populations at Day 20, Figure 35).

Libraries were then sequenced, giving a total of 25,032 cells (Figure 21A) after quality-control as performed by Stephanie Strohbuecker of the Bioinformatics and Biostatistics STP (BABS). After unsupervised clustering and Uniform Manifold Approximation and Projection (UMAP) to visualise the data, 10 clusters were found based on the similarity of cells' transcriptomes (Figure 21A). Before attempting to find HUWE1-knockouts, I first needed to understand and orient myself to the UMAP. The 10 unbiased clusters were thus grouped into 4 major cell-types (Figure 21B) by looking at cell-cycle states (Figure 21C), expression of canonical markers (Figure 21D), and the differentially-expressed genes that define each unsupervised cluster (Figure 21E).

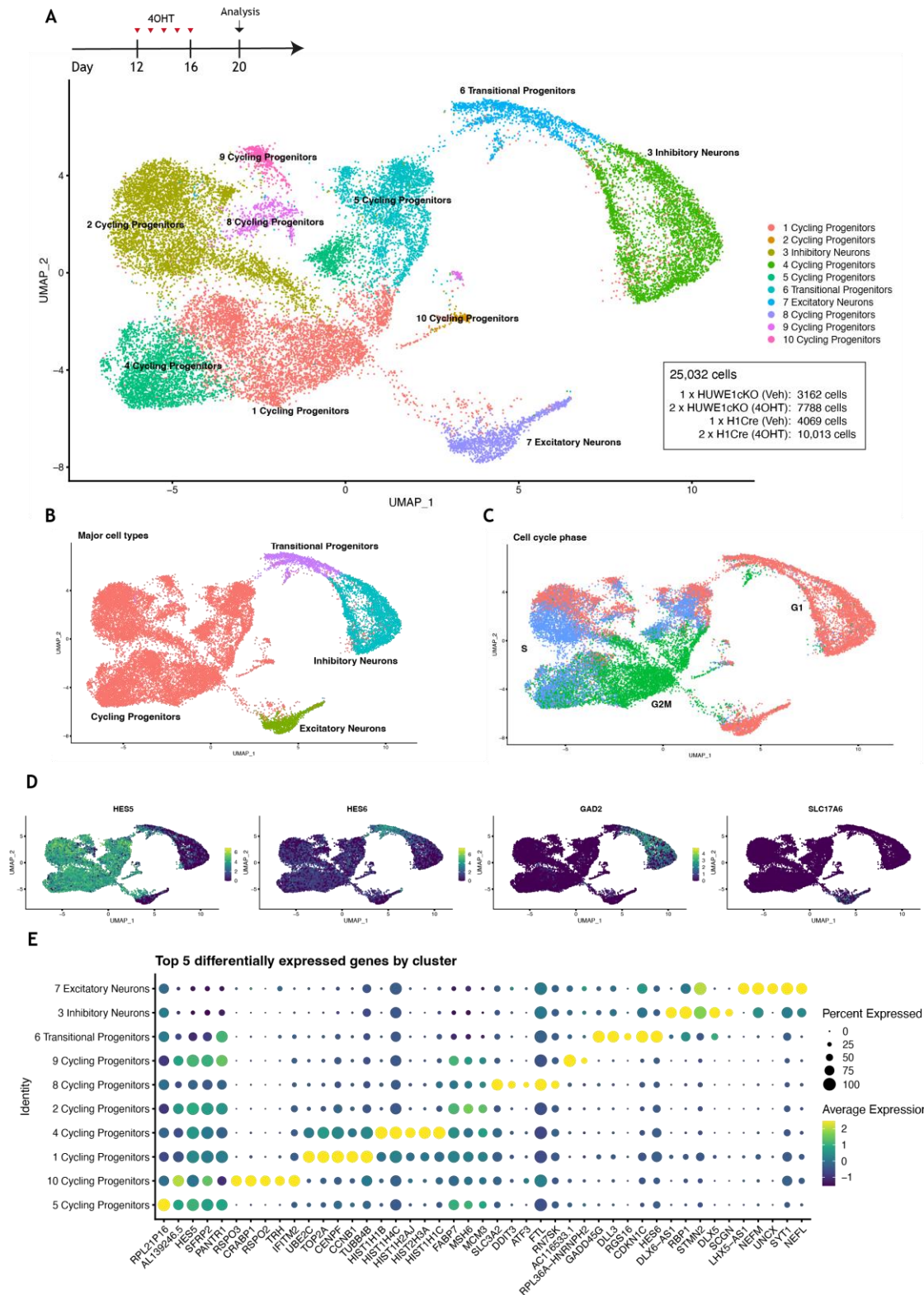


Figure 21. Single-cell RNA sequencing of Day 20 cultures identifies 4 major cell types.

A) scRNAseq was performed on H1Cre and HUWE1cKO cells at Day 20 following 4OHT or vehicle treatment and analysed using Seurat. Uniform Manifold Approximation and Projection (UMAP) plot and unsupervised clustering of single-cell transcriptomes from 25,032 cells. Dots represent single cells. Colours represent the different clusters identified on the right.

- B)** Clusters from **A)** were grouped into 4 broad cell-types based on cell-cycle and the expression of canonical markers (see **D**).
 - C)** Predicted cell-cycle states from Seurat's cell-cycle analysis. Neurons and transitional progenitors are in the G1 state.
 - D)** Representative canonical markers for the 4 annotated major cell-types: *HES5* (Cycling neural progenitors), *HES6* (differentiating progenitors), *GAD2* (inhibitory neurons), *SLC17A6* (*VGLUT2*, excitatory neurons).
 - E)** Top 5 differentially-expressed genes for each of the 10 unbiased clusters in **A)**, and the variation in their expressions across the clusters. Dot size indicates proportion of cells in each cluster expressing a gene, shading indicates the relative level of gene expression.
-

Cycling progenitors were defined as clusters in S, G2M phases, with the highest expression of the neural progenitor markers *HES5*, *VIM*, *NES*, *HES1*, *FABP7*, and cell-cycling genes. Neurons were defined as clusters in G1/0, and expressing neuronal markers such as *STMN2*, *MAP2*, *TUBB3*, *NEFM*, *NEFL*.

Neurons were then subdivided into inhibitory and excitatory neurons as there were 2 distinct neuronal clusters that differed from each other by their unique expression of *GAD2* and *SLC17A6* (*VGLUT2*, Figure 21D).

Cluster 6, which was in G1, was found to uniquely express or be enriched for markers of neural differentiation such as *HES6*^{241–243} (Figure 21D), *SOX4*²⁴⁴, and *BTG2/TIS21*²⁴⁵ (Figure 22B) and cell-cycle arrest genes like *GADD45G* and *CDKN1C*. Simultaneously, it retained neural progenitor markers like *VIM*, and was negative for neuronal markers like *STMN2* (Figure 22A). These, along with its position as a bridge between cycling progenitors and neurons, led me to deduce that Cluster 6 represented transitional progenitors exiting the cell-cycle and undergoing terminal differentiation.

Interestingly, the transitional progenitors were uniquely-enriched for *ASCL1* and several of its bound-and-regulated direct transcriptional targets such as *RGS16*, *IGFBP5*, *BTG2*, and *DLL1*^{63,73,246} (Figure 22B). Given *ASCL1*'s well-studied roles in driving neuronal differentiation^{27,68,69,74} – particularly when its expression is stable and out of phase with *HES5*⁹⁰ as it appears here – I hypothesised that *ASCL1* might be driving the formation of transitional progenitors from cycling progenitors. Of course, this would require further proof that would come later, in the form of an *ASCL1*-knockout (Figure 27).

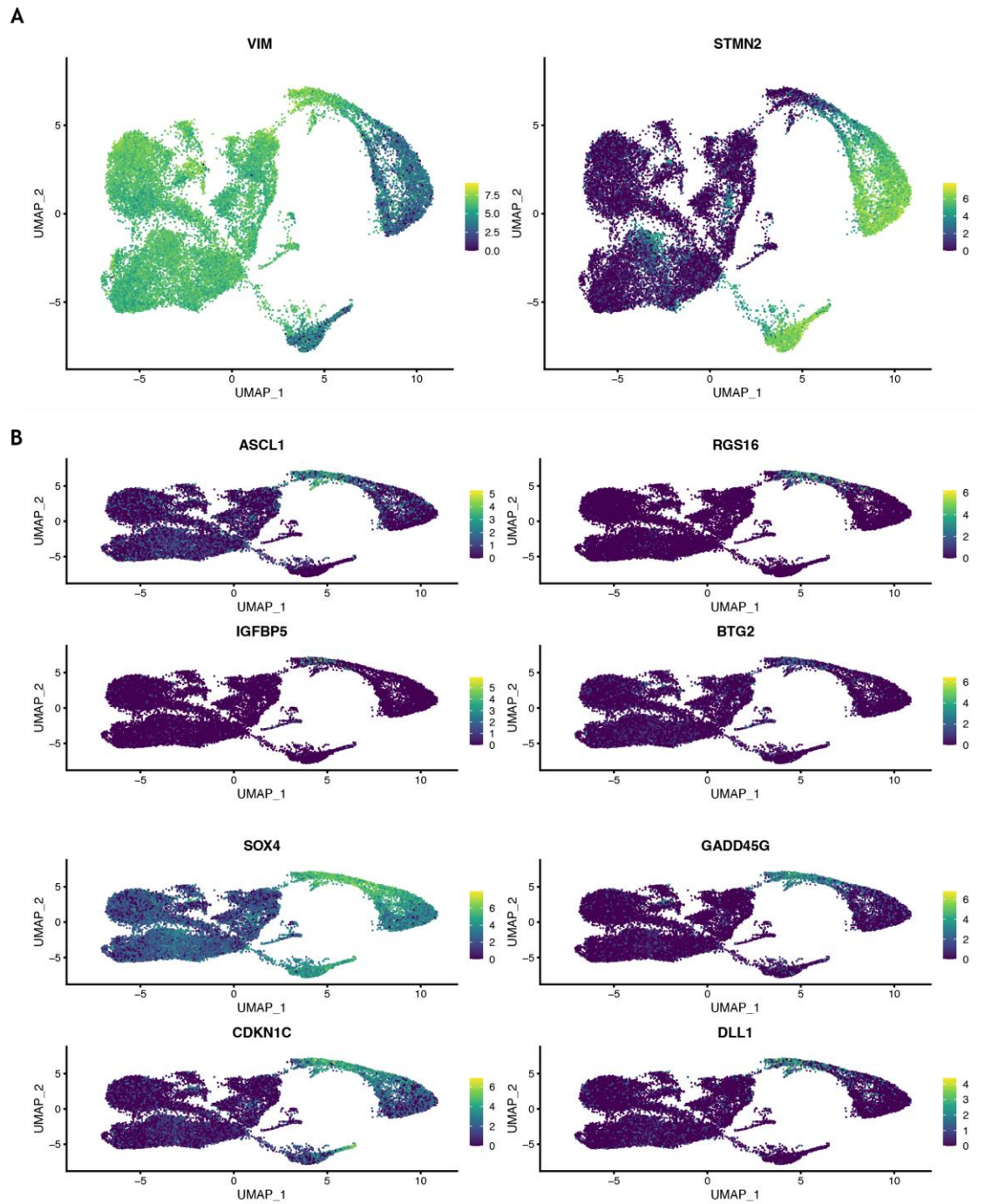


Figure 22. Transitional Progenitors are differentiating progenitors defined by *ASCL1* expression.

A) Transitional progenitors express the progenitor marker *VIM*, and not the neuronal marker *STMN2*.

B) Transitional progenitors are uniquely enriched for *ASCL1* and several of its previously-reported transcriptional targets such as *RGS16*, *IGFBP5*, and *BTG2*^{73,246}. They are also enriched for markers of terminally-differentiating neural progenitors like *SOX4*²⁴⁸ and *HES6*^{241,242} (Figure 21D). Additionally, they are uniquely enriched for the cell-cycle exit genes *GADD45G* and *CDKN1C*⁷³, and the Notch ligand *DLL1*.

The appearance of GABAergic neurons expressing *GAD2* and *DLX5* (Figure 21D, E) in such a high proportion was surprising given these neurons are thought to arise exclusively from ventral telencephalic neural progenitors, at least based on rodent studies²⁴⁷. Before attempting to identify *HUWE1*-knockouts within the UMAP, I thus thought to address this, and exclude the possibility of a misdirected, ventralized, differentiation.

I had previously shown by immunofluorescence that my cultures express the dorsal neural progenitor marker *PAX6* (*PAX6* is exclusively dorsal at early gestational weeks²⁴⁹, which I model here) and not the ventral marker *OLIG2* (Figure 20). This is also seen in the Day 20 scRNAseq dataset, where the cells also express the cortical progenitor markers *EMX2* and *FEZF2*^{42,250}. The Day 20 single-cells also did not express the ventral telencephalon marker *NKX2.1*²⁵¹, nor the medial ganglionic eminence (MGE) markers *LHX6* and *LHX8*³⁰, although they did express the lateral/caudal ganglionic eminence (LGE, CGE) markers *PROX1*²⁸, *DLX2/5*²⁴⁷, and to a smaller extent *GSX2*²⁵¹, *ISL1*²⁵¹, but not D1 and D2 dopamine receptors (*DRD1*, *DRD2*) – all markers of GABAergic striatal projection neurons derived from the LGE – was also expressed in my “inhibitory neurons”. A small fraction of these “inhibitory neurons” expressed calretinin (*CALB2*), but none of them expressed the main GABAergic interneuron subtype markers *PVALB*, *SST* or serotonin receptor subunit A (*HTR3A*)²⁴⁷. All markers mentioned above can be found in Figure 23 and Figure 24.

ASCL1 (*MASH1* in earlier literature) may arguably be considered a ventral marker due to its strong expression in the ganglionic eminences of mouse⁶⁸, where it confers a ventral identity^{27,65,72}. However, recent evidence has shown that *ASCL1* in humans is widely-expressed dorsally^{19,71}. Consequently, it may not be appropriate to consider it nor its downstream transcriptional targets such as *DLX1/2*²³⁴, *PROX1*²⁵², *ISL1*²⁵³ as strictly ventral markers in the human context. My cultures may thus not be ventralized, and this will be discussed in-depth in the Discussion chapter.

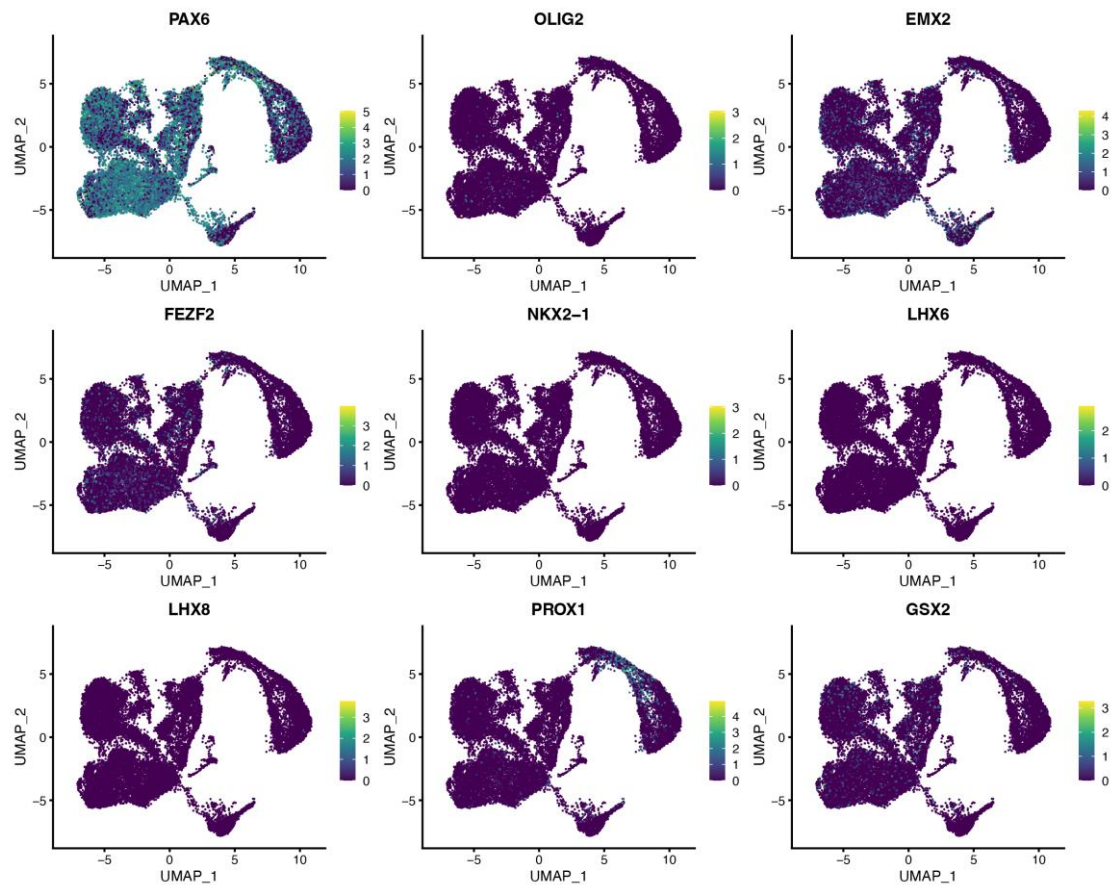


Figure 23. Expression of selected markers in Day 20 cultures. Day 20 cultures express the dorsal telencephalon progenitor markers *PAX6*, *EMX2*, and *FEZF2*, but not the ventral progenitor markers *OLIG2* and *NKX2-1*. They also do not express the MGE-progenitor markers *LHX6* and *LHX8*, although they express the LGE/CGE-associated progenitor markers *PROX1* and *GSX2*.

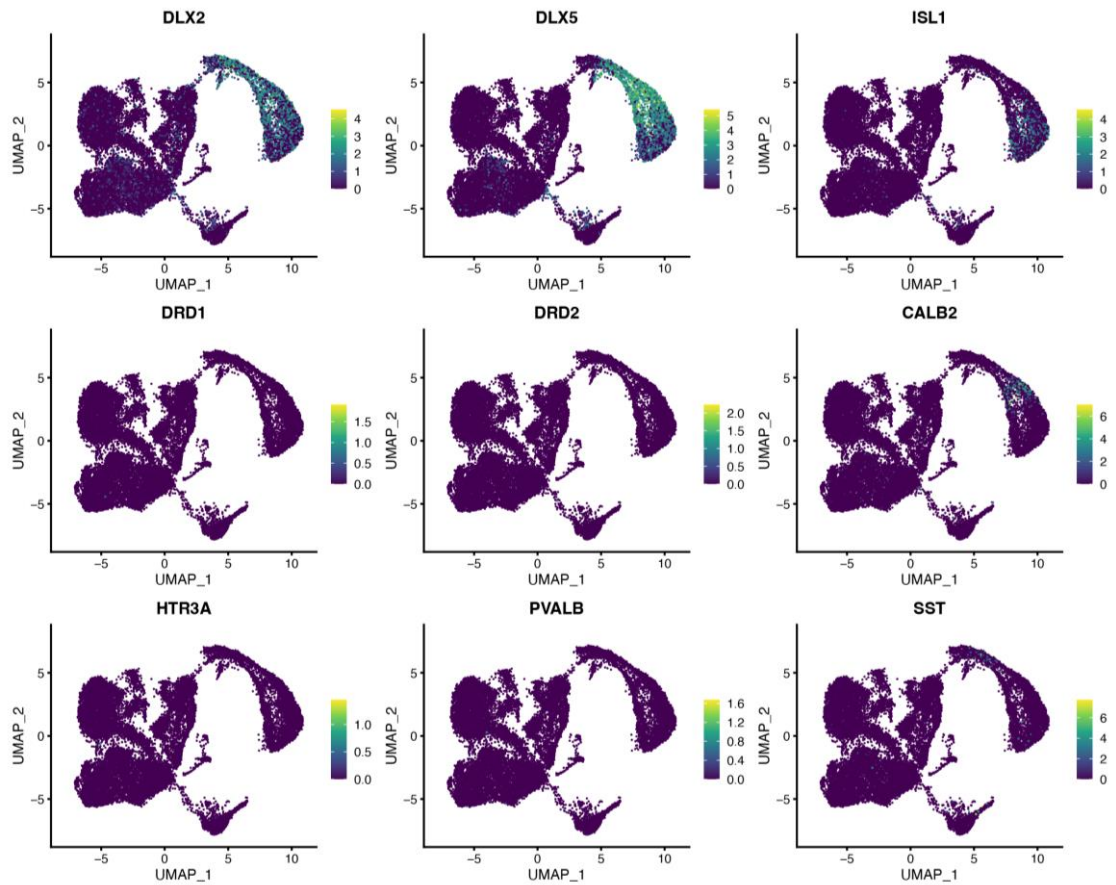


Figure 24. Expression of more selected markers in Day 20 cultures. Day 20 cultures expressed the LGE/CGE-associated progenitor markers DLX2, DLX5, and the LGE/CGE-derived striatal projection neuron marker ISL1. Despite ISL1 expression, the neurons did not express other striatal projection neuron markers DRD1 and DRD2. Of the major GABAergic subtypes, only CALB2 was expressed, and only in a small fraction of the “inhibitory neurons”.

The safest conclusion at this point was that the 2D differentiation model I use captures an *ASCL1*-relevant general neurogenesis at this timepoint, not necessarily into GABAergic neurons. Therefore, I next sought to identify a HUWE1-phenotype within my dataset.

Since I had previously shown that HUWE1 regulates *ASCL1* stability, I would therefore expect an increase in the proportions of “transitional progenitors”, “inhibitory neurons”, and “excitatory neurons” following 4OHT treatment in HUWE1cKOs as a quick population-level readout of a phenotype. This of course assumes *ASCL1* drives formation of transitional progenitors and is not just a marker, which I have not yet shown. Unfortunately, this was not the case, with no clear difference in the distribution of cell-types between Veh and 4OHT-treated

HUWE1cKOs (Figure 25A) – again very similar to all my previous genotype-agnostic analyses. I therefore needed to use the single-cell transcriptomes to identify HUWE1-knockouts within HUWE1cKO (4OHT-treated) samples.

The first approach I tried was to use a *HUWE1* transcript cut-off, since I previously showed HUWE1-knockouts should have drastically lower levels of *HUWE1* transcripts. In a mosaic culture, this would theoretically manifest as 2 discrete levels of *HUWE1* levels. Unfortunately, this was also not the case (Figure 25B, green), which is probably due to an insufficient read depth and the high dropout rate of scRNAseq²⁵⁴. Another approach I tried with the aid of Stephanie Strohbuecker (BABS) was to re-align the reads to a custom human genome to detect:

1. Reads that span exons 80 and 82, skipping the deleted exon 81.
2. Intron 80-81 reads that contain a single loxP while spanning across the deleted region (deleted region skip).
3. Reads that fall within the intron 80, 81, and exon 81 deleted region (Figure 25C).

Unfortunately, this approach didn't bear fruit either, with only 4 cells in total being identified as definitely HUWE1-knockouts (Figure 25C). Again, this was probably due to the dropouts and low sequencing-depth. Additionally, this approach would probably have been more successful if done on libraries with full-length transcripts such as those generated by SMART-Seq, and not ones prepared through 10X Chromium – although in that case, the resulting transcriptomes would not be quantitative and so also of limited use²⁵⁵.

Given the failure of single-cell RNA sequencing in allowing me to identify HUWE1-knockouts, I did not repeat the experiment further on HUWE1cKOs. However, scRNAseq showed that HUWE1cKO cells treated with 4OHT are represented in every cluster, indicating that loss of HUWE1 did not result in new or missing cell-types. Loss of HUWE1 also did not result in any major shifts in cell-type proportions on a population level. More importantly, scRNAseq revealed the existence of an *ASCL1*-defined transitional progenitor which I next sought to characterise better, as it could be the cell-type relevant to HUWE1's function given I now knew HUWE1 regulates *ASCL1* stability in humans too.

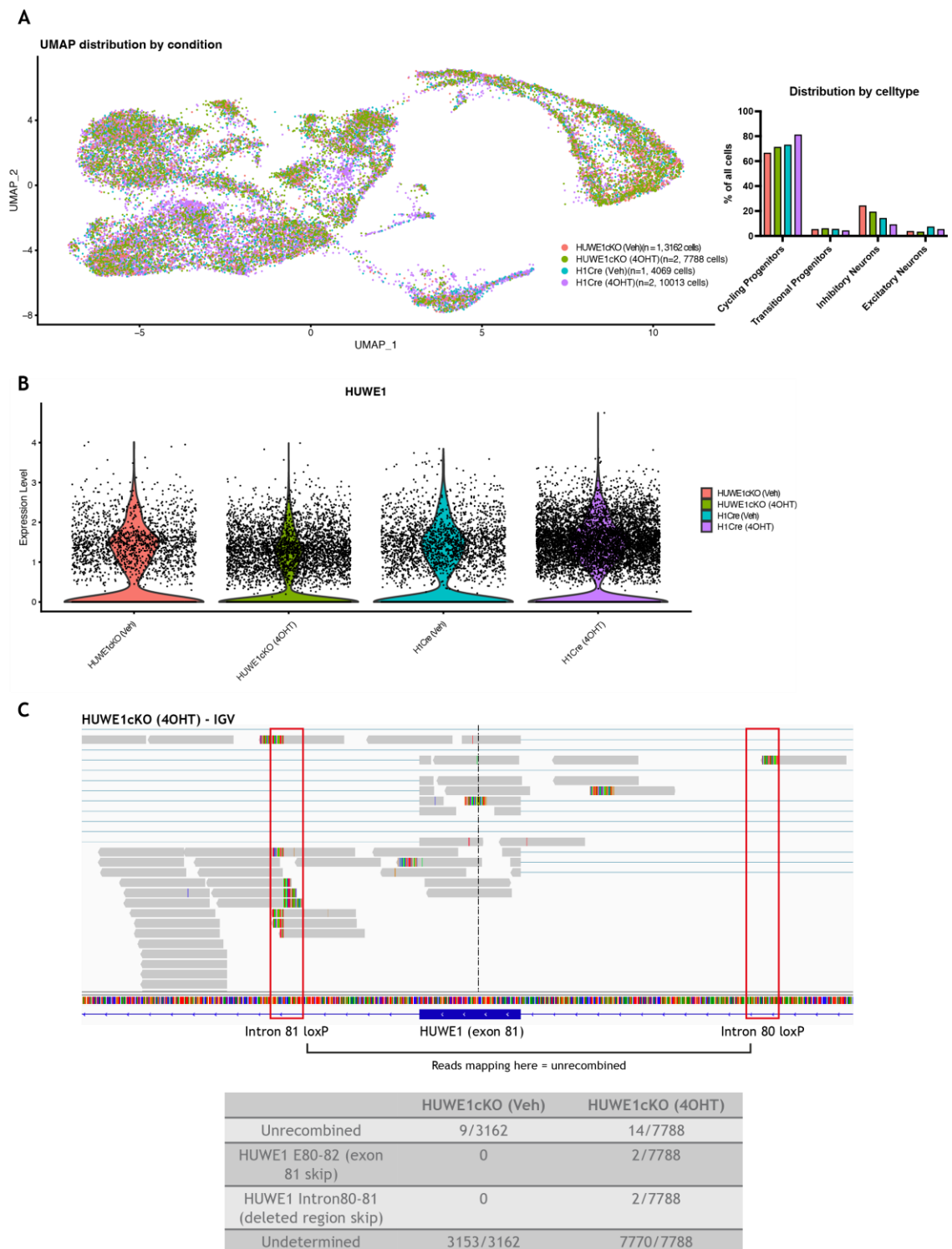


Figure 25. Single-cell RNA sequencing cannot identify HUWE1 KO cells.

A) UMAP from Figure 21A, showing distribution of cells by their genotype and 4OHT-treatment. There was no obvious effect of 4OHT on HUWE1cKO's distribution across cell-types, quantified on the right.

B) Normalised *HUWE1* read counts in individual cells from each of the conditions. HUWE1cKO (4OHT-treated) did not segregate into 2 discrete levels of *HUWE1*.

- C) Raw reads from HUWE1cKO (4OHT-treated) mapped onto the human genome at *HUWE1* exon 81. Reads that align to the reference are in grey, while mismatches (loxP sites or barcodes) are coloured. Red boxes indicate where the inserted loxPs are. Reads falling within the 2 loxP sites indicate a cell is wildtype and unrecombined, whereas reads skipping the deleted region or the deleted exon prove a cell is a HUWE-knockout. Only 4 such cells were found, all within HUWE1cKO (4OHT-treated).
-

5.2 *ASCL1* drives differentiation of cycling progenitors into transitional progenitors and neurons

While I had found that *ASCL1* appears to define a population of neural progenitors exiting the cell-cycle and initiating terminal differentiation, I had yet to demonstrate *ASCL1*'s role in these transitional progenitors. As others in the Guillemot lab were also working on *ASCL1*, there were both interest and opportunity to study the transitional progenitors further. The following work has been published in Păun et al. 2023²²⁰.

Siew-Lan Ang had previously generated 3 *ASCL1*-knockout clones in the KOLF2 hiPSC background, which would allow us to study on a single-cell level the effects of *ASCL1* loss on transitional progenitors. To do that, Oana Păun and I differentiated the 3 *ASCL1*-knockouts alongside 3 passages of wildtype KOLF2 to Day 24, and harvested the cells then for scRNAseq, 24hrs post-DAPT (Notch inhibitor) treatment. Concurrently, 3 wildtype KOLF2s differentiated to Day 24, without the addition of DAPT, were also harvested. Day 24 was chosen as *ASCL1* expression peaked then, following DAPT addition²²⁰, and Oana was interested in studying the mechanisms of endogenous *ASCL1*'s pioneer activity in humans which required a timepoint when *ASCL1* expression was maximal. Differentiations were performed in parallel and library-preparation and sequencing on the samples were also performed in parallel to minimize batch effects. Bioinformatic analyses on this dataset were performed by Stephanie Strohbuecker (BABS), Avinash Ghanate (BABS) and me (detailed breakdown can be found in Materials and Methods).

In total, 28,316 wildtype KOLF2s (+ DAPT) passed quality control, and unsupervised clustering of only these cells in Seurat^{208,240} resulted in 22 clusters, shown in Figure 26A. Clusters were then grouped together based on their expression of canonical progenitor and neuronal markers, and markers for transitional progenitors

discovered in Figure 22, giving 3 cell-type clusters (Figure 26B, D). RNA velocity analysis²¹⁶ showed 2 main trajectories – one within the cycling progenitors, reflecting the transition of cell-cycle states (Figure 26B, C), and another from cycling progenitors into transitional progenitors and subsequently neurons (Figure 26B). This adds confidence that transitional progenitors give rise to neurons – something which was previously only implied and inferred based on the literature surrounding neurogenesis and the expressed marker genes.

One difference between this new dataset and my Day 20 dataset is that there is only one neuronal cluster this time, made almost entirely of *GAD2+* cells, with some *GRIN2B+* cells at the end (not shown). The difference is probably because there are insufficient “excitatory neurons” to form their own cluster, and they have been grouped together with the other neurons. Whether this is due to the different genetic background or timepoint is unknown.

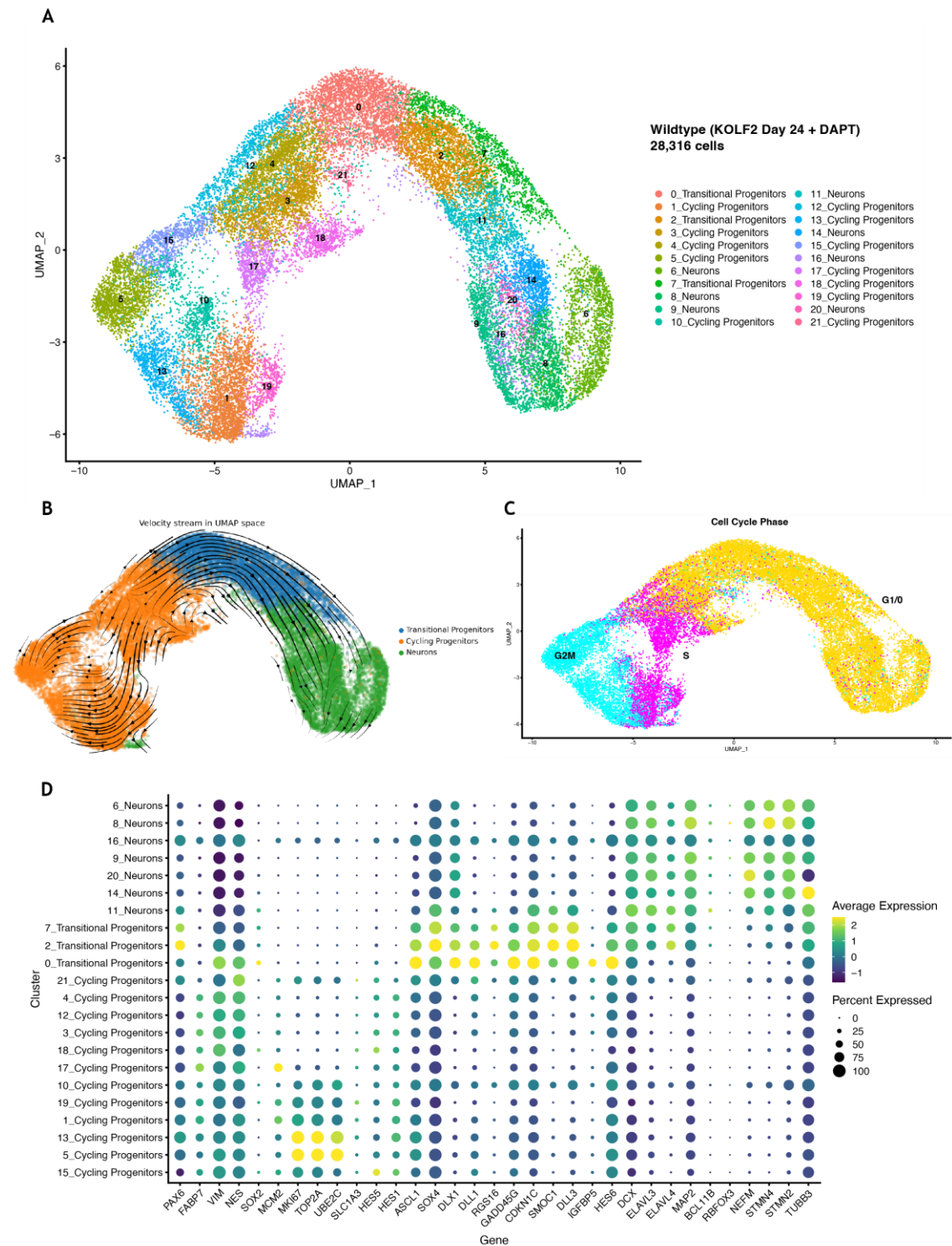


Figure 26. scRNAseq of wildtype KOLF2 at Day 24 also identifies transitional progenitors.

A) UMAP of 28,316 differentiated KOLF2 hiPSCs at Day 24 after unsupervised clustering. Dots represent single cells. Colours represent the different clusters identified on the right.

B) Clusters from **A)** were grouped together into 3 main cell-types. RNA velocity analysis was performed, with velocity vector streams overlaid, showing a trajectory from cycling progenitors to transitional progenitors and neurons. A second trajectory for the cell-cycle was also seen, going from S to G2M.

- C) Cell-cycle phases of cells in the UMAP, as predicted by Seurat based on gene expression.
 - D) Dot plot of the genes used to classify the clusters. Dot size represents the proportion of cells within a cluster expressing a gene, and shading represents the relative level of gene expression.
-

In any case, the transitional progenitors I previously saw were also present in this new dataset, characterised by the same markers such as an enrichment of *HES6*, *SOX4*, and *ASCL1*, and a unique expression of *RGS16*, *IGFBP5*, *BTG2*, and *GADD45G* (Figure 27A). Additionally, with this more-complete wildtype dataset, I could also look at *HUWE1* expression in humans, finding that it increased slightly as cells differentiated neuronally (Figure 27B), in agreement with Zhao et al. 2008¹⁷⁸. A quick look into *HUWE1* expression in other scRNAseq datasets from human fetal brains showed a similar trend: in van Bruggen et al. 2022²⁵⁶ (post conception week (pcw) 8-10 forebrains) *HUWE1* increased from an average SCT value of 0.185 in “Radial Glia” to 0.267 in “Neural Progenitors” and 0.296 in “Early Excitatory” neurons, while in Nowakowski et al. 2017²⁵⁷ (subset of pcw7-14, cortex-only cells), *HUWE1* also increased from an average TPM of 235 in radial glia to 316 in excitatory neurons and 261 in inhibitory neurons, although with a decrease to 213 in intermediate progenitors.

We also found that the proportion of transitional progenitors increased as a result of DAPT treatment (not shown here, but found in Păun et al. 2023²²⁰), suggesting that Notch signalling inhibits the formation of transitional progenitors from cycling ones. Since the transitional progenitors are also enriched for the delta ligands *DLL1/3* (Figure 26D) which would lead to Notch signalling in their neighbours, it is likely there is lateral inhibition occurring where transitional progenitors inhibit their neighbours from becoming transitional progenitors. This will prove relevant later, and in the next chapter.

When *ASCL1*-knockouts were projected onto the wildtype UMAP via label-transfer, there was a highly significant loss of transitional progenitors and neurons, with an accumulation of cycling progenitors (Figure 27C), suggesting *ASCL1* is required to drive the differentiation of cycling progenitors into transitional progenitors.

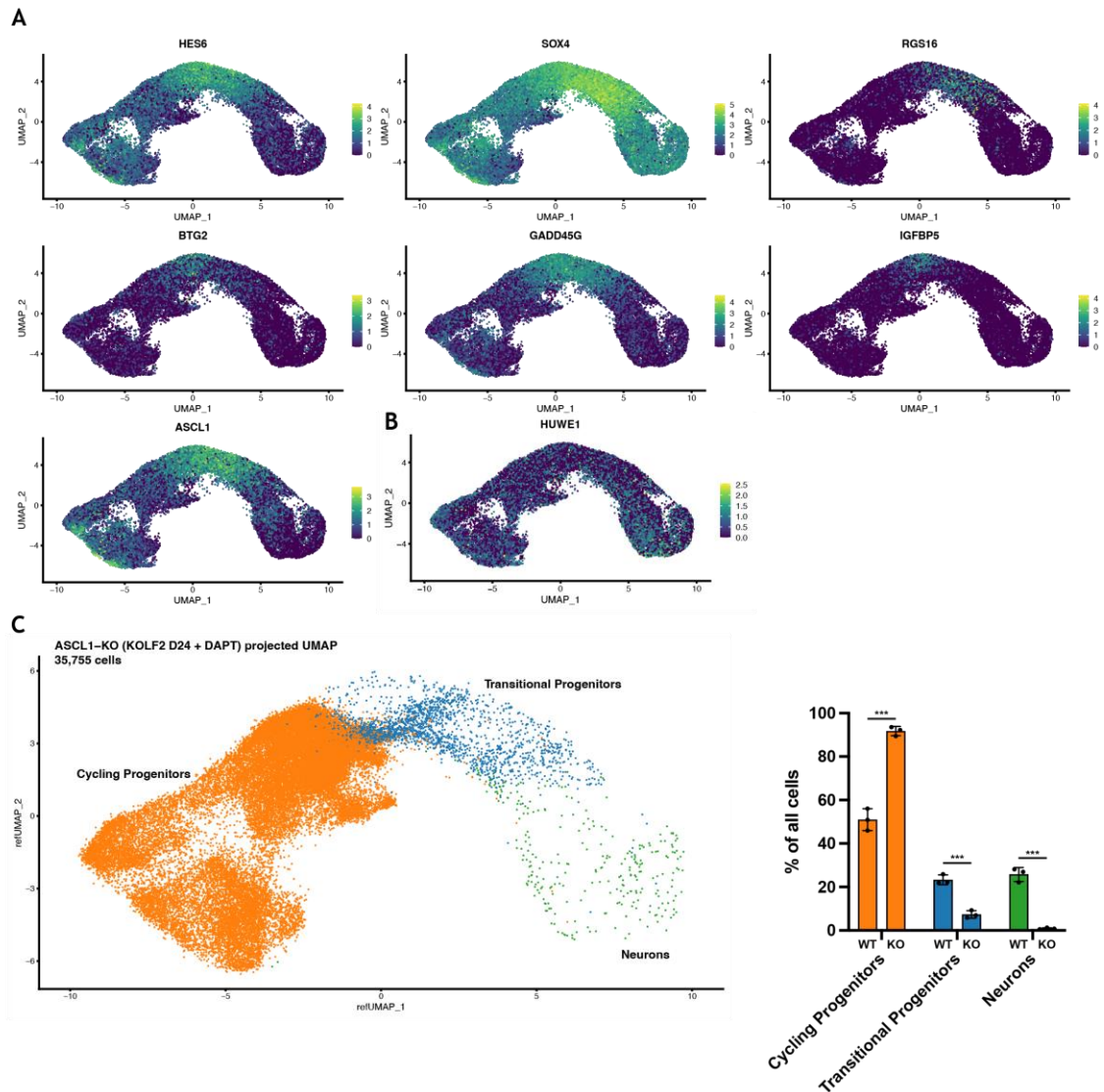


Figure 27. ASCL1 is required to drive differentiation of cycling progenitors into transitional progenitors.

A) Expression of transitional progenitor markers in the wildtype KOLF2 dataset.

B) *HUWE1* expression increases slightly as cycling progenitors become neurons.

C) Transcriptomes from *ASCL1*-knockout KOLF2s were projected onto the wildtype UMAP, revealing an accumulation of cells similar to cycling progenitors, and a significant loss of transitional progenitors and neurons. Student's t-test performed between genotypes within each cell-type.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

This was highly-similar to a study by Ma et al. 2021²⁵⁸, in which they generated *ASCL1*-knockout hESCs and showed a block in differentiation during both ventral and dorsal differentiation protocols, with an increase in cycling progenitor markers such as *HES1*, and a decrease in transitional progenitor and neuronal markers such

as *HES6*, *GADD45G*, and *STMN2*. This led them to postulate that *ASCL1* mediates the fate transition from radial glia to intermediate progenitors in both dorsal and ventral telencephalon, which we appear to see here, on a single-cell resolution.

To find *in vivo* correlates for our data, Stephanie Strohbuecker performed a label transfer using clustifyr²⁵⁹ with transcriptomes from the most comprehensive publicly-available dataset²⁶⁰ of micro-dissected fetal brains (only 5-10 post-conception weeks, forebrain, were selected) to date. Her analysis found that all cycling progenitors clusters were most similar to radial glia (Spearman correlation of 0.769 – 0.855), while transitional progenitors were most similar to neuroblasts (our cluster 0, correlation of 0.762), and neuronal intermediate progenitor cells (IPCs) (our clusters 2 and 7, correlation of 0.772 and 0.789) depending on which of the 3 clusters making up the transitional progenitors (Figure 26A) is considered. Surprisingly, our neurons were most similar to IPCs (our clusters 11, 14, 16; correlation of 0.774 – 0.809) and neurons (our clusters 6, 8, 9, 20; correlation of 0.780 – 0.807), suggesting that they are immature. This analysis can be found in Păun et al. 2023²²⁰. Considering the expected transition from radial glia to neuroblasts to IPCs and neurons appears in our dataset and follows exactly our RNA velocity trajectory, this would suggest that our data models *in vivo* neurogenesis well. It would also suggest that *ASCL1* indeed controls the transition from radial glia into neuroblasts. Additionally, it could also serve as supporting evidence that our “inhibitory neurons” are not mature GABAergic neurons. Consequently, their GABAergic signature could reflect a common lineage between GABAergic and glutamatergic neurons in humans, or be a signature of all immature neurons, and end up being transient.

Since all the above analyses were performed on a transcriptome level, I thought to further validate the idea that *ASCL1* marks transitional progenitors, on a protein-level. For this, I performed flow cytometry analysis on Day 24 wildtype KOLF2s co-stained for DAPI, the neuronal marker TUBB3 (TUJ1), the progenitor marker SOX2, and *ASCL1*. By plotting TUBB3 against SOX2, I found 3 clusters of cells (Figure 28A, left), which I presumed were neurons (SOX2-/TUBB3-high), differentiating progenitors (SOX2-low/TUBB3-high) and progenitors (SOX2-high/TUBB3-low). While it may be tempting to assume these correspond to the 3 cell-types found from scRNAseq (Figure 26B), I found that *ASCL1* expression peaked between the

progenitor and differentiating progenitor clusters (Figure 28A, middle). The differentiating progenitors revealed by TUBB3 and SOX2 are thus not exactly the same as transitional progenitors, but rather an overlapping group – likely to be the cells corresponding to IPCs in our dataset (clusters 2,7,11,14,16 of Figure 26A). In line with our scRNAseq data, however, is the observation that high levels of ASCL1 mark progenitors just prior to their losing SOX2 and increasing TUBB3 (terminal differentiation into neurons).

By comparing the DNA content, as measured by DAPI (Figure 28B, bottom), of these ASCL1-high cells against SOX2+ progenitors with low ASCL1 (Figure 28B, top-left), I was able to show that ASCL1-high cells were significantly more likely to be in the G1/0 and G2/M phases and less likely to be in S phase (Figure 28B, top-right). This suggests that ASCL1-high cells are no longer actively cycling, and are completing their final division prior to differentiation, again in line with behaviour expected of transitioning progenitors.

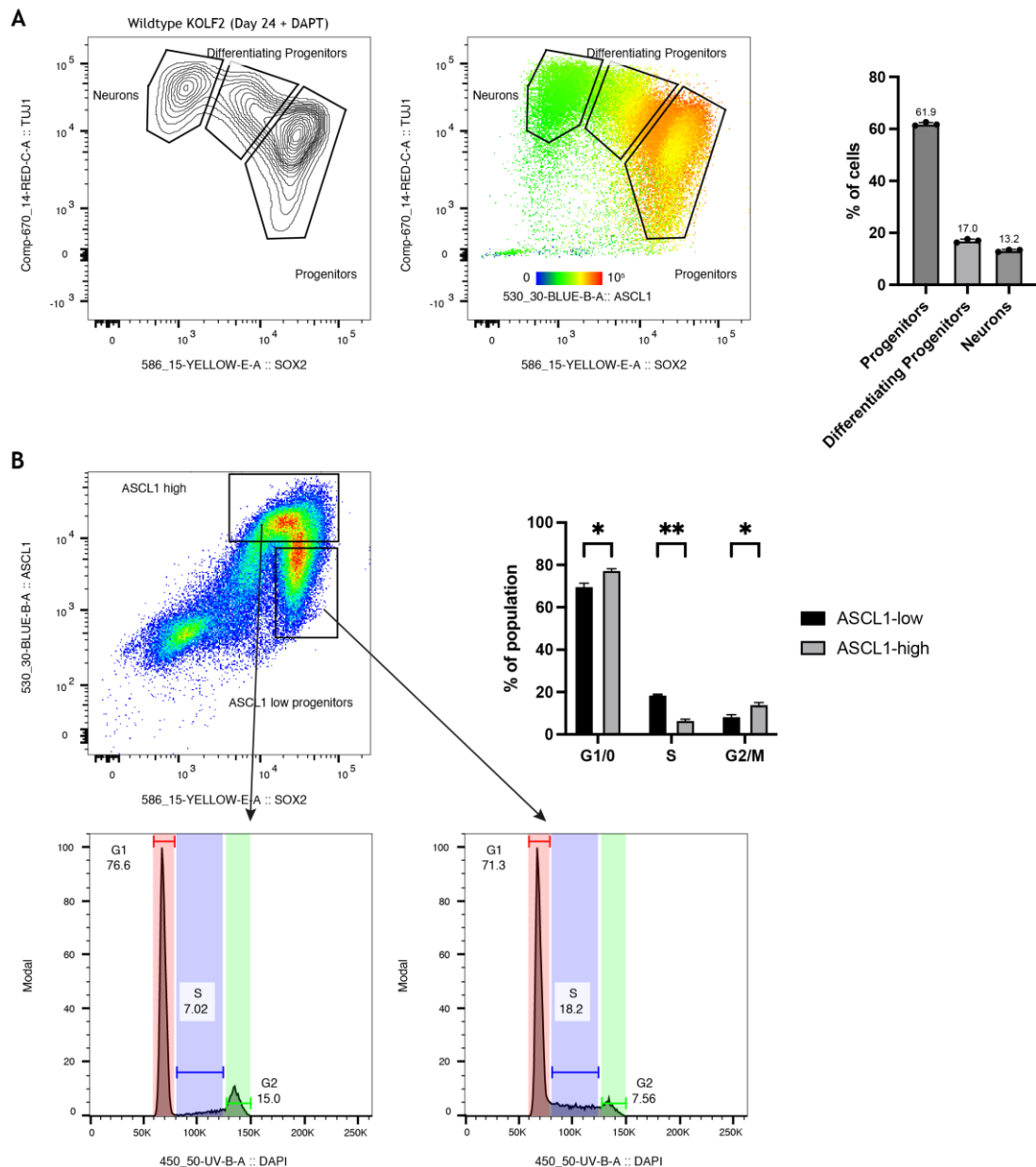


Figure 28. Flow cytometry analysis of ASCL1-high cells support its role as a marker of transitional progenitors.

A) Left: Flow cytometry contour plot of Day 24 KOLF2s for TUJ1 (TUBB3) against SOX2, showing 3 populations of cells with varying expression of both markers. Middle: ASCL1 overlaid onto the first plot as a colour axis, scale shown. The highest levels of ASCL1 are expressed in cells between progenitors and differentiating progenitors. Right: Quantification of the proportion of cells found in each of the 3 populations from the first plot.

B) Top left: DNA content of ASCL1-high cells was compared against ASCL1-low progenitors. Bottom: Histograms showing frequency of DAPI intensities of the 2 populations. Most cells were in G1, though ASCL1-high cells were more likely to be in G1 and G2 (doubled DNA content and hence DAPI intensity) phases. Top right: Quantification of the proportions of cells in each phase. Student's t-test performed for each cell-cycle phase. Data represent mean $\pm$ SEM from 3 independent differentiations.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

5.3 HUWE1 knockout phenotypes may involve transitional progenitors

Since I had now shown that transitional progenitors are both defined by, and dependent on, *ASCL1*, I was surprised that my Day 20 dataset did not show an increase in the proportion of transitional progenitors following 4OHT treatment in HUWE1cKO. This was despite *ASCL1* being strongly stabilized following loss of HUWE1. I therefore decided to revisit the Day 20 dataset and investigate specifically its transitional progenitors.

By performing a differential gene expression analysis (Seurat's^{208,240} FindMarkers function) between Vehicle and 4OHT-treated HUWE1cKOs, segmented by annotated cell-types, I found that *HUWE1* levels were most reduced between 4OHT and Vehicle-treated HUWE1cKO transitional progenitors ($p = 4.01 \times 10^{-5}$, $p_{\text{adj}} = 1$), and less so in the other cell-types (both p and p_{adj} ns) (Figure 29A). I interpreted this to mean that HUWE1-knockouts were over-represented in the transitional progenitor cell-type (for example being at a 1:1 ratio with wildtypes, instead of as a minority), enough so that their lower *HUWE1* transcript levels could be picked up as a differentially-expressed gene. In the other cell-types, the ratio of wildtypes to HUWE1-knockouts was probably higher, such that the difference in *HUWE1* levels compared to a 100% wildtype culture is not as great. This could for example be because HUWE1-knockouts are more likely to become transitional progenitors than wildtypes, or have difficulty turning into neurons from transitional progenitors, and so accumulate as transitional progenitors.

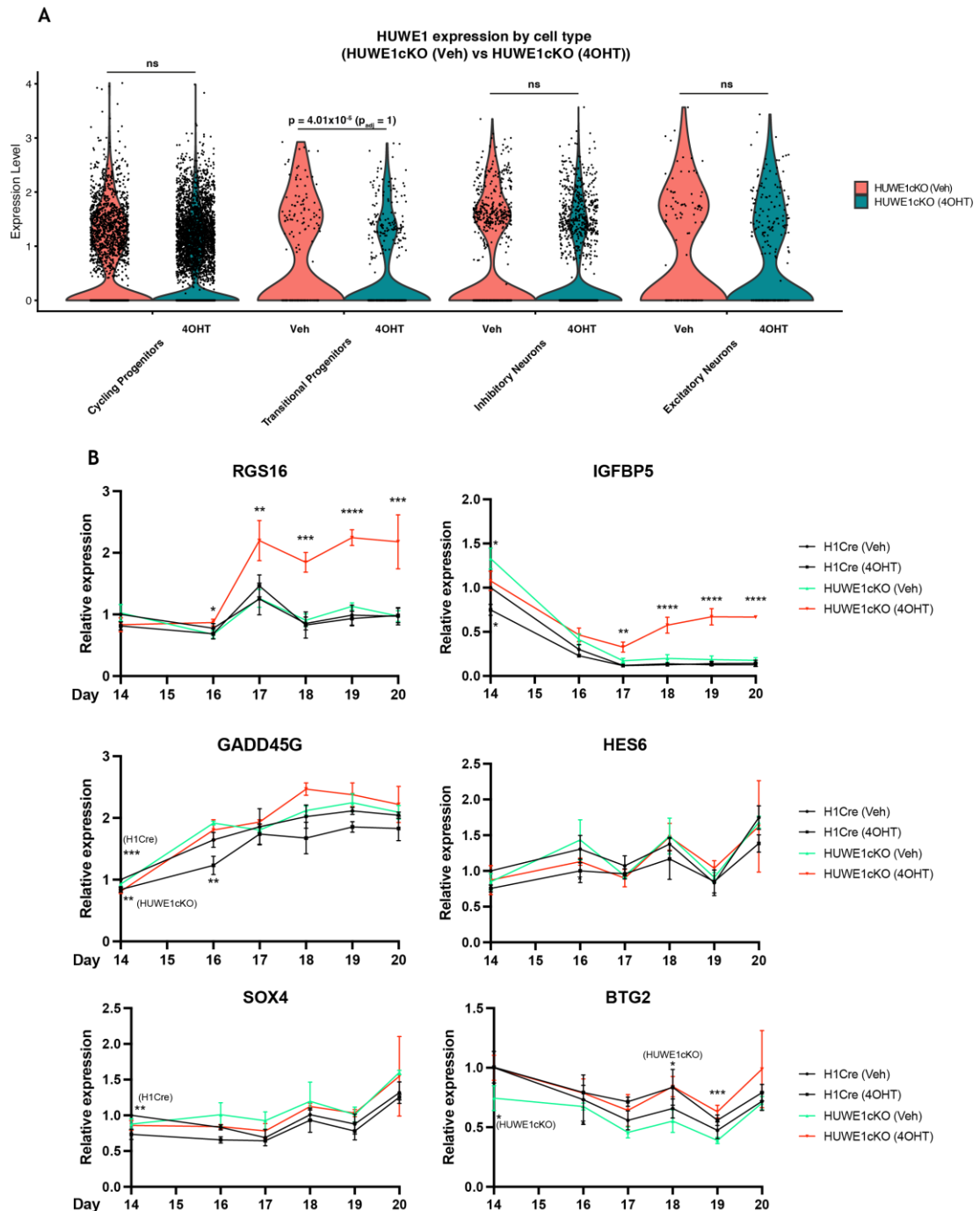


Figure 29. HUWE1-knockouts may be forming transitional progenitors at the expense of wildtype cells.

A) Differential gene expression analysis from the scRNAseq dataset of Day 20 HUWE1cKOs between vehicle and 4OHT-treated conditions, segregated by cell-types, found *HUWE1* levels to be most reduced in transitional progenitors following 4OHT treatment, suggesting there are more HUWE1-knockouts in this cell-type than in the others.

B) qRT-PCR at multiple timepoints from Days 14 – 20 for markers of transitional progenitors. Data represent mean $\pm$ SD. One-way ANOVA was performed at each timepoint with Sidak's multiple comparisons test on both pre-defined Veh vs 4OHT pairs. All ns unless indicated.

p>0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****)

In support of this, a qRT-PCR for markers specific to the transitional progenitor cell-type at several timepoints showed an increase in *RGS16* and *IGFBP5* in HUWE1cKO (4OHT-treated) only (Figure 29B), from Day 17, following expression and stabilization of ASCL1 (*ASCL1* mRNA, Figure 6; ASCL1 protein, Figure 17). In most circumstances, the simplest explanation would be an increase in the total number of transitional progenitors. However, this is a scenario that is unlikely since there was no increase in the proportion of transitional progenitors in HUWE1cKO (4OHT-treated) (Figure 25A); there were no population-level changes in neuronal markers (which we now know arise from transitional progenitors) in the previous chapter; and other markers for transitional progenitors such as *GADD45G*, *HES6*, *SOX4*, and *BTG2* do not show the same increase (Figure 29B). Instead, the greatly increased *RGS16* and *IGFBP5* probably reflects transitional progenitors with abnormally high ASCL1 levels (i.e., HUWE1-knockouts) in similar numbers to a 100% wildtype culture, leading to individual transitional progenitors with abnormally-strong ASCL1 transcriptional signatures.

This would also mean *RGS16* and *IGFBP5* are the strongest ASCL1 targets of those checked in Figure 29B, such that following ASCL1 stabilization in a minority of cells, their increase in transcription is still strong enough to be detected on a population-level. The other transcripts are either not direct ASCL1 targets, or are less strongly upregulated by ASCL1. *RGS16* (Regulator of G-protein Signalling 16) is a GTPase-activating protein and thus negative regulator of G-protein-coupled receptors. Little else is known about *RGS16* apart from a potential role in establishing sleep-wake circadian rhythms in mice and in humans²⁶¹. *IGFBP5* (Insulin-like Growth Factor Binding Protein 5) is a secreted protein that regulates the bioavailability of the insulin-like growth factors IGF1 and IGF2 by binding them with high affinity. This can either enhance or inhibit IGF-signalling depending on whether this binding is competing with the IGF-receptor (IGF1R) or if it is increasing the local extracellular matrix's concentration of IGFs. In mice, its overexpression led to growth delays while its loss did not result in overt phenotypes presumably due to compensation by the other IGFBPs²⁶². While nothing is known yet about whether *RGS16* and *IGFBP5* directly affect neurogenesis, the Guillemot lab^{63,73,220,246} and my data have shown they

appear to be transcriptional targets of ASCL1, and thus serve as markers of ASCL1-enriched cells in my project.

Another implication of this scenario of HUWE1-knockouts becoming over-represented in transitional progenitors, while the total number of transitional progenitors remains unchanged, is that the differentiation of HUWE1-knockouts is happening at the expense of the wildtype cells within the same culture. Since we have shown that transitional progenitor formation also responds to inhibition of Notch signalling²²⁰ within cycling progenitors, and transitional progenitors are the source of delta ligands *DLL1/3* (Figure 26D) that would trigger Notch signaling in neighbouring cells, we also have a mechanism to explain this whereby any cell that preferentially (for example by stabilizing ASCL1 due to HUWE1 loss) becomes a transitional progenitor can inhibit neighbours from doing so.

Unfortunately, at this point, these are all speculative and require the identification of HUWE1-knockouts to prove – something which scRNAseq was unable to provide. I thus had to search for an alternative method to detect HUWE1-knockouts and investigate their differentiation, guided by the possibility of an expected premature differentiation at the expense of wildtype neighbours hinted at from this chapter.

Results 4: Flow cytometry reveals premature differentiation of HUWE1-knockouts following ASCL1 stabilization

Previous studies of ASCL1 function, primarily conducted in developing mouse brains, have shown it to promote the general differentiation of neural progenitors in the ventral²⁷ and dorsal^{68,74} telencephalon. Separately, it promotes a GABAergic identity⁷² in, and the migration⁷⁵ of, new-born neurons, although subtype specification was found to be context-dependent and not universal⁶⁸. Ascl1 was also most highly expressed in mouse neural progenitors transitioning from proliferating progenitors to differentiating neurons, as inferred from a lineage tracing experiment⁶⁹. These observations, together with demonstrations that ASCL1 overexpression is sufficient to drive neuronal differentiation in multiple cell-types due to its pioneer activity^{136–139,233}, support the possibility of ASCL1 performing a similar pro-differentiation role in human neural progenitors during development.

While Ascl1 has been shown to be predominantly expressed ventrally in the mouse telencephalon, with few cells expressing it at low levels in the cortex⁶⁸; in humans ASCL1 has been recently shown to be widely expressed dorsally, where it appears to also label progenitors in the glutamatergic lineage^{71,263}, although it is also more strongly-expressed ventrally. The role of ASCL1 in these cortical progenitors is unclear, especially given ASCL1's expected role in specifying GABAergic lineages.

Having shown in the previous chapter that endogenous ASCL1 also promotes neural differentiation in human progenitors – which Oana Păun from the Guillemot lab subsequently showed to be due to co-operation with the SWI/SNF complex for its pioneer activity at neuronal genes²²⁰ – the expected consequence of the increased ASCL1 levels seen in HUWE1cKOs treated with 4OHT was premature differentiation into transitional progenitors and neurons following ASCL1 stabilization. However, this premature differentiation was not seen on a population-level through analyses like qRT-PCR and microscopy that could not segregate cells by genotype, despite a

clear stabilization of ASCL. Prima facie, this could have been due to dilution of any mild phenotypes by the larger proportion of wildtype cells. An alternative method capable of identifying and analysing HUWE1-knockouts specifically within mosaic HUWE1cKO (4OHT-treated) cultures was needed to eliminate this possibility.

In this chapter, I first attempt to label HUWE1-knockouts specifically through introducing a Cre-activity reporting fluorescence plasmid, with partial success. This effort led to flow cytometry-based analyses in which I first validated HUWE1 antibodies further, then used the technique to identify HUWE1-knockouts within mosaic HUWE1cKO (4OHT-treated) cultures. Through comparing HUWE1-knockout cells to wildtype cells from the same mosaic culture, I was finally able to demonstrate premature differentiation of HUWE1-knockouts.

6.1 Labelling HUWE1-knockouts through reporting Cre-activity

Since single-cell RNA sequencing was unable to confidently identify cells of either genotype in meaningful numbers (Figure 25), I required an alternative method to do so. Previously, I had also tried HUWE1 (CST#5695) antibodies during differentiation, but was unable to obtain 2 distinct levels of expression which would have suggested a wildtype and a knockout population (Figure 19) – a failure I attributed to the stacking of cells since the same method worked in hESCs (Figure 14).

Reasoning that since only HUWE1cKO cells that respond to 4OHT can be knockouts, an equivalent alternative to detecting knockouts through HUWE1 staining could be to look at CreERT2 activity instead. To that end, I set out to introduce pEF1a-EGFP-P2A-FLEX-tagRFP, a Cre-activity reporter, into the HUWE1cKO line. pEF1a-EGFP-P2A-FLEX-tagRFP, designed with Molly Strom of the Viral Vector Core at the Crick, is a lentiviral transfer plasmid with an EF1a promoter driving EGFP expression. Following Cre activity, to be provided by the AAVS1-integrated CAG-CreERT2 of the HUWE1cKO line in the presence of 4OHT, tagRFP expression is turned on permanently as well (Figure 30A). HUWE1cKO cells that respond to 4OHT, and therefore possess CreERT2 activity, will be both EGFP⁺, tagRFP⁺, and are presumably HUWE1-knockouts. Cells in the same 4OHT-treated culture that are

EGFP⁺ but tagRFP⁻ prove they lacked CreERT2 activity throughout the 4OHT exposure (Figure 30B), and are presumably wildtype.

In theory, the accuracy of the HUWE1cKO-reporter system could then be validated by either the HUWE1 (CST#5695) antibody or fluorescence in situ hybridisation (FISH) (since HUWE1-knockouts should lack *HUWE1* transcripts and the deleted region) combined with immunofluorescence. Regarding retrying HUWE1 (CST#5695): previously it looked somewhat promising in discriminating between the two genotypes, but only by eye (Figure 19), suggesting there was the possibility to create a better-resolution metric (for example, by comparing a cell's HUWE1 intensity to its neighbours; or an edge-detection-based/colocalization-based method to identify cells without HUWE1 within their cell outlines). This would however require some ground-truth or additional biological justification to support the chosen metric (in this case, evidence of Cre-activity during the time of 4OHT treatment), to avoid arbitrarily creating a metric to fit visual expectations. Future experiments with HUWE1cKO-reporter could then skip validation steps, or HUWE1cKO could use the now-verified metric.

pEF1a-EGFP-P2A-FLEX-tagRFP was delivered via lentiviruses into both H1Cre hESCs and HUWE1cKO hESCs, and infected cells became EGFP⁺/tagRFP⁻, unless 4OHT was added to the culture (Figure 30C). Infected cells were then enriched by fluorescence-activated cell-sorting (FACS) and cultured as a non-clonal pool, henceforth referred to as Cre-reporter lines (or H1Cre-reporter and HUWE1cKO-reporter). Post-sorting, about 60% of cells were EGFP⁺ in both Cre-reporter lines, with cells only becoming EGFP⁺/tagRFP⁺ after 4OHT treatment (Figure 30D).

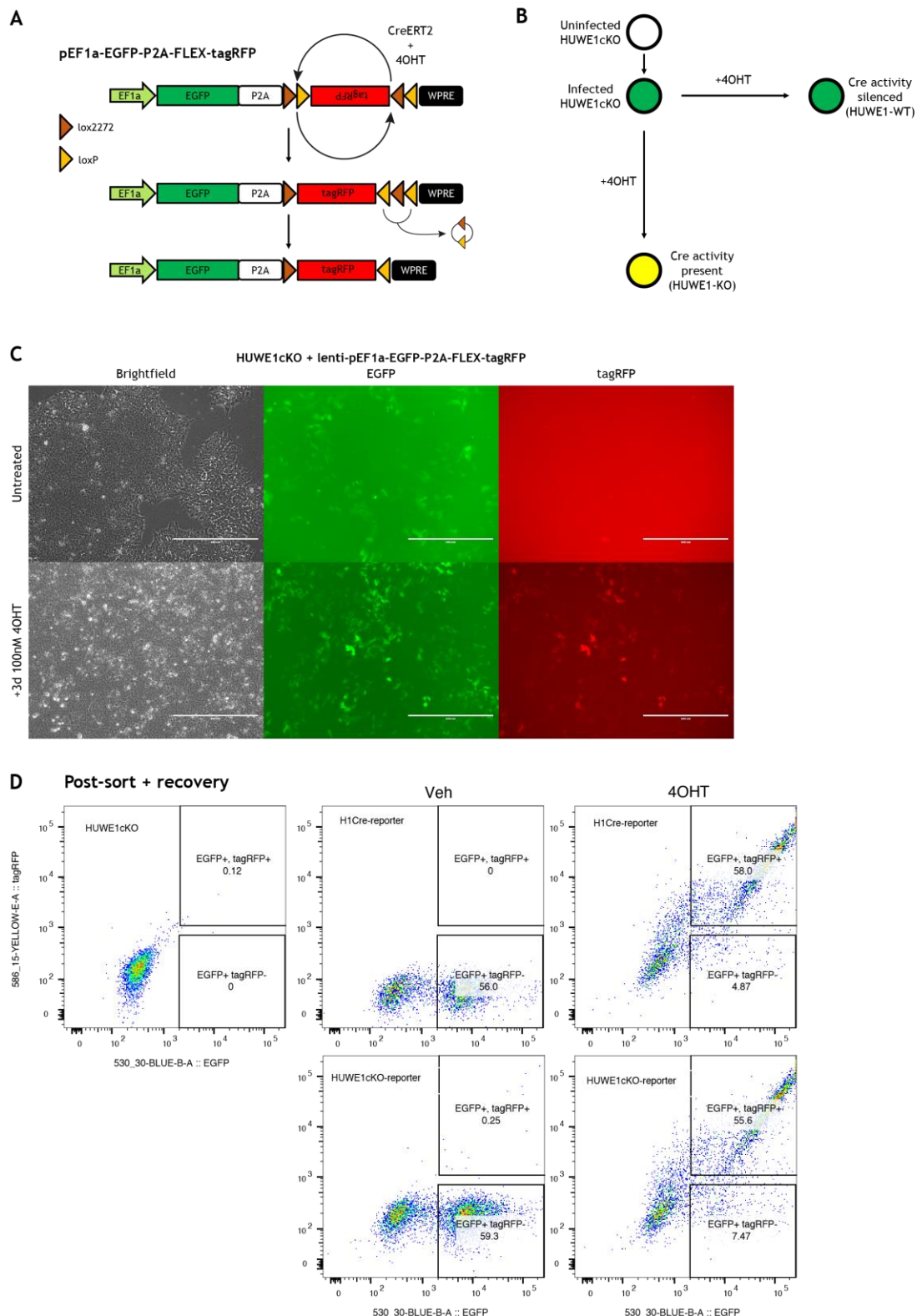


Figure 30. Generation of HUWE1cKO-reporter, a Cre activity-reporting cell line to identify HUWE1 knockouts.

A) pEF1a-EGFP-P2A-FLEX-tagRFP is a lentiviral transfer plasmid that expresses EGFP under an EF1a promoter to label infected cells. In the presence of active Cre (in this case CreERT2 in the presence of 4OHT), the inverted tagRFP reading frame is restored permanently through stepwise recombination of specific lox-site pairs, and such cells co-express tagRFP.

- B)** Using the system in **A)**, HUWE1cKO cells can theoretically be labelled such that cells which silenced CreERT2 expression or failed to respond to 4OHT during differentiation remain EGFP+/tagRFP-. Such cells lacked Cre activity at all times during 4OHT treatment and should be wildtype. Conversely, EGFP+/tagRFP+ cells following 4OHT treatment demonstrate Cre activity and are presumably also HUWE1 knockouts.
- C)** HUWE1cKO was infected with lentivirus carrying pEF1a-EGFP-P2A-FLEX-tagRFP. Infected cells were EGFP+, and only expressed tagRFP in the presence of 4OHT. Scale-bar: 400µm.
- D)** EGFP+ H1Cre and HUWE1cKO infected with pEF1a-EGFP-P2A-FLEX-tagRFP were FACS-sorted and allowed to recover. The resulting Cre-reporter lines (H1Cre-reporter and HUWE1cKO-reporter) were then analysed by flow cytometry. ~60% of cells were EGFP+, and tagRFP co-expression only occurred after 4OHT exposure in both lines. The parental HUWE1cKO line was included as a negative control to aid in gate-setting.

The Cre-reporter lines were then differentiated like before, with 100nM 4OHT added daily into the media from Days 12-16 inclusive. Live images were taken of the cultures at Day 6 using an EVOS FL (ThermoFisher) (Figure 31), showing that the majority of cells were EGFP+, with no tagRFP+ cells. However, even EF1a-EGFP-P2A-FLEX-tagRFP was silenced during differentiation, similar to CAG-CreERT2, such that very few cells were EGFP+ by Day 17 (Figure 32A, B).

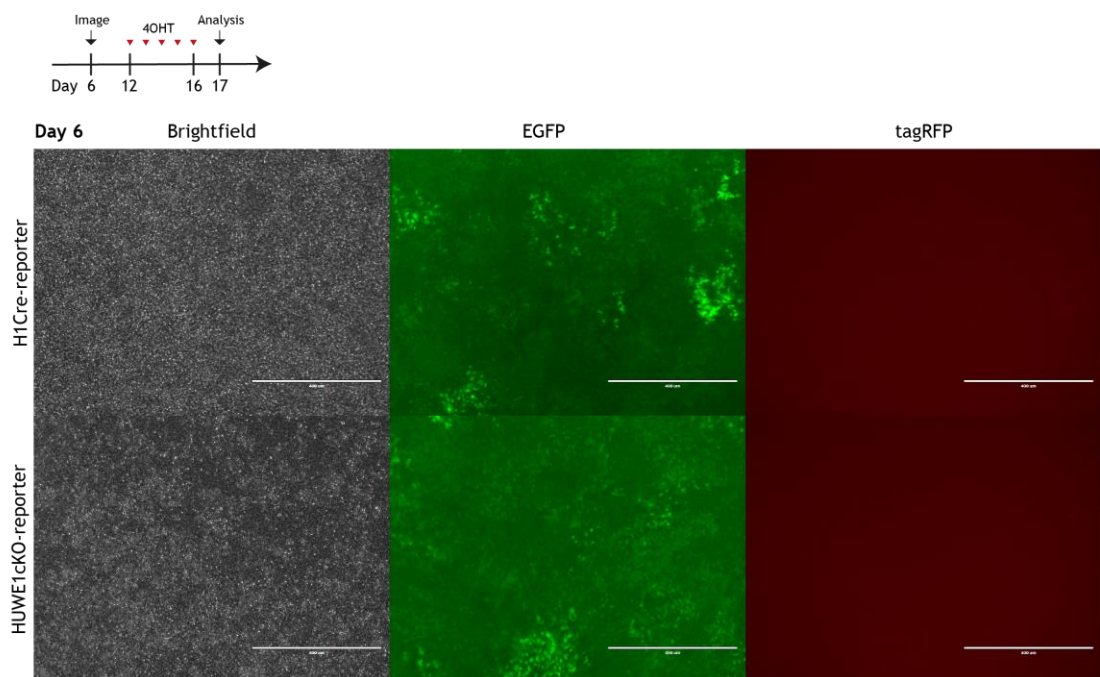


Figure 31. Cre-reporter lines express EGFP and not tagRFP during early neural differentiation. Live-cell images taken at Day 6 of differentiation for both H1Cre-reporter and HUWE1cKO-reporter. Both lines express only EGFP and not tagRFP prior to 4OHT exposure (and CreERT2 activity) at Day 12. Scale bar: 400µm.

Given that the HUWE1 (CST#5695) antibody was the closest I had gotten to segregating the cells by genotype, and is a simpler technique than combining FISH with immunofluorescence, I revisited this approach first to validate the reporter lines. On Day 17, both reporter lines were fixed and stained for HUWE1 (CST#5695), and imaged on an SP5 confocal microscope (Leica). Similar to previous stainings at Day 20 (Figure 19), HUWE1 levels were more varied visually in HUWE1cKO-reporter (Figure 32A) than H1Cre-reporter (Figure 32B). Despite the majority of cells being EGFP⁻, there were sufficient EGFP⁺ cells to analyse, such that even some EGFP⁺/tagRFP⁻ cells could be identified.

In Figure 32A and 3B, 3 cells are labelled in each with white arrows, with the middle cell being EGFP⁺/tagRFP⁻ in both. The left cell in Figure 32A, which is an EGFP⁺/tagRFP⁺ HUWE1cKO-reporter, appears to be HUWE1-negative – lacking a HUWE1 stain corresponding to its nuclear outline or EGFP/tagRFP outlines – while the middle EGFP⁺/tagRFP⁻ cell is HUWE1-positive as expected. Unfortunately, the right cell which is EGFP⁺/tagRFP⁺ is clearly also HUWE1-positive. For comparison, all labelled H1Cre-reporter cells in Figure 32B have HUWE1 regardless of EGFP/tagRFP status. By analysing and quantifying intensities in only the EGFP⁺ cells of both reporter lines, I found that most EGFP⁺ cells would also be tagRFP⁺, and that HUWE1 levels were distinctly lower in high tagRFP⁺ (tagRFP intensity >5000) HUWE1cKO-reporter cells compared to in H1Cre-reporter (Figure 32C) – even almost separating into 2 populations. However, because within HUWE1cKO-reporter itself there are also tagRFP-low cells with very-low HUWE1 levels similar to tagRFP-high cells, and mostly because of the visual confirmation that tagRFP⁺ cells can still be HUWE1-positive, tagRFP is labelling HUWE1-negative cells preferentially at best, but not exclusively.

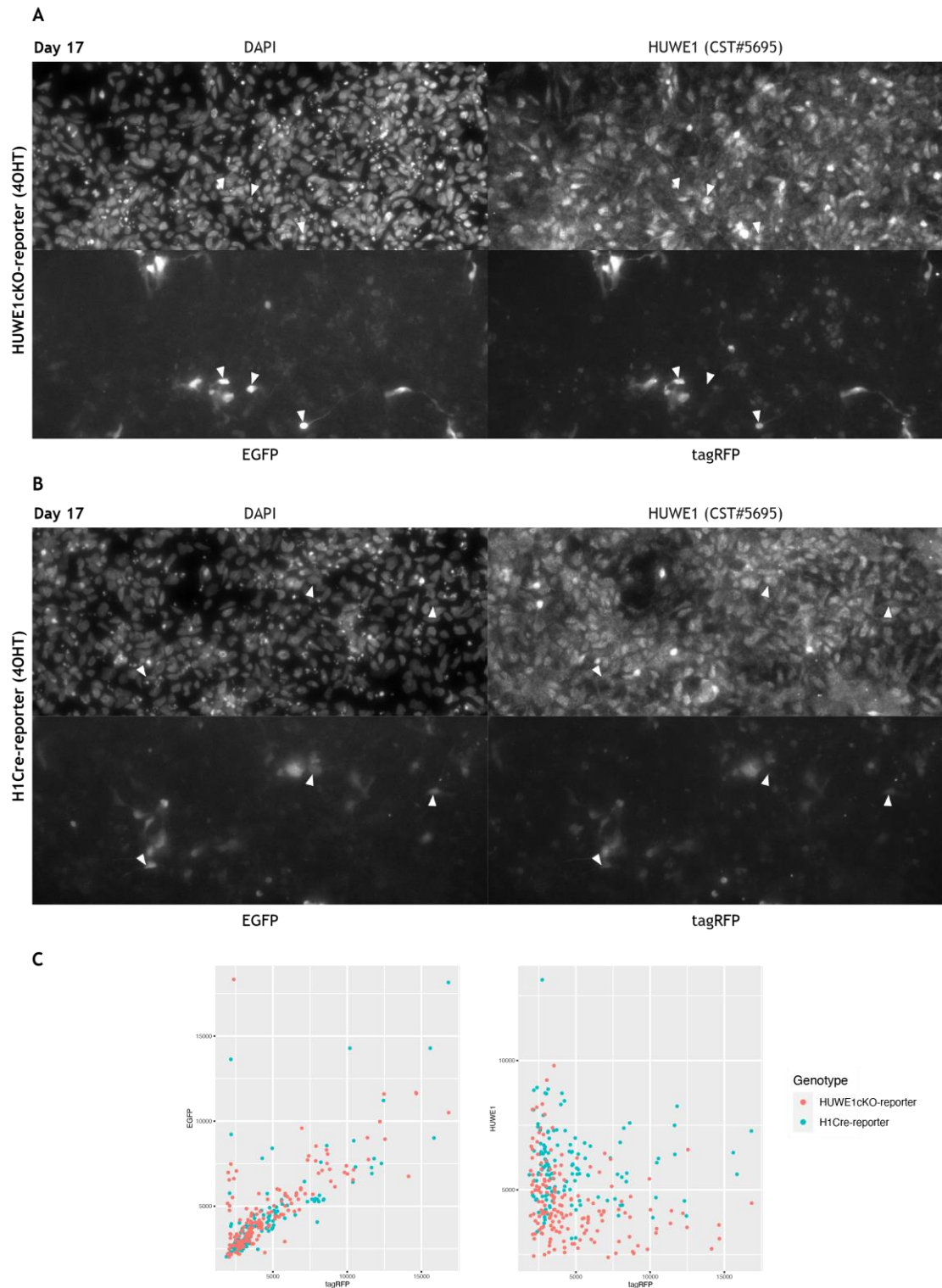


Figure 32. HUWE1cKO-reporter may label HUWE1 knockouts imperfectly.

A) Confocal microscopy images of HUWE1cKO-reporter (+4OHT) at Day 17 of differentiation, stained for DAPI and HUWE1. Very few cells are still expressing EGFP at this time, suggesting silencing of the construct. Arrows indicate 3 cells that are EGFP+, with 2 of them also being tagRFP+. The EGFP+/tagRFP- cell appears to have HUWE1, while one (rightmost) of the EGFP+/tagRFP+ cell also appears to have HUWE1.

- B)** Confocal microscopy images of H1Cre-reporter (+4OHT) at Day 17 of differentiation, stained for DAPI and HUWE1. Arrows indicate 3 cells that are EGFP+, with 2 of them also being tagRFP+. All 3 cells appear to have HUWE1 within their cell boundaries.
- C)** EGFP+ cell bodies were manually traced, and fluorescence intensities for all channels measured within these regions of interest. The majority of EGFP+ cells are also tagRFP+, though a few remain EGFP+/tagRFP- in both reporter lines. HUWE1 appears to decrease in intensity with tagRFP expression in the HUWE1cKO-reporter, but no justifiable cut-off in HUWE1 levels to distinguish the knockouts from wildtypes was apparent. 6 fields of view from 1 differentiation each. Images taken on an SP5 II confocal.
-

During the course of analysis, I found that cells' EGFP, tagRFP, and HUWE1 intensities varied significantly within each field of view, which I suspected to be dependent on the local cell density and focal plane. While ten 1µm stacks were taken, and maximum-intensity projections analysed, this approach was still inadequate as denser-packed regions always appeared brighter than their peripheries. Consequently, weakly staining antibodies (such as HUWE1) or poorly expressed proteins could have biologically-relevant intensity differences obscured by confluency-dependent intensity variations. Since this would be an inherent limitation of microscopy on our confluent culture model if true, I decided to try flow cytometry on HUWE1 as a seeding-independent workaround.

6.2 Flow cytometric detection of HUWE1-knockouts

To test the use of flow cytometry in distinguishing HUWE1 genotypes, I differentiated the Cre-reporter lines like before to Day 16, collecting and fixing the dissociated cells then, instead of replating them. Cells were then stained for HUWE1 (CST#5695) and analysed on a Fortessa flow cytometer (BD Biosciences). After gating for live, single-cells (not shown, strategy identical to Figure 35A), Cre-reporter lines were gated into quadrants based on EGFP and tagRFP levels (Figure 33A).

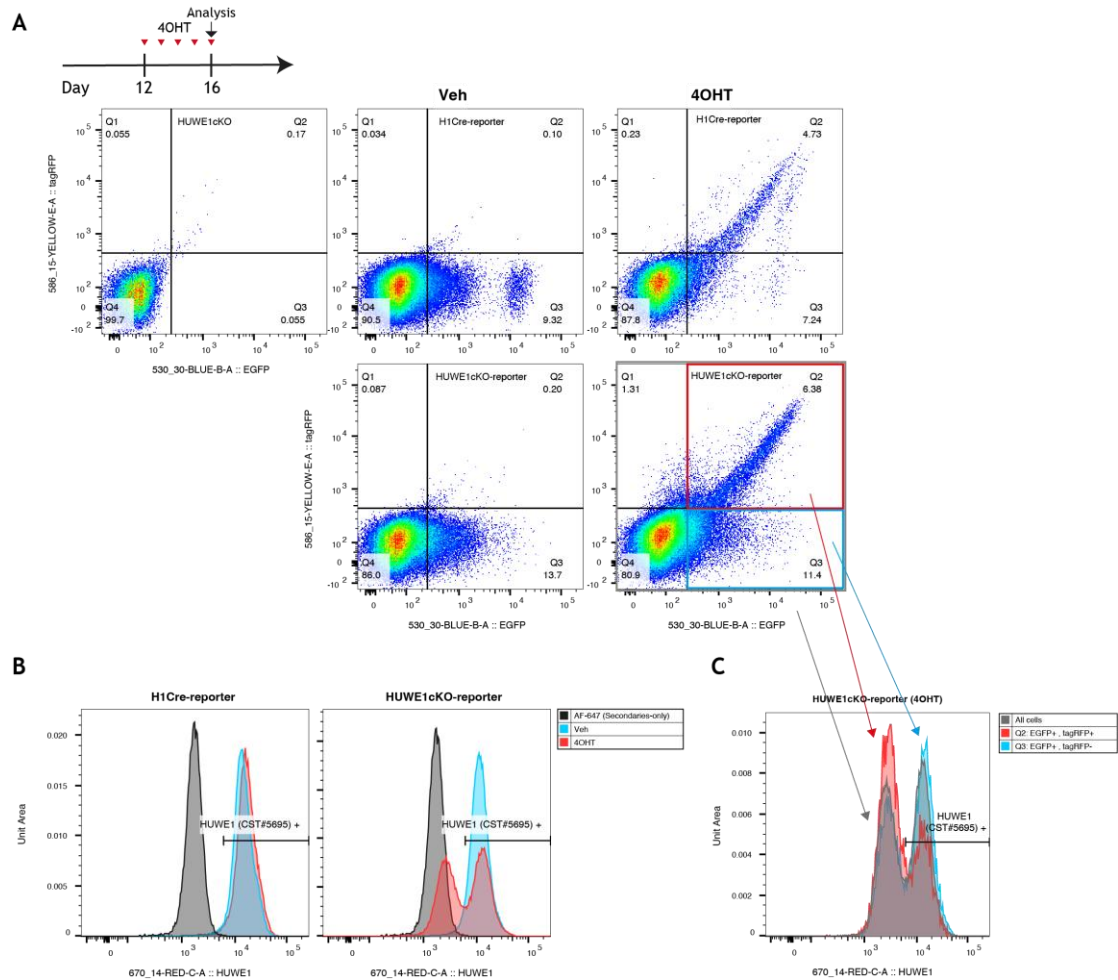


Figure 33. HUWE1cKO-reporter preferentially labels HUWE1 knockouts, but flow cytometry can identify knockouts.

- A)** Cre-reporter lines were subjected to 4OHT treatment from Day 12 and differentiated to Day 16, before dissociation and fixation for flow cytometry. Most cells were no longer EGFP+ in both lines, and tagRFP expression required prior 4OHT exposure to enable CreERT2 activity. Gates were set using a HUWE1cKO differentiated to Day 16.
- B)** HUWE1 (CST#5695) levels in H1Cre-reporter was unchanged upon 4OHT exposure (left). In HUWE1cKO-reporter, HUWE1 decreased to levels equal to the negative control in about half of the cells – with the other half being unchanged – giving two distinct populations (right).
- C)** The EGFP+/tagRFP+ HUWE1cKO-reporter population from **A)** (red) has a higher proportion of HUWE1 knockout cells (64.4%) than the EGFP+/tagRFP- (blue, 42.2%) and ungated (grey, 48.7%) populations, demonstrating some degree of success in the labelling strategy.

Compared to the initial ~60% of cells being EGFP+ at the hESC stage (Figure 30C), there were only ~10% EGFP+ cells at Day 16 (Figure 33A, Veh), again suggesting silencing of EF1a-EGFP-P2A-FLEX-tagRFP. 4OHT was required for cells to express tagRFP. With regards to HUWE1 levels, 4OHT had no effect on H1Cre-reporter's HUWE1, but resulted in 2 distinct populations of HUWE1 intensities in HUWE1cKO-

reporter, with the lower-expressing population having intensities similar to a secondary-only negative control (Figure 33B), suggesting it lacks HUWE1 altogether. Meanwhile, the higher-expressing population retains HUWE1 expression similar to its Vehicle-treated self. This observation is consistent with a mosaic culture of 4OHT-dependent HUWE1-knockouts (that are protein-null as expected from Western Blots and qRT-PCR) and wildtype cells, much to my relief.

Since the samples run here were from Cre-reporter lines, I then gated HUWE1cKO-reporter (4OHT-treated) further, comparing EGFP+/tagRFP+ cells (Figure 33C, red) against EGFP+/tagRFP- cells (blue) and the ungated culture as a whole (grey). Based on Figure 32C, the expectation was that tagRFP+ cells would be enriched for HUWE1-knockouts while EGFP+/tagRFP- cells would be enriched for HUWE1-wildtypes, assuming the HUWE1-negative population revealed by HUWE1 (CST#5695) immunostaining in only the HUWE1cKO-reporter line (Figure 33B) is truly from HUWE1-knockouts. Indeed, tagRFP+ cells had a greater proportion of HUWE1-negatives (64.4%) than EGFP+/tagRFP- (42.2%) and the ungated culture (48.7%). This provides additional evidence that the HUWE1-negative population represents HUWE1-knockouts, and meant I might finally have the means to isolate and analyse them. Careful optimism was taken as this differentiation and cytometry was only done once.

Using the same Day 16 HUWE1cKO-reporter sample containing ~50% knockouts, I proceeded to test the other HUWE1 (HPA002548) antibody to expand the combination of antibodies I could co-stain for, since it is raised in rabbit. Unfortunately, HPA002548 was unable to cleanly resolve the two populations of genotypes (Figure 34A), instead showing a smear of lower-HUWE1 cells, with intensities above the secondaries-only negative control, suggesting some degree of unspecific staining from this polyclonal antibody. To my surprise, however, HUWE1 (CST#5695) was also unable to give two populations of genotypes (Figure 34B), despite this being the same samples from Figure 33. The only changes this time were that I co-stained using both HUWE1 antibodies, and that the secondary antibody used with CST#5695 was changed to anti-mouse Alexa-Fluor 350 (AF350). Alternatively, as the previous successful run was only done once, the results there could have been a fluke.

Given the importance of identifying and limiting analyses to HUWE1-knockouts, I had to address this discrepancy and figure out if HUWE1-knockout cells could truly be visualised, and the conditions for reliably doing so.

The quickest and cleanest way to test and establish the conditions for successful HUWE1 staining was to use pseudo-mosaic cultures from HUWE1cKOs at the hESC stage, where I had demonstrated complete loss of HUWE1 on a population-level following 4OHT treatment. I thus mixed dissociated Veh and 4OHT-treated HUWE1cKO hESCs in a 1:1 ratio and stained the mixture singly with each antibody-secondary pair for flow cytometry. The setup performed as hoped, with HUWE1cKO (4OHT-treated) (Figure 34C, red) consisting nearly entirely of HUWE1-negative cells, and HUWE1cKO (Veh control) (blue) consisting of a distinct HUWE1-positive population. The simulated 1:1 mosaic made from the two could be resolved into its component populations by CST#5695 combined with AF647 as a secondary (green). Likewise, AF488 and AF568 were able to do so, but not AF350 (Figure 34C, 2nd to 4th histograms). The failure to resolve knockouts from wildtypes in Figure 34B was thus likely due to AF350. Mechanistically, this would be because I paired the dim AF350 fluorophore with the weakly-expressed/stained HUWE1, such that positive cells did not have sufficient signal to separate from the negatives²⁶⁴.

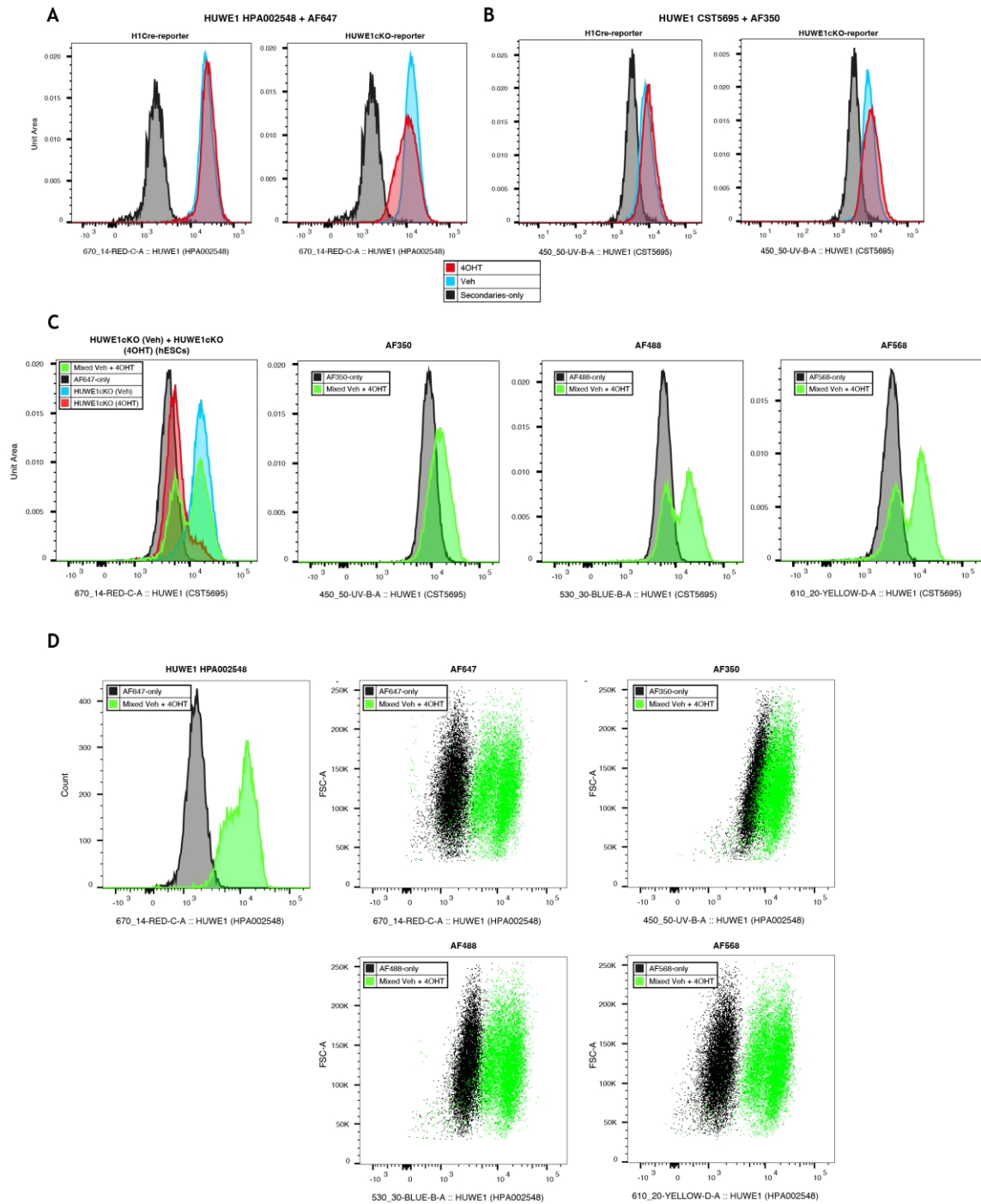


Figure 34. HUWE1 antibodies discriminate knockouts only in conjunction with bright fluorophores.

- A)** The same HUWE1cKO-reporter (Day 16, +4OHT) samples from Figure 33 failed to show two distinct populations of HUWE1 intensities when stained using HUWE1 HPA002548, instead only showing a smaller decrease in intensity.
- B)** HUWE1 CST#5695 was unable to resolve any differences in HUWE1 levels in the same HUWE1cKO-reporter sample where it previously worked, when coupled with Alexa Fluor 350 secondaries.
- C)** HUWE1cKO treated with ethanol (blue) and HUWE1cKO treated with 4OHT (red) were dissociated and fixed for flow cytometry at the hESC stage, when CreERT2 recombination is

most efficient. Equal numbers of each were mixed to simulate a mosaic culture (green), then stained for HUWE1 with CST#5695. All Alexa Fluor secondaries were able to resolve the HUWE1-knockouts from the wildtypes except AF350. Grey histograms are secondaries-only controls, without CST#5695.

- D)** HPA002548 was unable to cleanly resolve the two HUWE1-genotypes in the same mosaic-simulation. When plotting forward scatter area (FSC-A) against HUWE1, 2 populations of HUWE1 intensities can be seen, though with significant overlaps. The low-HUWE1 population (presumably HUWE1-knockouts) has higher HUWE1 intensity than the secondaries-only control (higher background), and cannot be resolved using the dim AF350 secondary.
-

Using the simulated-mosaic, I tested HPA002548 again, finding yet again that it could not resolve the mosaic into its constituent populations (Figure 34D, left). Switching displays to forward scatter area (FSC-A) against HUWE1 (Figure 34D, right), however, 2 populations could be made out. Again, the lower-HUWE1 population had intensities above the secondary-only control, indicating unspecific background staining from HPA002548. Also consistent with the importance of fluorophore brightness, AF350 had the poorest resolution between the 2 populations. In all cases though, HPA002548 with its higher background and poor resolution make it undesirable for analysing HUWE1cKO mosaic cultures.

6.3 HUWE1-knockouts prematurely differentiate following ASCL1 stabilization

Having found that flow cytometry was capable of achieving the genotype segregation I sought, I returned to using the HUWE1cKO and H1Cre lines instead of the Cre-reporter lines to free up the EGFP and tagRFP spectra for co-stains.

I differentiated H1Cre and HUWE1cKO as usual, splitting the culture at Day 12 into Veh-treated and 4OHT-treated isogenic pairs for 5 consecutive days of 4OHT exposure. Cells were dissociated, fixed, and collected for flow cytometry on Days 14, 16, 18, 19, and 20. During all collections for flow cytometry, a fixable viability dye (NIR Live/Dead, Invitrogen L34975) was used. Briefly, the dye stains only cell-surface amines in live-cells, but can additionally stain cell-interior amines in permeable dead cell, resulting in higher fluorescence in dead cells. Crucially, the dye is not quenched by fixation.

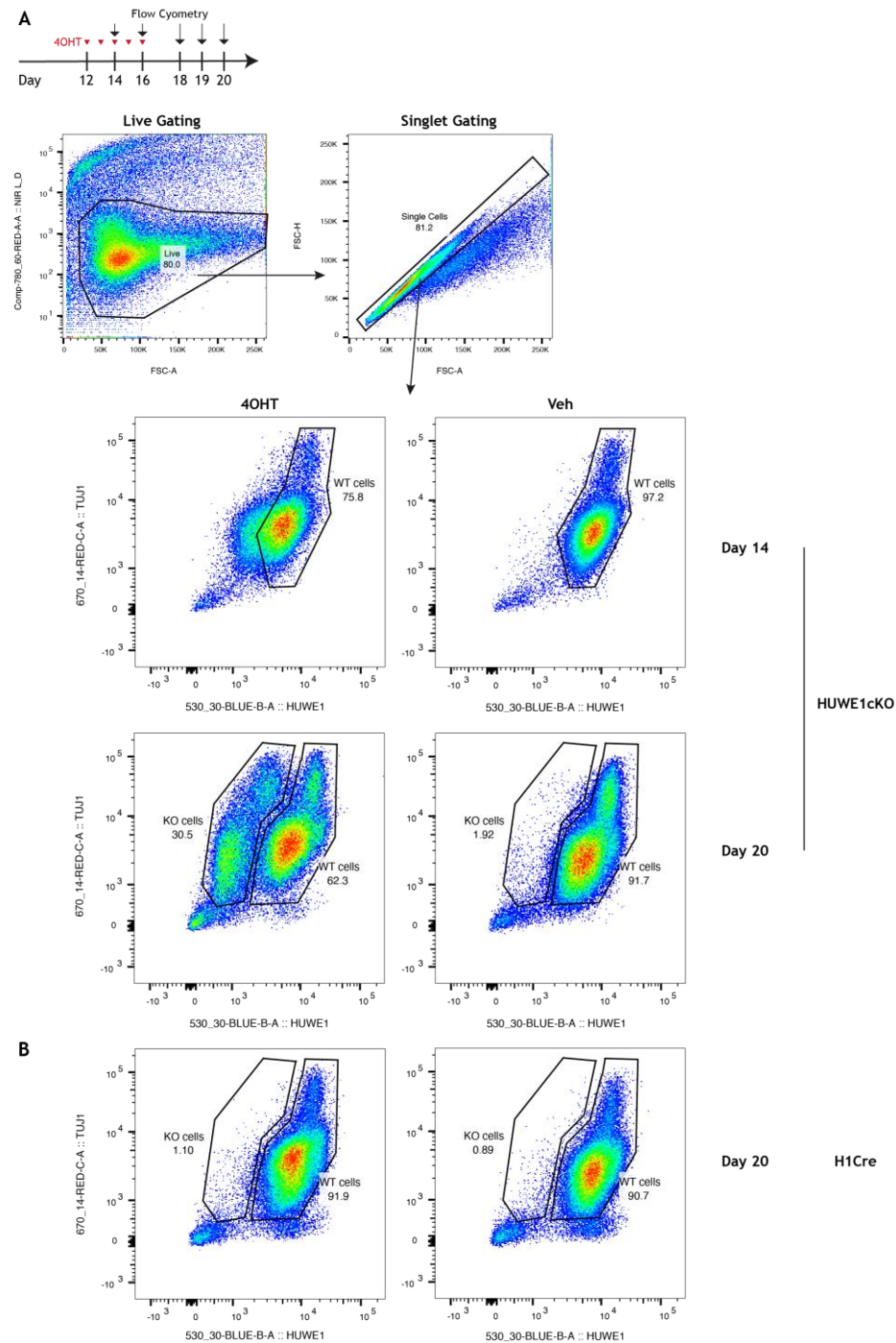


Figure 35. Flow cytometry enables limiting analyses to HUWE1-knockouts within mosaic HUWE1cKO cultures.

A) HUWE1cKO and H1Cre were dissociated and fixed for flow cytometry at Days 14, 16, 18, 19, and 20, during and following 4OHT treatment. Cells alive at the point of fixation were identified and gated using a fixable live stain (NIR L/D), then subject to a singlet gate. The live single cells at each timepoint were then genotype-gated based on HUWE1 (CST#5695) levels, with gates drawn using vehicle-treated controls. A clear HUWE1-negative (HUWE1-knockout) population absent in vehicle-treated controls could be seen only upon 4OHT exposure at all timepoints after Day 14.

B) The HUWE1-negative population is absent in H1Cre treated with 4OHT, with no difference in HUWE1 levels seen from between Veh and 4OHT conditions in this control line.

After gating for live cells, singlets are gated for through a diagonal gate consisting of cells with proportional FSC-Height against FSC-A, to obtain live, single-cells at the point of fixation (Figure 35A, top). Briefly, doublets and multiplets are largely excluded this way as they are particles with the same maximum forward scatter intensity (FSC-H) of other cells, but have a longer flight-time through the laser path, resulting in a greater-than expected cumulative forward scatter intensity (FSC-A). By gating for live, single-cells, I thus exclude highly-auto-fluorescent debris, and multiplets with higher total staining intensities.

When investigating HUWE1 intensities at each timepoint, I found that at Day 14, the knockout cells had not yet formed a distinct HUWE1-negative population, instead forming a smear of cells with varying degrees of reduced HUWE1 levels compared to the Vehicle-treated condition. At Days 16-20, the 2 genotype populations could be resolved (Figure 35A), with ~2 wildtype cells for every knockout at Day 20, suggesting that HUWE1 is still being depleted at Day 14. HUWE1-knockouts found using this gating strategy is also specific to HUWE1cKO (4OHT-treated), and not the result of 4OHT alone as the HUWE1-negative population is not seen in H1Cre following 4OHT treatment (Figure 35B).

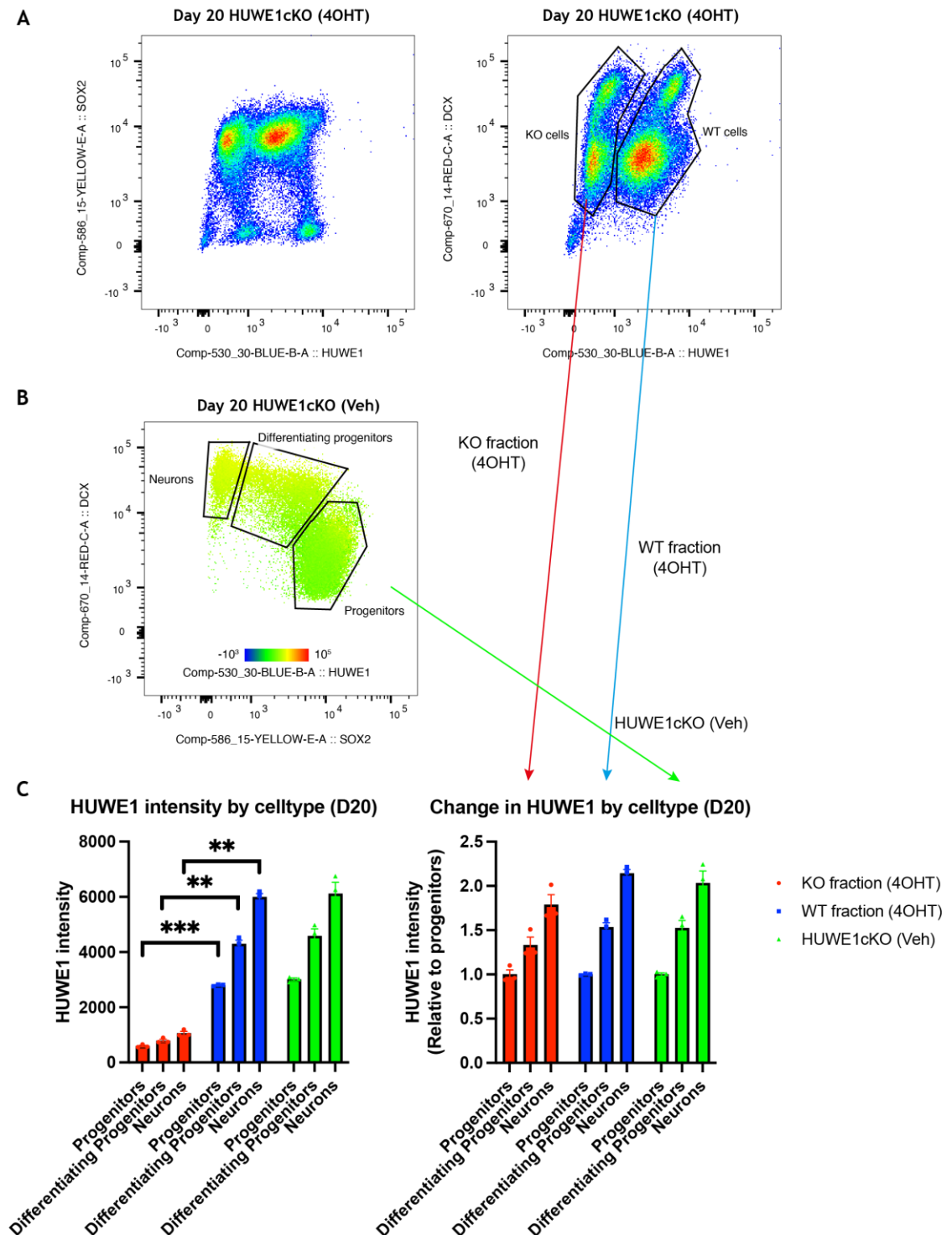
An additional observation from Figure 35 is that HUWE1 appears to increase in cells with higher levels of the neuronal marker TUJ1 (TUBB3), hence the diagonally-slanted gates. This would be consistent with my previous observations of *HUWE1* transcripts increasing in neurons (Figure 6, Figure 27B), and observations of *Huwe1* increasing from the ventricular zone to the cortical plate in mice¹⁷⁸. Also, this means HUWE1-knockouts can indeed survive and differentiate if *HUWE1* is lost after Day 12, but that will be discussed in Figure 38.

To better investigate the increase in HUWE1 with differentiation, I performed cytometry on Day 20 HUWE1cKO cells using another early-neuronal marker DCX and the neural progenitor marker SOX2, finding an increase in HUWE1 in cells with less SOX2, and those with more DCX (Figure 36A). Since DCX and SOX2 were co-stained, I could also identify the 3 cell-type populations similar to the approach in

Figure 28A, showing that HUWE1 increases with differentiation from progenitors to neurons (Figure 36B).

An advantage of specifically analysing HUWE1-knockouts within mosaic cultures is that the wildtypes within the same cultures can serve as perfect controls. They are not only isogenic (apart from *HUWE1* exon 81), but also experienced the same culture conditions including 4OHT, and more importantly the same staining and cytometry steps. By subsequently comparing the wildtype cells from mosaic cultures against vehicle-treated cells, I can look for 4OHT-dependent effects as well as non-cell-autonomous effects of HUWE1-knockouts on wildtype cells. 4OHT-specific effects can be controlled for as usual by comparing H1Cre (Veh control) cells against H1Cre (4OHT-treated) cells. Following this line of reasoning, all subsequent statistical comparisons were performed on predefined pairs of: knockout fraction against wildtype fraction within HUWE1cKO (4OHT-treated); wildtype fraction against HUWE1cKO (Veh control); and H1Cre (Veh control) against H1Cre (4OHT-treated). A consistent colour scheme of KO fraction (red), WT fraction (blue), HUWE1cKO Veh (green), H1Cre Veh (black triangles) and H1Cre 4OHT (black diamonds) will also be used.

By quantifying HUWE1 levels in each of the 3 cell-types in the KO fraction (red), WT fraction (blue), and HUWE1cKO (Veh control) (green), and performing a Repeated-Measures one-way ANOVA with Sidak's multiple comparisons test on predefined pairs as described above, I was able to show that HUWE1 levels were significantly lower in the knockout-fraction, but unchanged in both WT cells (Figure 36C, left). This is of course unsurprising, since HUWE1 could even be used to gate the knockout-fraction. Interestingly however, HUWE1 was found to increase in level in not only the WT cells as they differentiated, but also in the knockout-fraction, and to a similar extent (Figure 36C, right). This is consistent with the increase in *HUWE1* transcripts I saw as cells differentiated neuronally.



- C)** Quantifications of HUWE1 levels by cell-type from **B)**, coloured by condition, showing increase in HUWE1 with differentiation. HUWE1-KO cells (KO fraction, red) in the mosaic 4OHT-treated culture have less HUWE1 regardless of cell-type, while WT cells (WT fraction, blue) in the same culture have similar levels of HUWE1 in each cell-type to their WT counterparts from a homogenous culture (HUWE1cKO (Veh control), green). A similar relative increase in HUWE1 with differentiation also occurs in KO cells (right, normalised to HUWE1 levels in progenitors). Data represent mean $\pm$ SEM from 3 paired Veh vs 4OHT differentiations. Repeated-Measures one-way ANOVA with Sidak's multiple comparisons test performed on predefined pairs within each cell-type (KO vs WT fractions, and WT fraction vs HUWE1cKO (Veh control)). All pairs ns unless stated.

$p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

6.4 HUWE1-knockouts show premature differentiation following ASCL1 stabilization

Previously, there was a discrepancy between the absolute proportions of HUWE1-knockouts as measured by the genotyping gel method (Figure 16) and the qRT-PCR method (Figure 17A), though both agreed that HUWE1-knockouts were gradually being replaced by wildtype cells over time. Seeking to resolve this now that I could identify knockout cells, I measured the change in knockout proportion over time, finding that it decreased from ~40% to 30% between Days 16-20 (Figure 37A), with the decrease starting from Day 18. This was more similar to the numbers reported by qRT-PCR, although this time it was also clear that at Day 20 HUWE1-knockouts are still present.

Another prior observation was a stabilization of ASCL1 starting from Day 16, through to Day 20, with the gradual loss in knockout cells beginning right after ASCL1 stabilization (Figure 17). The possibility was also open that ASCL1 was increased throughout the culture, and not only within HUWE1-knockouts. Looking into the knockout-fraction, I found a specific increase in ASCL1 (Figure 37B, left: red vs blue, and center) at all timepoints other than Day 16, with no change in ASCL1 levels in the wildtype-fraction (Figure 37B, right, blue vs green). This confirms on a single-cell resolution that HUWE1 regulates ASCL1 levels in human cells undergoing neuronal differentiation. The increase in ASCL1, this time starting from Day 18 (although statistical-significance was not reached) also coincided with the onset of the loss of knockouts, which would be consistent with a model whereby the increased ASCL1 drives neuronal differentiation specifically within HUWE1-knockouts leading to their reduced proliferation and supplantation by wildtype cells.

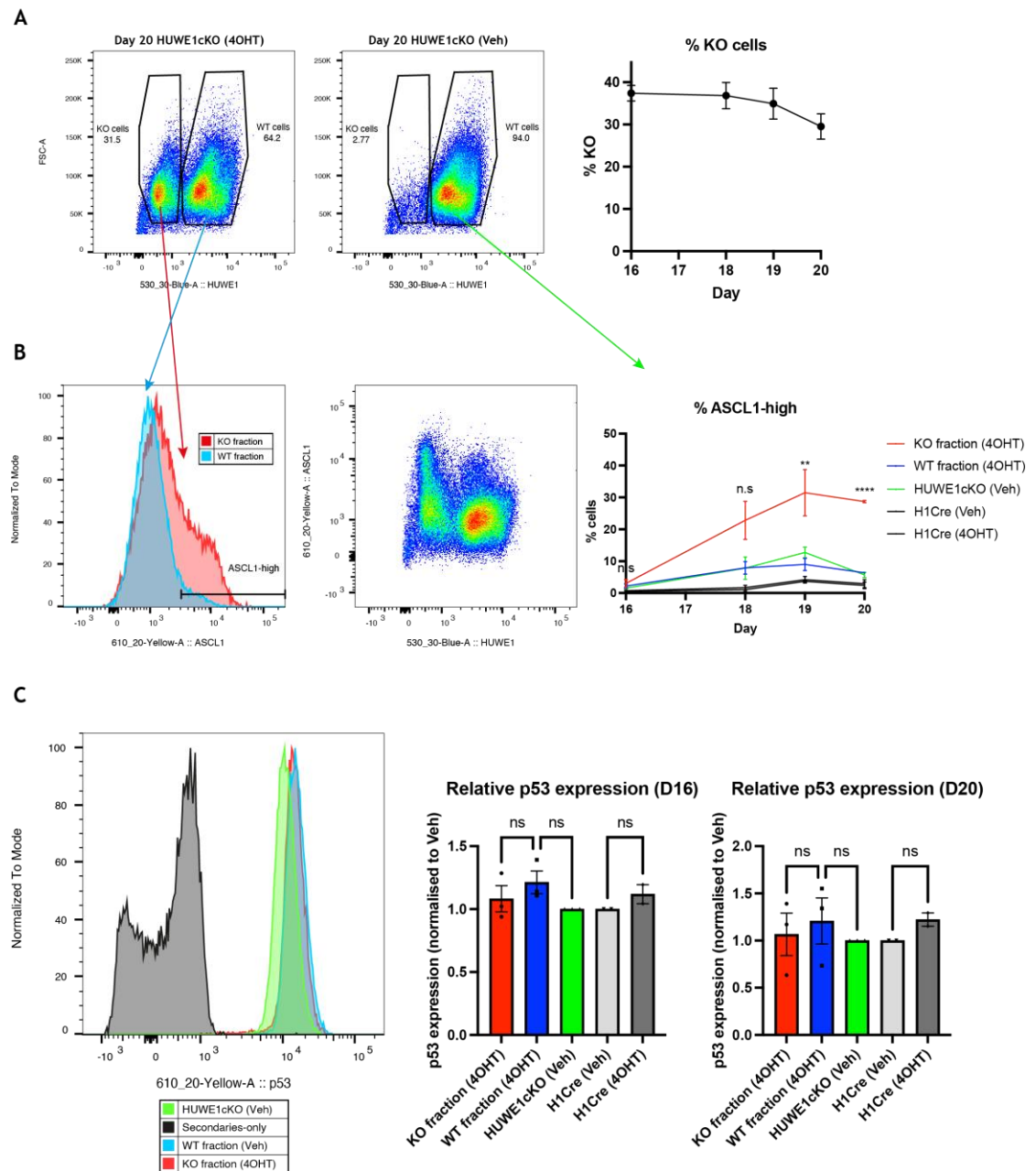


Figure 37. HUWE1-knockouts specifically upregulate ASCL1 and not p53.

A) HUWE1-knockouts were identified at Days 16-20 using the gating strategy from Figure 35A, and the proportion of KO cells quantified over time (right). HUWE1-KO cells are gradually lost. Data represent mean $\pm$ SEM from 3 paired Veh vs 4OHT differentiations.

B) Left: ASCL1 (ab211327) levels were greatly increased in the KO fraction of HUWE1cKO (4OHT-treated) (red) compared to the WT fraction (blue). Centre: Alternative visualisation showing ASCL1 is increased in HUWE1-KO cells. Right: Quantification of % of ASCL1-high cells over time in each group. 4OHT itself had no effect on ASCL1 levels (H1Cre Veh vs 4OHT, black lines), and there was no difference in ASCL1 levels between WT cells cultured homogeneously (green) or with KO cells (blue) at all timepoints. ASCL1 was increased specifically in HUWE1-KOs (red) from Day 18.

- C)** Left: p53 (Novus NB200-171) levels were increased mildly but equally in both HUWE1-KO and HUWE1-WT fractions following 4OHT treatment, compared to HUWE1cKO Veh. Right: Quantifications at Days 16 and 20 of changes in p53 levels in both H1Cre and HUWE1cKO lines relative to Veh-treated conditions. There was a small, non-statistically-significant, increase in p53 due to 4OHT treatment, but no contribution from loss of HUWE1.

Data represent mean $\pm$ SEM from 3 paired HUWE1cKO Veh vs 4OHT and 2 paired H1Cre Veh vs 4OHT differentiations. One-way ANOVA with Sidak's multiple comparisons test performed on 3 predefined pairs within each timepoint (KO vs WT fractions; WT fraction vs HUWE1cKO (Veh control); and H1Cre Veh vs 4OHT). All pairs ns unless stated. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

I had also shown previously that p53 levels were unaffected by loss of HUWE1 at Day 16 by Western Blot (Figure 17B), supported by qRT-PCR of its transcriptional target p21 (Figure 18B). It could have been argued then that the result was a false-negative as a mild HUWE1-KO specific stabilization of p53 in a minority knockout culture would be diluted out. With flow cytometry, this too could be addressed. Similar to before, I found that 4OHT itself could perhaps increase p53 levels slightly in both H1Cre and HUWE1cKO at both Days 16 and 20 (Figure 37C, not-significant). Critically, the knockout-fraction had similar p53 levels to the wildtype-fraction (Figure 37C, red vs blue), demonstrating that p53 is not a substrate of HUWE1 at these timepoints.

Based on a working hypothesis that increased ASCL1 in HUWE1-knockouts would drive their cell-cycle exit and premature differentiation following ASCL1 expression and stabilization, I next investigated the expression of differentiation markers within the knockout-fraction. After gating on HUWE1, by co-staining for the neuronal markers TUBB3 (TUJ1), or DCX, and the progenitor marker SOX2, I could see the distribution of each genotype into the 3 cell-types (Figure 38A, C) previously demonstrated in wildtype KOLF2 differentiations (Figure 28A). There was a clear shift in the knockout-fraction's distribution, with many more cells than expected being differentiating progenitors, along with a smaller increase in the proportion of neurons. As would be expected from the working hypothesis, this shift in cell-type proportions started from Day 18 (Figure 38B, D), with a decrease in progenitors and corresponding increase in differentiating progenitors and neurons starting then.

Interestingly, HUWE1-knockout cells are blocked from differentiating at Day 16 prior to ASCL1 expression, with significantly fewer knockout neurons than expected. This

could indicate that the differentiation block also seen in HUWE1 patient-derived iPSCs¹⁶⁸ is not entirely p53-driven, since at Day 16 p53 levels are not elevated but a differentiation block still occurs. Additionally, this finding might relate to why HUWE1 tends to be under-expressed in brain tumours¹⁷¹, and involve stabilization of another substrate not studied in this project, with no stabilization of ASCL1.

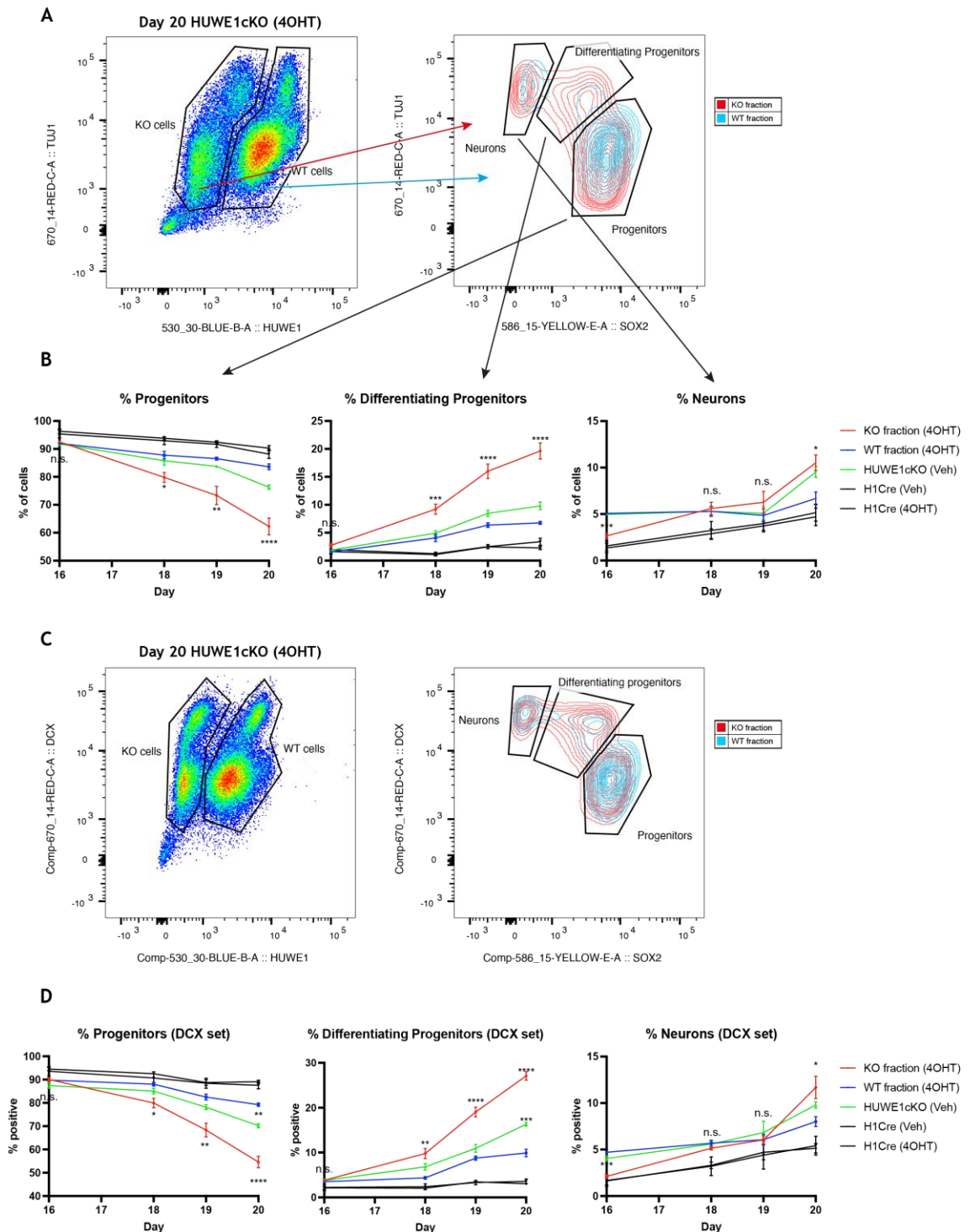


Figure 38. HUWE1-knockout cells differentiate prematurely following increase in ASCL1.

A) Within Day 20 mosaic HUWE1cKO (4OHT-treated) cultures, HUWE1-KOs were overwhelmingly likelier to be differentiating progenitors than WT cells. Cell-types in this case were defined by TUJ1 (TUBB3, neuronal marker) and SOX2.

B) Quantifications of the 3 cell-types from **A)** over time. 4OHT itself had no effect on differentiation (black lines). The KO fraction turned from progenitors into differentiating progenitors and neurons faster than the WT fraction starting from Day 18, indicating accelerated differentiation (red vs blue lines). There appeared to be a non-cell-autonomous component to this phenotype as the WT fraction differentiated slower than WT cells from a

homogenous culture (blue vs green lines), though statistical-significance was not reached. HUWE1-KOs also appeared to be blocked from differentiating before Day 16, with a lower proportion of neurons.

- C)** The same phenotype was observed when using DCX instead of TUBB3 to define cell-types.
- D)** Quantifications for **C)** over time, showing a near-identical result to **B)**. This time, delayed differentiation in the WT fraction relative to HUWE1cKO (Veh control) was statistically-significant at Day 20 (blue vs green).

Data represent mean $\pm$ SEM from 3 paired HUWE1cKO Veh vs 4OHT and 2 paired H1Cre Veh vs 4OHT differentiations. One-way ANOVA with Sidak's multiple comparisons test performed on 3 predefined pairs within each timepoint (KO vs WT fractions; WT fraction vs HUWE1cKO (Veh control); and H1Cre Veh vs 4OHT). All pairs ns unless stated. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Another interesting observation was that HUWE1-knockouts were accumulating as differentiating progenitors, where the difference from the wildtype-fraction was greatest. Despite the disproportionately-high numbers of differentiating progenitors, the proportion of neurons did not subsequently increase to a similar extent at the later timepoints. This suggested that HUWE1-knockouts might even be trapped as differentiating progenitors.

A potentially-concerning result from Figure 38B, D, is that the wildtype-fraction appears to be inhibited from differentiating (blue vs green traces), with fewer wildtype differentiating progenitors and neurons than expected, when cultured in the presence of HUWE1-knockouts. This was not the result of 4OHT application as H1Cre (Veh control) and H1Cre (4OHT-treated) differentiated identically (black traces). This would fit a model whereby HUWE1-knockouts express more Notch ligands (DLL1, DLL3 – both transcriptional targets of ASCL1^{63,73,265}) relative to their wildtype neighbours, through stabilized ASCL1. This would inhibit neurogenesis laterally⁴ in the wildtypes, meaning HUWE1-knockouts differentiate prematurely at the expense of wildtype cells sharing the culture. A natural follow-up question is therefore whether the premature differentiation of HUWE1-knockouts is cell-autonomous? I therefore attempted to address this in the next chapter.

To further support the working hypothesis of loss of HUWE1 stabilizing ASCL1 and in turn driving premature differentiation, I measured the proliferation of knockouts over time. For this, I pulsed cultures for 2hrs with 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analogue that incorporates into the DNA of actively-dividing cells during

the S phase, prior to collection for flow cytometry. EdU can then be detected by click chemistry to AF647, to label cells that entered S phase during the 2hr window. By co-staining with DAPI to measure the total DNA content of each cell, the G1/0, S, and G2/M (having completed DNA replication and doubling their DNA content) cell-cycle phases can be distinguished in a 'horseshoe' shape (Figure 39A, right). When segregating by genotype in such an analysis, I found that the knockout-fraction was less proliferative overall (Figure 39A, B), with proportionately more cells in G1/0 and fewer cells in S and G2/M. This phenotype was strongest starting from Day 18, in line with the working hypothesis, and could also explain the gradual takeover of HUWE1cKO (4OHT-treated) cultures by the wildtype-fraction.

At Day 16, however, S phase was not yet different between KO and WT fractions, though G1/0 was significantly increased, and G2/M reduced. This would indicate a mild reduction in proliferation prior to ASCL1 stabilization. If true, then there might be a short time window between preventing p53-driven cell-death and delaying ASCL1-driven differentiation whereby HUWE1 is necessary for promoting proliferation and differentiation through unknown, p53 and ASCL1-independent, mechanisms.

Another observation in support of HUWE1-knockouts inhibiting their wildtype neighbours' differentiation non-cell-autonomously is that the WT fraction is more proliferative than expected of wildtype cells (Figure 39B, blue vs green), with fewer cells in G1/0, and more cells in S phase, at Day 19. This would be consistent with them being more likely to be progenitors than in HUWE1cKO (Veh control).

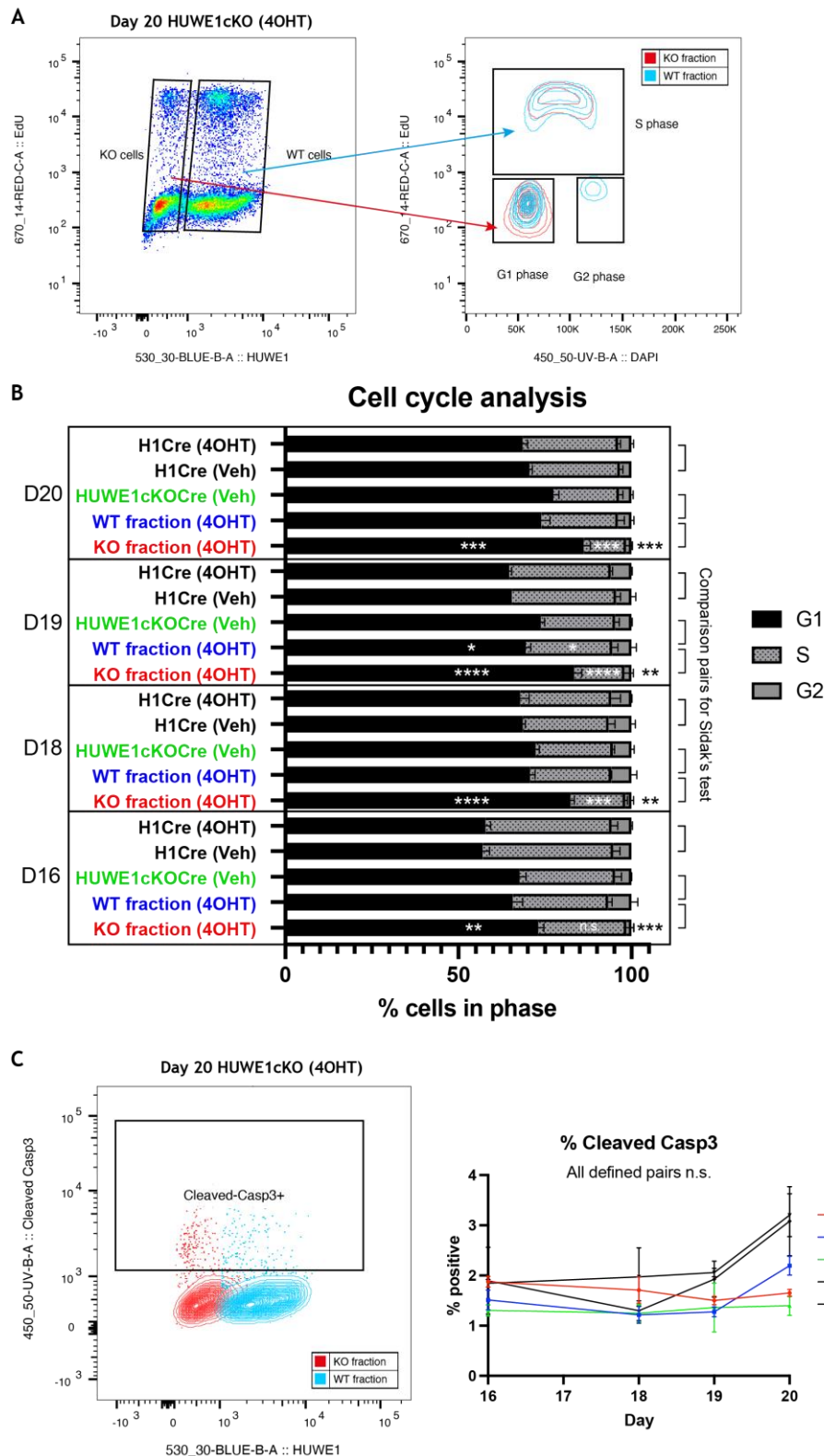


Figure 39. HUWE1-knockouts have reduced proliferation but are not more apoptotic.

A) Cells were pulsed for 2hrs with EdU prior to dissociation for flow cytometry. Cells entering S phase during the 2hr window incorporate EdU into their DNA, which can be conjugated by click chemistry to AF647 for detection. HUWE1-knockouts were less proliferative than WT cells within the same culture, with proportionately fewer cells entering S phase. Fewer KO cells were also in G2 phase as revealed by DAPI staining.

- B)** Quantification of proportion of cells in each cell-cycle phase between Days 16-20, based on gating from **A**). Consistent with overall differentiation of the culture, the proportion of cells in G1 increased with fewer in S phase over time. KO cells were significantly more likely to be in G1 phase with fewer KO cells in G2 at every timepoint. Fewer KO cells were in S phase from Day 18. 4OHT had no effect on any cell-cycle phase as seen in H1Cre Veh vs 4OHT comparisons. There appeared to be a small, non-significant, increase in proliferation of the WT fraction, compared to the homogenous HUWE1cKO (Veh control) at every timepoint, with statistical-significance reached at Day 19.
- C)** After genotype-gating, the proportion of live single cells that were undergoing early apoptosis, as marked by cleaved-Caspase 3 staining, was quantified. Very few live cells were apoptotic in any of the cultures at all timepoints, and loss of HUWE1 did not affect the proportion of cells in early apoptosis. Use of a fixable viability dye in combination with cleaved-Caspase 3 to identify early apoptosis events was adapted from Vossenkamper and Warnes 2018²⁶⁶.

Data represent mean $\pm$ SEM from 3 paired HUWE1cKO Veh vs 4OHT and 2 paired H1Cre Veh vs 4OHT differentiations. One-way ANOVA with Sidak's multiple comparisons test performed on 3 predefined pairs for each phase at each timepoint (KO vs WT fractions; WT fraction vs HUWE1cKO (Veh control); and H1Cre Veh vs 4OHT). For cleaved-Casp 3, one-way ANOVA with Sidak's multiple comparisons test was performed on the 3 predefined pairs at each timepoint. All pairs ns unless stated. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Since the loss of knockout cells over time could potentially also be explained by preferential cell-death, I briefly explored this by staining for the apoptosis marker cleaved-Caspase 3. This analysis was done on the gated live single-cells like before, to identify viable cells that were initiating apoptosis (Figure 39C) – a strategy from Vossenkamper and Warnes 2018²⁶⁶. Additionally, this was because it was not possible to accurately segregate dead cells by genotype using cleaved-caspase 3 or the viability dye since all dead/late-stage apoptotic cells had lower levels of protein (including HUWE1) in general and would appear as HUWE1-knockouts. Through this strategy, I found that loss of HUWE1 had no effect on the likelihood of initiating apoptosis (Figure 39C).

Having shown that HUWE1-knockouts are disproportionately likely to be differentiating progenitors (and neurons to a smaller extent) following ASCL1 stabilization, a question that arose was whether ASCL1 was stabilized ectopically in HUWE1-knockouts. Based on our KOLF2 scRNAseq²²⁰ and flow cytometry results (scRNAseq, Figure 26; Flow cytometry, Figure 28A), ASCL1 is not expressed in neurons on both transcript and protein levels, and thus should also not be elevated in HUWE1-knockout neurons.

Comparing knockout against wildtype fractions from Day 20 cultures, I found that ASCL1 was increased in knockouts only in cells with intermediate-high SOX2 levels (Figure 40A, right). By quantifying the proportion of ASCL1-high cells in each of the 3 cell-types, I found that ASCL1 was only elevated in HUWE1-knockout progenitors and differentiating progenitors, but not HUWE1-knockout neurons (Figure 40B). This, combined with the observation that HUWE1-knockouts appear trapped as differentiating progenitors, would fit a model whereby neurons require the downregulation of ASCL1 to form from differentiating progenitors. In such a model, HUWE1-knockout differentiating progenitors take longer to complete neuronal differentiation, as their ASCL1 persists at a higher level until it is transcriptionally-silenced.

Given what I now know – that HUWE1-knockouts are present in a sizable minority (30-40%) between Days 16-20, and are between 2-4 times more likely to become differentiating progenitors in the same timeframe (Figure 37, Figure 38) – one would expect a detectable ~2 fold increase in the proportion of differentiating progenitors at Day 20 when comparing HUWE1cKO (4OHT-treated) against HUWE1cKO (Veh control) as a population. However, there were also hints that HUWE1-knockouts were inhibiting the differentiation of wildtypes within their culture non-cell-autonomously, which would counteract the expected phenotype above. Furthermore, prior population-level analyses showed no detectable shifts in differentiation markers (qRT-PCR, Figure 18; IFs, Figure 20), other than an increase in *RGS16* and *IGFBP5* that was not matched by a similar increase in other transitional progenitor markers (Figure 29B).

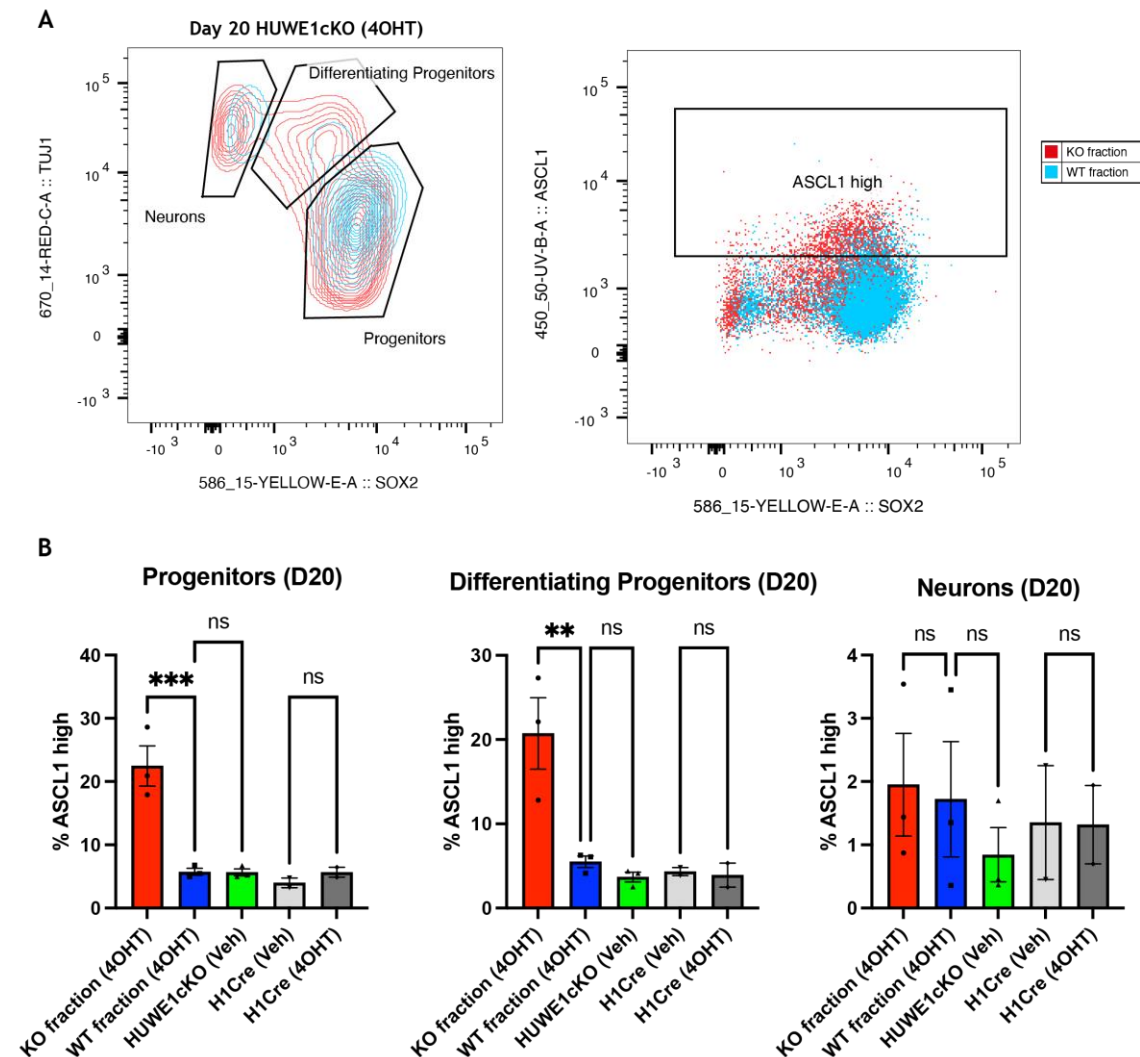


Figure 40. ASCL1 overexpression is restricted to progenitors in HUWE1-knockouts.

A) ASCL1 levels in each of the 3 cell-types from Figure 38 were checked at Day 20. ASCL1 levels were higher in the KO fraction than the WT fraction as previously seen in Figure 37, but were similar between both fractions in SOX2⁺ cells (right).

B) The % of ASCL1-high cells in each cell-type was quantified. Only HUWE1-KO progenitors and differentiating progenitors had significantly more ASCL1-high cells than in their WT counterparts, with no difference in neurons.

Data represent mean $\pm$ SEM from 3 paired HUWE1cKO Veh vs 4OHT and 2 paired H1Cre Veh vs 4OHT differentiations. One-way ANOVA with Sidak's multiple comparisons test performed on 3 predefined pairs (KO vs WT fractions; WT fraction vs HUWE1cKO (Veh control); and H1Cre Veh vs 4OHT). All pairs ns unless stated. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

I thus thought to investigate the potential non-cell-autonomous effects by revisiting the data from Figure 38C, D, and removing the genotype-gates to look at the proportion of the 3 cell-types over time in the cultures as a whole. In an indication that the cultures are differentiating similarly overall, the proportions of progenitors,

differentiating progenitors, and neurons, were identical between HUWE1cKO (4OHT-treated) and HUWE1cKO (Veh control) (Figure 41A, B) at every timepoint. This confirmed the necessity of a genotype-gate to identify HUWE1-dependent phenotypes in my system. More critically, this, combined with the prior population-level analyses, strongly indicate a non-cell-autonomous inhibition of differentiation by HUWE1-knockouts on neighbouring wildtypes, resulting in premature differentiation and depletion of HUWE1-knockouts but overall unaffected neural differentiation.

Two key questions arise from the findings in this chapter:

1. Given the likely non-cell-autonomous effects at play, is the premature differentiation of HUWE1-knockouts itself non-cell-autonomous, and dependent on having wildtype neighbours?
2. The premature differentiation phenotypes in HUWE1-knockouts coincide with stabilization of ASCL1 – which has a strong literature supporting this relationship as causal. However, I have yet to demonstrate this causality. Would preventing the increase in ASCL1 rescue the HUWE1-knockout phenotypes?

In the next chapter, I attempt, unsuccessfully, to address these questions.

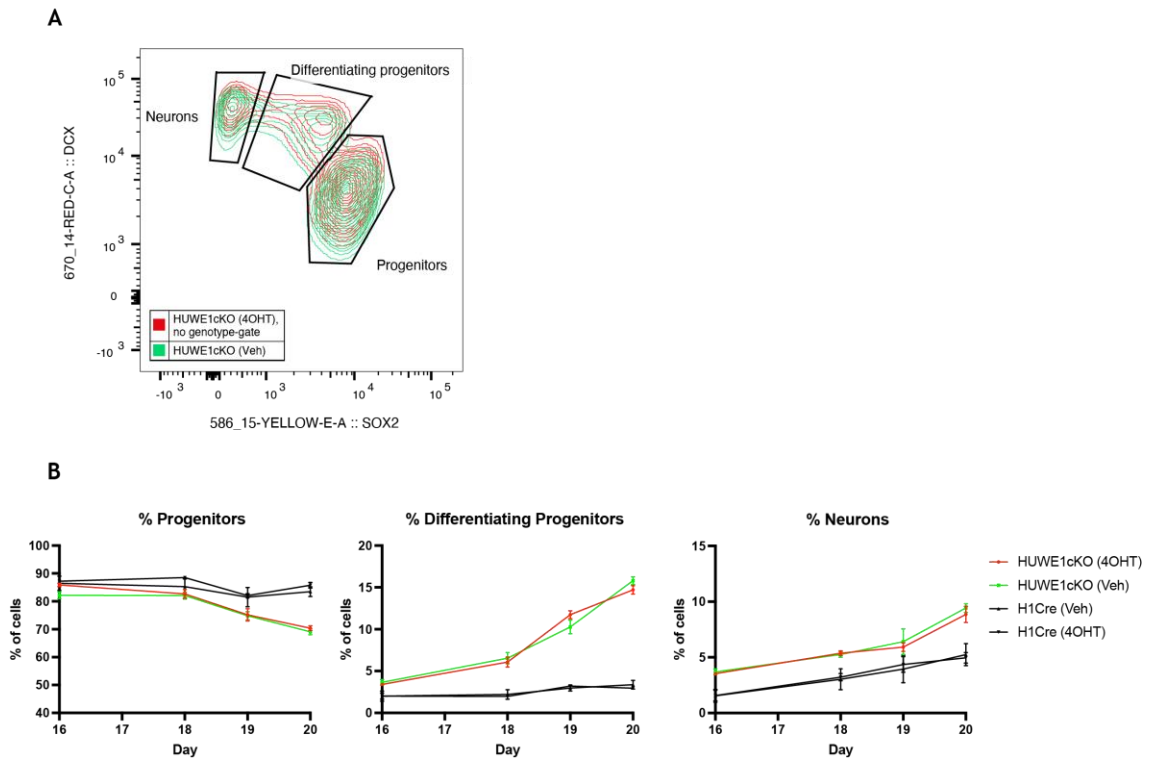


Figure 41. 4OHT-treated HUWE1cKO differentiates similarly to vehicle-controls overall.

- A)** Without the use of a HUWE1-stain genotype gate, the mosaic HUWE1cKO (4OHT-treated) culture differentiates similarly to its vehicle-treated homogenously WT pair, despite having 30-40% KOs. Data is the same as Figure 38C, with the genotype gate removed.
- B)** Quantifications of the proportion of cells in each of the 3 cell-types in **A)**, coloured by condition. No difference was observed between Veh and 4OHT conditions at all timepoints in both H1Cre and HUWE1cKO when a genotype gate was not applied.

Data represent mean $\pm$ SEM from 3 paired HUWE1cKO Veh vs 4OHT and 2 paired H1Cre Veh vs 4OHT differentiations. One-way ANOVA with Sidak's multiple comparisons test performed on 3 predefined pairs at each timepoint (KO vs WT fractions; WT fraction vs HUWE1cKO (Veh control); and H1Cre Veh vs 4OHT). All pairs ns unless stated. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Results 5: Unsuccessful attempts to address questions on cell autonomy and *ASCL1*

One key characteristic of proneural transcription factors is the ability to restrict their own expression in neighbouring cells non-cell-autonomously through lateral inhibition^{4,267}. This is achieved by promoting expression of extracellular Notch ligands, such as *DLL1/3*, that signal to neighbouring cells' Notch receptors, resulting in receptor cleavage and upregulation of transcription factors, such as *HES1/5*, that repress proneural gene expression within the target cell^{90,94}. Consequently, a comparison of Notch ligand levels occurs between neighbouring neural progenitors, such that small differences in proneural gene expression (and the resulting ligand levels) can be amplified to result in segregation, by proneural gene expression levels, of neighbours into discrete progenitor populations²⁶⁷.

Two key questions arose in the previous chapter revolving around this hallmark of proneural genes. Firstly, were *HUWE1*-knockout cells' premature differentiation caused by *ASCL1* stabilization? Assuming so, will this *ASCL1* stabilization and premature differentiation occur in a homogenous *HUWE1*-knockout culture? One might imagine such a culture could behave similarly to a wildtype one through lateral inhibition, as the stabilization of *ASCL1* in a knockout cell could still repress *ASCL1* transcription in neighbouring *HUWE1*-knockout cells, and inhibit their differentiation. With repressed *ASCL1* transcription, the loss of *HUWE1* could have no effect on *ASCL1* levels in these neighbours. Addressing this second question thus has wider implications for understanding *HUWE1*-related genetic disorders, which can occur both as a hemizygous (and therefore homogenous genotype) condition in males, and as a result of mosaicism in females¹⁶⁰.

In this chapter, I first attempted unsuccessfully to inhibit *HUWE1* activity uniformly during neural differentiation through the use of a small molecule inhibitor. I next attempted to increase the knockout efficiency by using a cell-permeable recombinant Cre, but found it to be toxic. In relation to the causality of *ASCL1* in driving the premature differentiation seen in *HUWE1*-knockout cells, I tried to answer this by

knocking down *ASCL1* transcript levels with a lentiviral-delivered short-hairpin RNA (shRNA). When this was unsuccessful, I switched to using electroporation during neural differentiation to deliver the *ASCL1* shRNAs, unfortunately finding that they were ineffective. This however revealed a new HUWE1-knockout phenotype that will require further characterisation.

7.1 HUWE1 small molecule inhibitor BI8622 did not affect neural differentiation

Seeking a way to model a homogenous HUWE1 loss-of-function (LOF), I chanced upon HUWE1 small molecule inhibitors (BI8622 and BI8626) first developed and reported in Peter et al. 2014²⁶⁸. Both were discovered in a screen of compounds capable of preventing HUWE1's HECT domain's auto-ubiquitination, in which BI8622 had an IC₅₀ of 3.1µM while BI8626 had an IC₅₀ of 0.9µM, and subsequently validated in HeLa cells²⁶⁸. BI8622 was also used in a later study in relation to Huwe1 and Myst2 in mouse embryonic stem cells²⁶⁹, where its use at 5 and 10µM mildly increased Myst2 levels, similar to shRNA against *Huwe1*. Since BI8622 was used independently to apparent success, I decided to try it (BI8622, MedChemExpress HY-120929) on my neural differentiation. This approach, if successful, could both allow for an orthogonal method of verifying my HUWE LOF observations while potentially addressing the issue of cell autonomy by achieving a uniform inhibition of HUWE1 activity.

I first performed a pilot to establish the working concentration for BI8622 in my system, adding it into the differentiation medium daily from Days 12 to 20 at 3 doses (2/5/10µM) (Figure 42A). Since this was an orthogonal validation that did not require HUWE1cKOs, I used unedited H1 hESCs. 10µM BI8622 was found to be toxic to the cells within 24hrs of application, whereas all other doses and a DMSO-control matched to the amount used in the 10µM dose remained confluent and capable of reaching Day 20 (Figure 42A). Interestingly, one other indication that BI8622 had some effect on the cultures was that media that included it was consistently more yellow during culture (Figure 42B). While the reason for this was unknown, this colour

change could serve as a positive control that suggests an active compound was present, should downstream assays prove to be no different from the DMSO control.

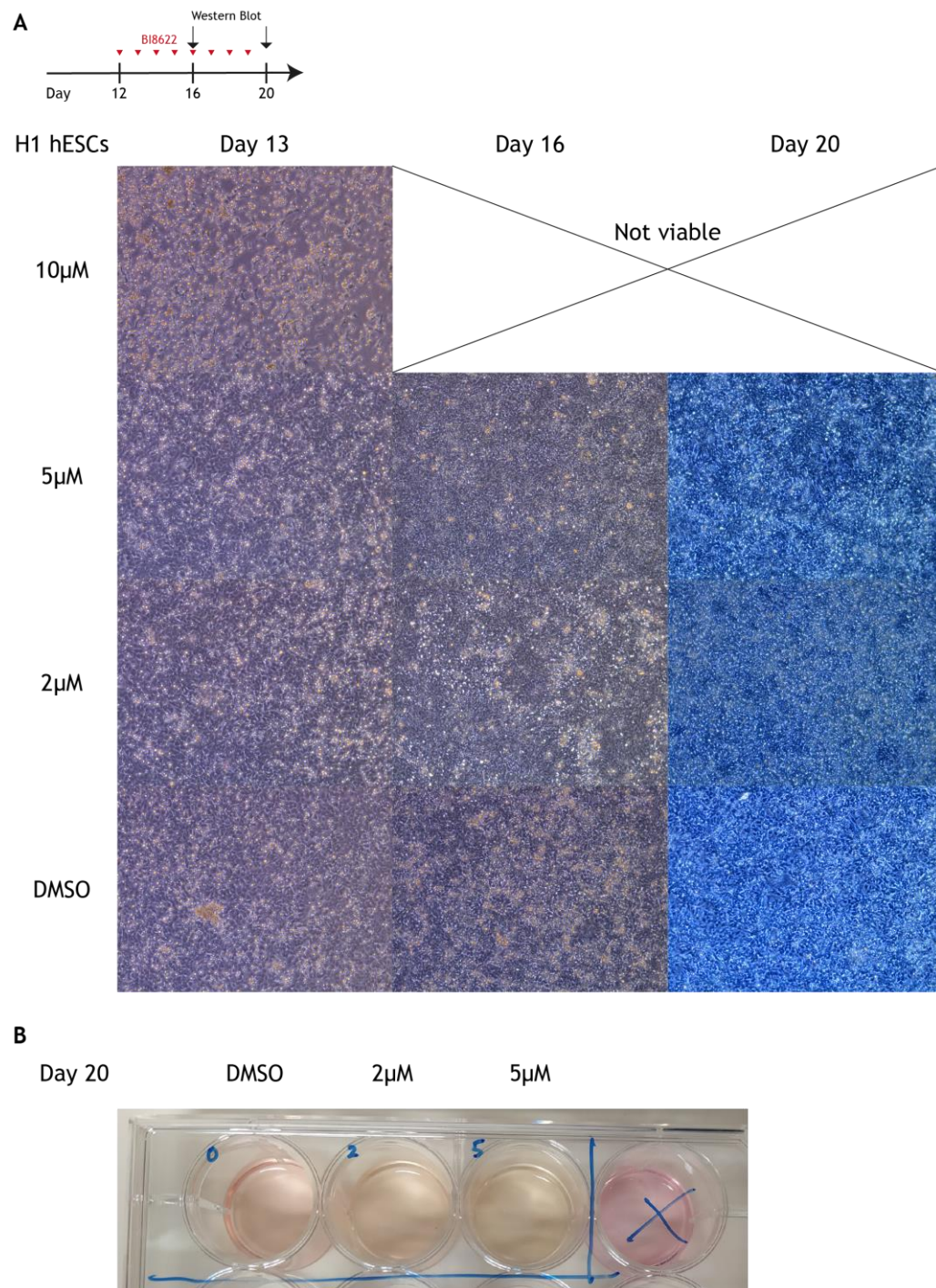


Figure 42. BI8622 is toxic to neural progenitors at 10µM, and results in a change of media colour.

A) The HUWE1 small molecule inhibitor BI8622 was added daily to neural differentiations from Days 12 – 20 at 0/2/5/10µM. 10µM BI8622 resulted in extensive cell death within 24hrs whereas all other concentrations were viable.

B) The media colour of cells treated with BI8622 was consistently more yellow throughout differentiation, serving as a positive control for its presence.

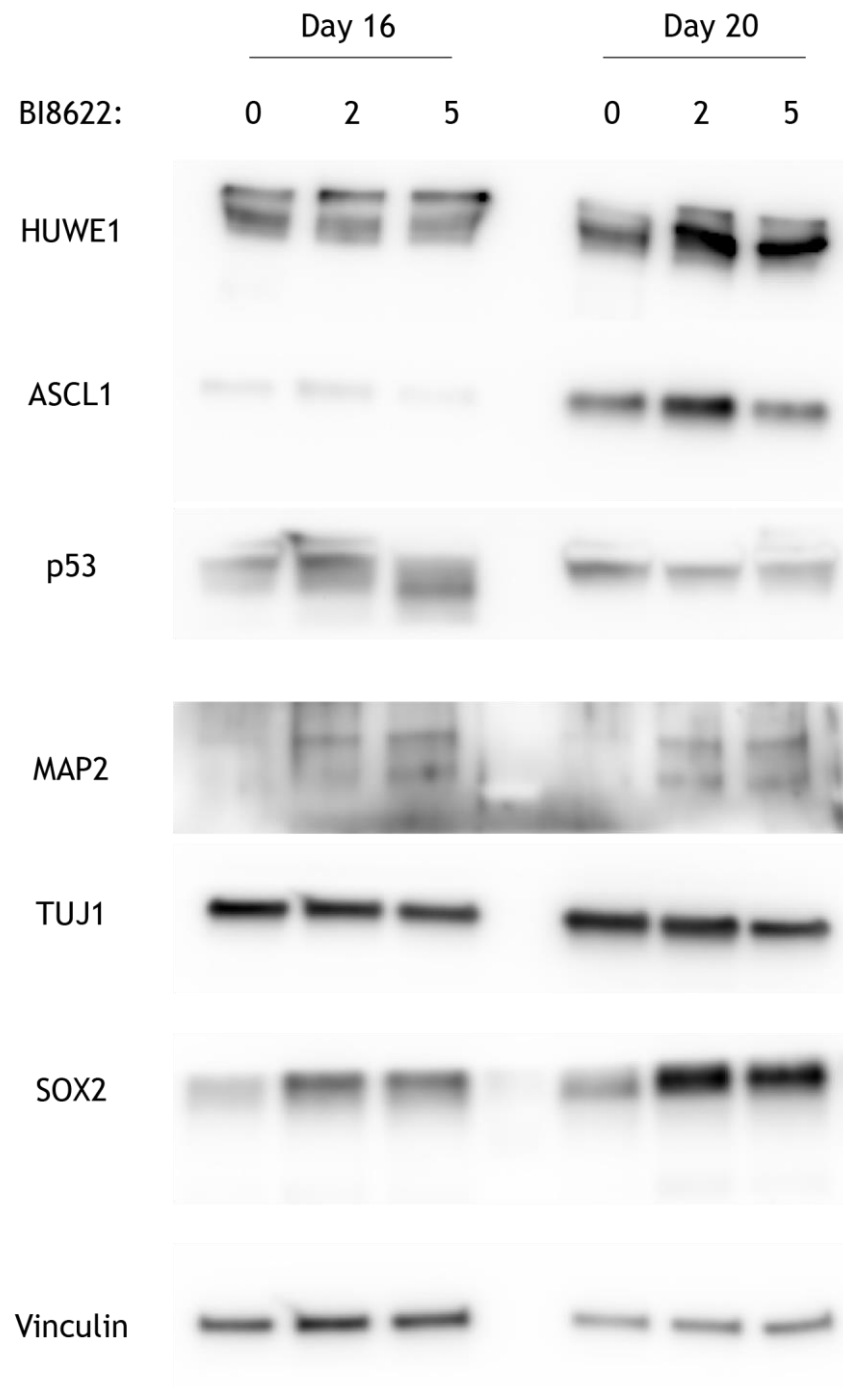


Figure 43. Western Blot for BI8622-treated neural differentiations at Days 16 and 20. Cells treated with 2 or 5 μ M BI8622 were harvested at Days 16 and 20, and 20 μ g of protein lysates subjected to Western Blotting. Blots were probed for HUWE1 and its reported substrates ASCL1 and p53. The neuronal markers MAP2 and TUBB3 (TUJ1), and the neural progenitor marker SOX2 were also included. Vinculin (VCL) served as the loading control. Pilot experiment of one.

The 2/5 μ M BI8622-treated cells were then harvested for Western Blotting at Days 16 and 20 to determine whether BI8622 worked, and the concentration to use, for subsequent experiments. Bearing in mind that this Western was from one differentiation, I found that HUWE1 was greatly increased at Day 20 at both doses although not at Day 16 (Figure 43). Since HUWE1 was known to auto-ubiquitinate^{174,176} and tag itself for proteasomal degradation²⁷⁰, this could indicate successful inhibition of HUWE1, leading to its accumulation. In line with this, at 2 μ M (though not at 5 μ M), ASCL1 levels were increased which might again suggest successful inhibition of HUWE1 (Figure 43). The neuronal marker MAP2's (though not TUBB3's) (Figure 43) expression also increased following BI8622 treatment which might indicate the HUWE1 LOF-related premature differentiation I was expecting. This was however accompanied by a paradoxical increase in the levels of the progenitor marker SOX2 (Figure 43). While the BI8622-induced changes in expression were inconsistent (for ASCL1 and HUWE1) and contradictory (for MAP2, TUBB3, and SOX2), I could not exclude the possibility that these reflected the phenotypes of a uniform HUWE1 inhibition, nor make too many conclusions on a single replicate. There was also the possibility that transient changes in protein levels caused by BI8622 were lost since the cells were being harvested ~24hrs after their last BI8622 treatment.

Instead, because the safest conclusions from this pilot were that BI8622 appeared to be active; was toxic at high doses; and at 2 μ M increased both HUWE1 and ASCL1 levels (suggesting inhibition of HUWE1), I repeated the differentiations at 2 μ M BI8622. Since all previous phenotypic characterisations were done by flow cytometry, I did the same, collecting BI8622-treated cells from Days 16-20 (Figure 44A). This time, I renewed their media with 2 μ M BI8622 for 2hrs before collection for flow cytometry.

By plotting DCX against SOX2 like before, I was able to quantify the proportions of the 3 cell-types, finding the expected increase in the proportion of differentiating progenitors and neurons over time, and a decrease in the proportion of progenitors. At all timepoints however, 2 μ M BI8622 had no effect on the proportions of the 3 cell-types (Figure 44A). It also had no effect on ASCL1 levels (Figure 44B) nor HUWE1

levels (Figure 44C), as though no inhibitor was added. However, the media colour of BI8622-treated wells was again more yellow at every timepoint (not shown).

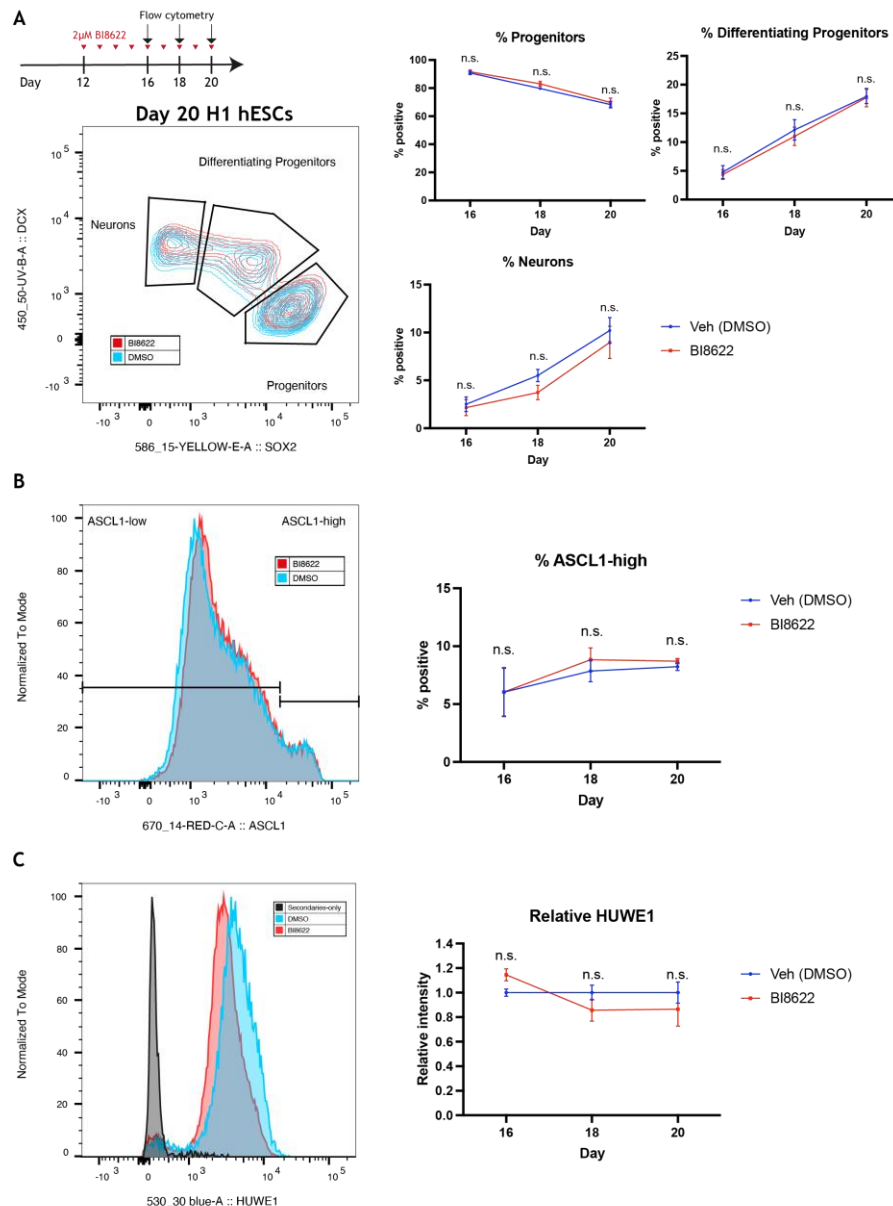


Figure 44. 2µM BI8622 has no effect on neuronal differentiation.

- A)** Treatment with BI8622 had no effect on the proportions of progenitors, differentiating progenitors and neurons, quantified on the right.
- B)** Treatment with BI8622 had no effect on the proportion of ASCL1-high cells, quantified on the right.
- C)** Treatment with BI8622 had no effect on the levels of HUWE1, quantified on the right.

Data represent mean $\pm$ SEM from 3 BI8622-treated H1, and 3 DMSO-treated H1 differentiations. Unpaired Student's t-tests performed at each timepoint. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Since such a colour change could also suggest more proliferation, and since proliferation is also a readout for changes in differentiation, I next looked at the distribution of cell-cycle phases (assessed by DAPI) following BI8622 treatment (Figure 45, top). Like previously, DAPI was used to measure the total DNA content within individual cells, with cells in G2/M phases having completed DNA replication and thus double the DAPI intensity of G1/0 phase cells. S phase cells are in the process of DNA replication and thus have intermediate DAPI intensity. In agreement with the results from Figure 44, there was no effect of BI8622 on the proliferation of neural cells at the timepoints collected (Figure 45, bottom).

Taken together, it looked like BI8622 did not inhibit HUWE1 after all, at least not in neurally-differentiating H1 hESCs, and at 2 μ M concentration. This might be because BI8622 (and BI8626) were discovered from experiments on tumour cell lines²⁶⁸, which the authors concede have higher levels of the endogenous HUWE1 inhibitor ARF, meaning HUWE1 activity in those lines might already be inhibited sufficiently such that any further weak antagonists can tip the scales (and result in a lower IC₅₀). Additionally, the effect size of BI8622 in Cho et al. 2020 was small, and similar to that of a ~45% *Huwe1* knockdown in mESCs, whereas the premature differentiation phenotype I was screening for was first observed from full HUWE1-knockouts. The level of partial HUWE1 inhibition by BI8622 might just not be sufficient to elicit the expected phenotype in human neural progenitors.

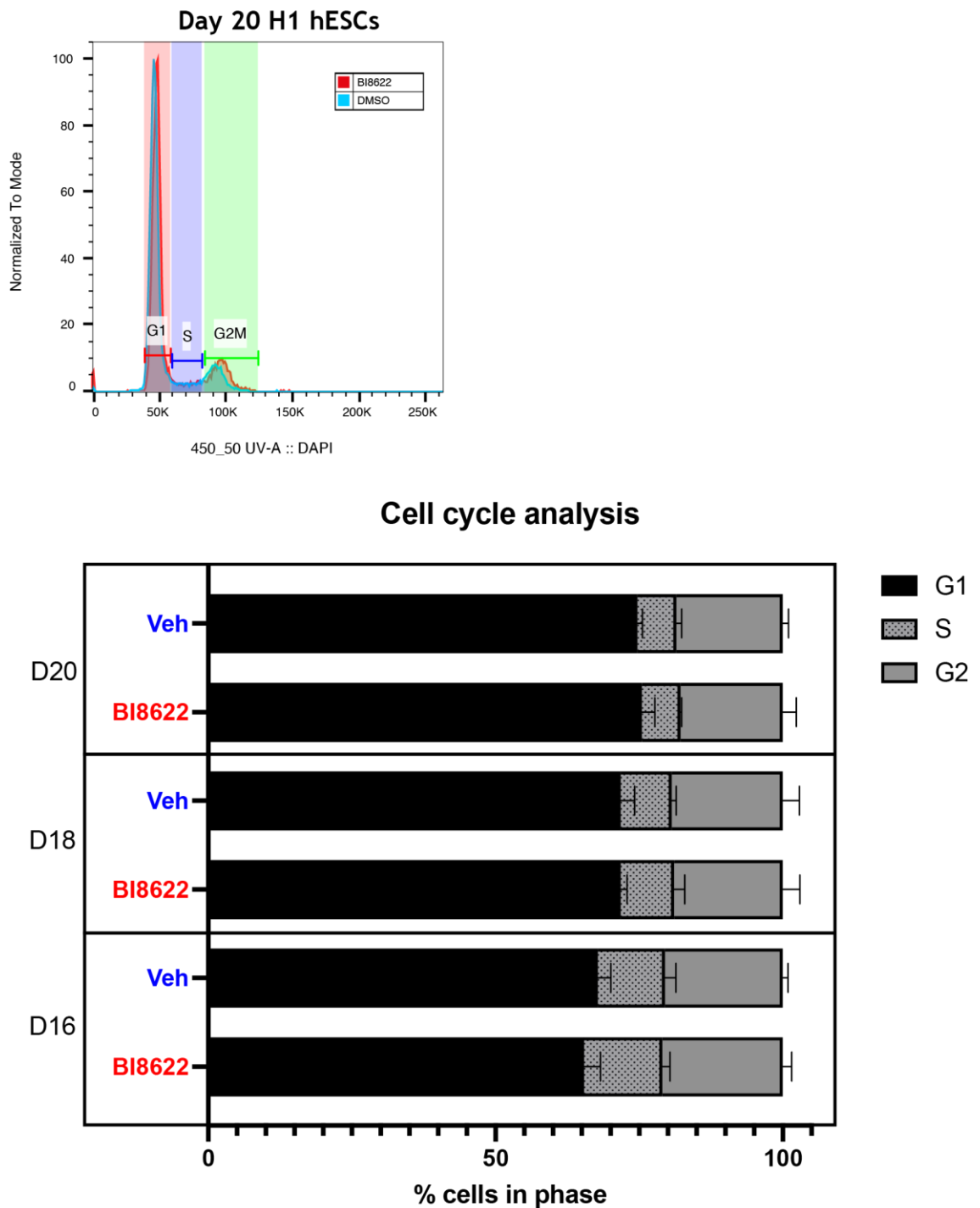


Figure 45. BI8622 did not affect cell proliferation. Top: Histograms of DAPI intensities from Day 20 H1 neural cultures treated with 2 μ M BI8622 (red) or DMSO control (blue), with cell-cycle phase gates shown. Bottom: Quantification of proportions of cells in each gate, at Days 16, 18, and 20. Data represent mean $\pm$ SEM from 3 BI8622-treated H1, and 3 DMSO-treated H1 differentiations. Unpaired Student's t-tests within cell-cycle phases performed at each timepoint. All ns.

7.2 TAT-Cre is toxic to neural cultures

An alternative approach to address the issue of cell-autonomy was to increase the knockout efficiency in HUWE1cKO to approach 100%. Since I suspected that the mosaic recombination was due to silencing of the CAG-CreERT2 I had inserted into the *AAVS1* locus, I just required a way to introduce additional Cre into HUWE1cKO to raise the knockout efficiency. For this, I settled on TAT-Cre (Merck #SCR508), a cell-permeable Cre recombinase fused to HIV's translocation peptide (TAT) and a nuclear localization sequence. TAT-Cre had even successfully been used before in hESC-derived neural precursors to achieve a ~80% recombination efficiency²⁷¹, and I aimed to layer this on top of the ~40% knockout efficiency I was getting with 4OHT.

To test if TAT-Cre was effective on HUWE1cKOs, and the concentration to use it at, I applied a serial dilution (0/2/4/10/20/100 Units/ml) of it onto HUWE1cKO hESCs for 24hrs, then fixed and imaged them a further 48hrs later (100Units/ml is defined by the manufacturer as the amount of TAT-Cre needed for 50% recombination in a HEK293T reporter assay). ~40% of cells lost HUWE1 expression at 100U/ml compared to 5% at 20U/ml (and 2% at 0U/ml using the same cutoff and quantification method, Figure 46A). All lower concentrations tested did not have any visible HUWE1 loss (not shown).

Considering that I required a near 100% deletion efficiency, and that the highest dose tested here (100U/ml) would only result in a ~64% (40% from 4OHT + (40% of remaining 60% from TAT-Cre)) deletion in conjunction with 4OHT in a best-case scenario, I decided to try a higher dose of 166.5U/ml for 3hrs during differentiation. Unfortunately, within 3hrs of TAT-Cre treatment, there was extensive cell death, which the cells never recovered from in both Veh and 4OHT-treated conditions (Figure 46B, n = 3, only Veh shown). The surviving cells were not analysed as there was insufficient material for cytometry.

Due to time constraints, my attempts to address the question of cell-autonomy had to stop here.

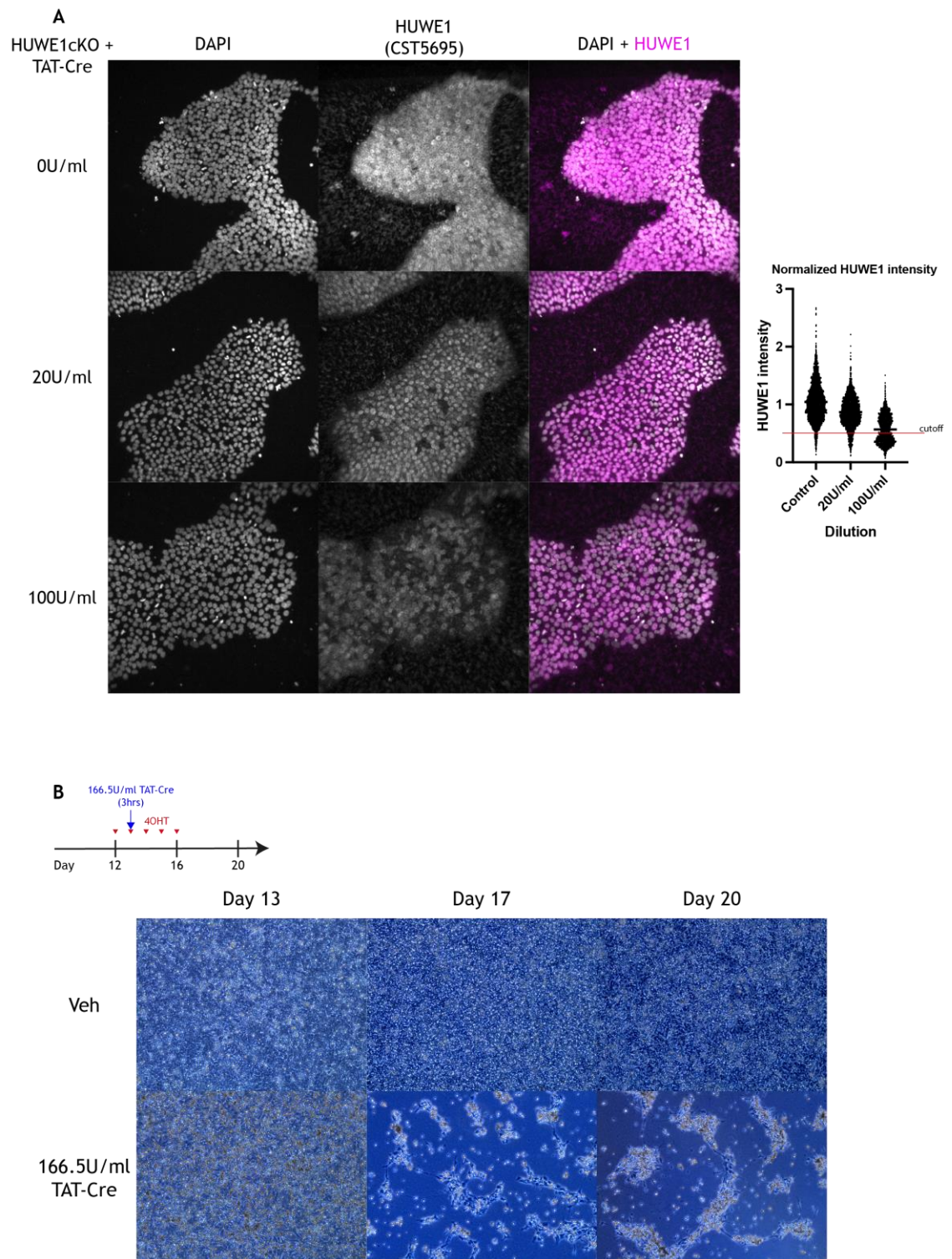


Figure 46. TAT-Cre treatment is toxic to neural progenitors.

A) HUWE1cKO hESCs were treated with different doses of TAT-Cre for 24hr, then fixed, stained, and imaged 48hrs later. 3 fields of view, 1 coverslip each. Images taken on an SP5 II confocal microscope. HUWE1 intensity was normalised to DAPI in this case, and a cutoff of 0.45 for calling knockouts used. At 100U/ml, TAT-Cre was able to give a 40% recombination efficiency.

- B)** 166.5U/ml TAT-Cre was added to neural differentiations at Day 13 for 3hrs, within which there was extensive cell death in both Veh and 4OHT-treated HUWE1cKOs. Cultures never recovered despite washing TAT-Cre off after the 3hr treatment. n = 3 differentiations.
-

7.3 HUWE1-knockouts are vulnerable to lentivirus treatment

At the same time that I was attempting to address the issue of cell-autonomy, I was also trying to establish that ASCL1 was the primary mechanism through which HUWE1 regulated neural differentiation in neural progenitors. For this, I had to show that without the increase in ASCL1, loss of HUWE1 would not result in premature differentiation. To perform this rescue experiment, I required a way to reduce ASCL1 levels in HUWE1-knockout cells.

This experiment is however a complicated one. Ideally the rescue is performed with 100% efficiency in a 100% HUWE1-knockout neural progenitor culture. This is because of the non-cell-autonomous lateral inhibition that differences in ASCL1 levels itself can create, as discussed previously. A mosaic suppression of ASCL1 would therefore open itself to similar questions of cell-autonomy as my mosaic HUWE1cKO whereby one might ask if a rescue of the premature differentiation phenotype in ASCL1-suppressed cells will still be possible in a uniformly suppressed culture. For this uniform ASCL1-suppression, a uniform HUWE1 genetic background, unlike HUWE1cKO mosaic cultures, would also be necessary. Consequently, several steps needed to be in place for this experiment:

1. A method to acutely achieve or simulate a 100% HUWE1-knockout culture.
2. A method to reduce ASCL1 levels.
3. A method to deliver ASCL1 reduction at high efficiency into neural progenitors, or at least to label these cells.

To satisfy conditions 2 and 3 within a short timeframe, I thus thought to use lentiviruses to deliver published shRNAs against *ASCL1* while delivering a fluorescent reporter. Condition 1, which was simultaneously being worked on through my previous efforts above, ultimately was not satisfied.

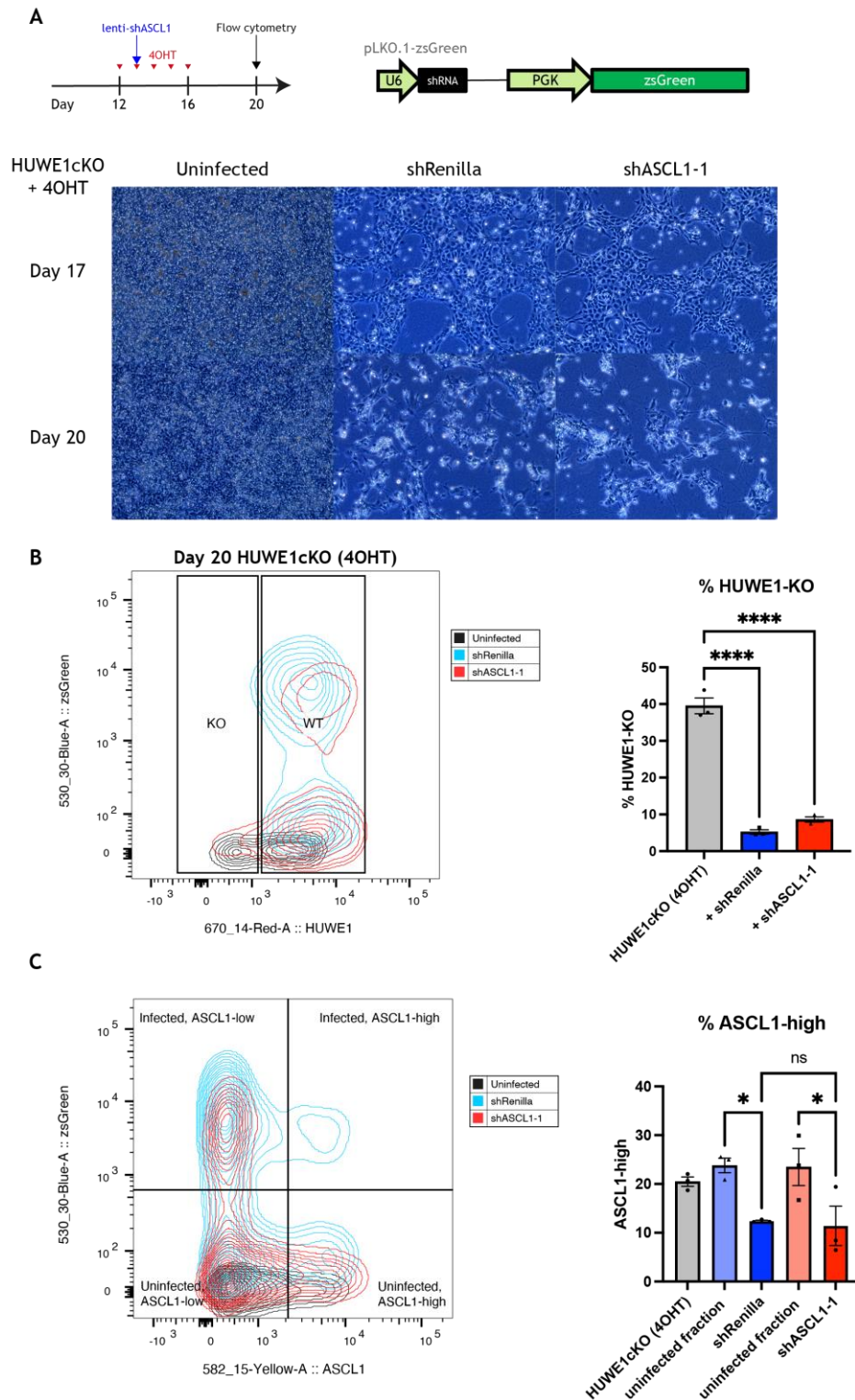


Figure 47. Lentivirus-delivered shRNA causes selective loss of HUWE1-knockouts.

A) Lentiviruses were used to deliver shRNA against *ASCL1* via pLKO.1-zsGreen into HUWE1cKOs (4OHT) on Day 13 of differentiation and harvested at Day 20 for flow cytometry. Infected cultures gradually lost confluency.

B) Contour plot of Day 20 HUWE1cKO (4OHT) cells. Infected cultures showed a specific loss of HUWE1-knockouts, quantified on the right, even though infection efficiency (zsGreen+) was not 100%. One-way ANOVA with Dunnett's multiple comparisons test.

- C)** Contour plot of zsGreen against ASCL1 levels. Infected cells had lower levels of ASCL1, even when infected with the control shRenilla shRNA. One-way ANOVA with Sidak's multiple comparisons test for pre-defined comparison pairs as shown.

Data represent mean $\pm$ SEM from 3 HUWE1cKO (4OHT) cultures. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

With the aid of Molly Strom and Ana Cunha (Viral Vector Core), pLKO.1 puro (Addgene #8453) was modified to replace puromycin resistance with the fluorescent reporter zsGreen under a PGK promoter, and shRNA sequences cloned in under the control of the U6 promoter (Figure 47A). Infected cells would thus be zsGreen+. The two shRNA sequences (shASCL1-1 and shASCL1-2) against *ASCL1* used can be found in the Materials and Methods, but were obtained from Wang et al. 2019²⁰⁵ and also correspond to The RNAi Consortium's²⁷² (TRC) TRCN0000013550 and TRCN0000235657. A control non-targeting shRNA against Renilla luciferase was also used.

HUWE1cKO was differentiated to Day 12 as per usual, then split into 3 conditions (uninfected, shRenilla, shASCL1-1) and infected the next day. Only 4OHT-treated HUWE1cKOs were used, and only one shRNA against *ASCL1* was tested. Wells where lentivirus was added gradually became less confluent (Figure 47A) over time, independent of the shRNA sequence. When the surviving cells were analysed by flow cytometry on Day 20, there was a specific loss of HUWE1-knockouts in infected wells but not in the uninfected wells (Figure 47B). This was despite lentivirus infection efficiencies of $17.2 \pm 2.83\%$ (shASCL1-1) and $55.4 \pm 1.83\%$ (shRenilla).

Since neither lentivirus had a 100% infection rate, it was unlikely HUWE1-knockouts were all infected – this specific loss of HUWE1-knockout cells thus did not appear to require infection by a lentivirus, and could be due to the temporary addition of polybrene on Day 13 to facilitate infection. However, there remains the possibility that HUWE1-knockout cells are more likely to be infected than wildtype cells and also less tolerant of either integration of the pLKO.1 construct or the lentivirus components.

As I still had surviving cells infected with shASCL1, I could still assess if the shRNA was successful in knocking down ASCL1 levels. Unfortunately, infected cells had

similar proportions of ASCL1-high cells regardless of shRNA sequence, though these were lower than in uninfected cells (Figure 47C), suggesting that the 1st shRNA against *ASCL1* was ineffective. Strangely, infection itself may reduce ASCL1 levels, or permit the survival of only ASCL1-low cells (such as cycling progenitors).

Because of the selective loss of HUWE1-knockouts, I needed another way to deliver the pLKO.1-zsGreen shRNAs into HUWE1-knockouts (Condition 3, above), regardless of whether the shRNAs themselves worked (Condition 2). For this, I tried an electroporation method optimised by Oana Păun on my cultures during the splitting step at Day 16. Briefly, to account for and mitigate the cell death inherent to the procedure, 2 wells worth of cells are combined into 1, and ROCK inhibitor is used liberally. This method was successful, resulting in zsGreen+ cells within 24hrs, and more surviving cells at Day 20 for flow cytometry than with the lentivirus (Figure 48A).

When analysed by flow cytometry, the HUWE1-knockouts were present this time, with no selective loss of knockouts (Figure 48B). Surprisingly, the electroporation method also appeared to result in nearly every cell taking up the pLKO.1-zsGreen plasmid and turning zsGreen+, with zsGreen levels above that of the un-electroporated control (Figure 48B).

Unfortunately, the proportion of ASCL1-high cells was again no different between shRenilla and the shASCL1 sequences, even when analysing only the cells with the highest levels of zsGreen (Figure 48B, should one suspect that those are the cells that are truly electroporated). Only Vehicle-treated HUWE1cKOs (i.e., wildtype cells) were analysed to avoid complicating analysis as the knockouts had more ASCL1 regardless of shRNA. Since zsGreen was still expressed in the cells, the plasmid was still present despite the transient nature of electroporation. This would thus suggest that neither shRNA sequence against *ASCL1* was able to reduce ASCL1 protein levels to any meaningful degree in this system.

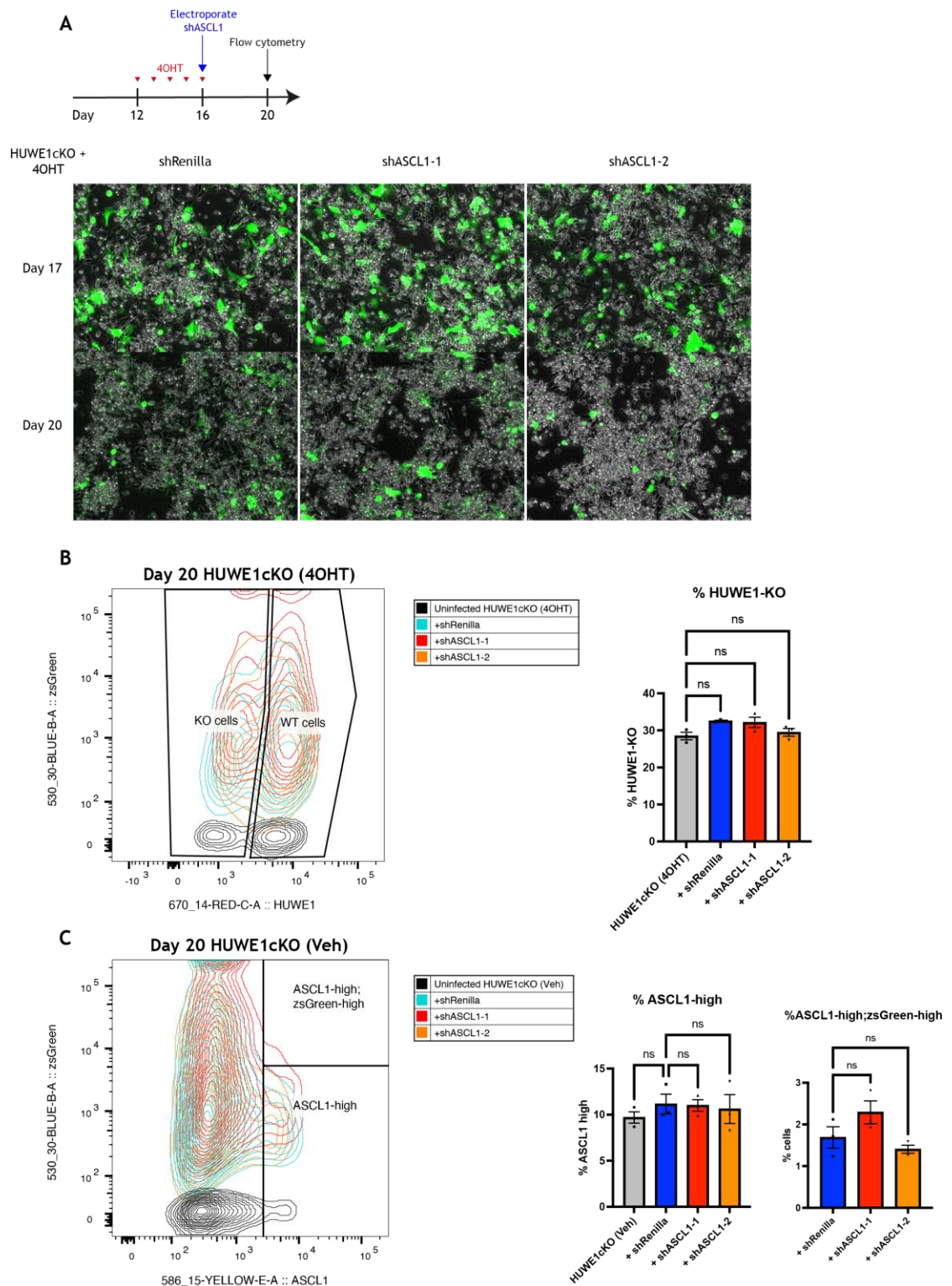


Figure 48. Electroporation delivers shRNA into HUWE1cKO successfully, but shASCL1 sequences are ineffective.

A) HUWE1cKOs were electroporated at Day 16 with pLKO.1-zsGreen plasmids to deliver shRNAs against Renilla or ASCL1. Cells were zsGreen+ within 24hrs, and did not progressively lose confluency by Day 20.

B) Electroporation resulted in nearly every cell being zsGreen+ (all contours above the un-electroporated control in black). HUWE1-knockouts were present in all electroporated

conditions, in the expected proportions, as quantified on the right. One-way ANOVA with Dunnett's multiple comparisons test.

- C)** shASCL1-electroporated cells had similar proportions of ASCL1-high cells as un-electroporated or shRenilla-electroporated cells. There was no difference in the proportions of ASCL1-high cells between the electroporated conditions when looking only within zsGreen-high cells. One-way ANOVA with Dunnett's multiple comparisons test.

Data represent mean $\pm$ SEM from 3 HUWE1cKO differentiations each. $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

Revisiting the 3 conditions I listed as necessary for addressing the issues of cell-autonomy and causality of ASCL1:

1. A method to acutely achieve or simulate a 100% HUWE1-knockout culture.
2. A method to reduce ASCL1 levels.
3. A method to deliver ASCL1 reduction at high efficiency into neural progenitors, or at least to label these cells.

Meeting the 3 criteria turned out to be too onerous and sadly, I was only able to find a method to satisfy the 3rd condition. Potential workarounds will be discussed in the Discussion chapter.

Discussion

Throughout this project, I encountered multiple challenges while trying to identify the functions of HUWE1 during human neurogenesis. Although ultimately somewhat successful, several questions remain and several more have been uncovered by my results. While I have briefly discussed many of these issues where pertinent in their Results chapters, I shall discuss them again, and others, here.

8.1 HUWE1 degrades p53 and ASCL1 at different stages of neurogenesis to expand the neural progenitor pool

Starting off with the an overview of the central findings of my project, I showed that human cells lacking HUWE1 are unable to survive early neurogenesis, accompanied by an increase in levels of HUWE1's substrate, p53 – in agreement with results from Aprigliano et al. 2021¹⁶⁸ in patient cells where they showed this relationship was causal to the cell death phenotype. The extent to which this occurs *in utero* is unknown since the expected phenotype would be incompatible with life, yet patients are born alive. This was discussed in Results chapters 1 and 2, and will be again later. It is however plausible that some lesser degree of p53-regulated cell death normally occurs during early neurogenesis, which HUWE1 helps to suppress – thereby expanding the neural progenitor pool.

Mechanistically, this may be through preventing the Notch signalling-induced, p53-dependent, apoptosis of neuroepithelial cells²⁷³. Cells with higher HUWE1 activity should tend to have lower ASCL1 activity than their neighbours – and thus have more Notch signalling. As such, they would also require HUWE1 to simultaneously suppress p53 levels. This would mean HUWE1 helps neuroepithelial cells achieve both Notch signalling-induced proliferation (by keeping ASCL1 levels low in these cells), and prevention of the associated apoptosis. The same paper²⁷³ also showed a cell-type (and temporal) specificity for this p53-dependency, showing that neurons with high Notch signalling did not experience this p53-induced apoptosis, much like how deleting *HUWE1* at later timepoints does not entail p53-related cell death. It could therefore be interesting to investigate if the p53-dependent apoptosis seen in

my HUWE1-knockouts and Aprigliano et al. 2021¹⁶⁸ is actually non-cell-autonomous, and can be attenuated through Notch inhibition.

At later stages of neurogenesis, in neural progenitor cells (NPCs), regulation of p53 by HUWE1 no longer occurs, and instead HUWE1 regulates the proneural transcription factor ASCL1 to maintain proliferation. As a result, human NPCs lacking HUWE1 have increased ASCL1 levels, and prematurely differentiate into transitional progenitors, instead of remaining in the cell-cycle. Completion of terminal differentiation from transitional progenitors into neurons itself requires removal of ASCL1, which is normally lost on a transcriptional level, but also appears to involve HUWE1's facilitation, such that transitional progenitors lacking HUWE1 are also slower to remove ASCL1 and become neurons. Perhaps the increase in HUWE1 levels I saw on both protein and transcript levels in neurons reflects this second function. Loss of HUWE1 in human NPCs thus leads to an accumulation of transitional progenitors and neurons, and a depletion of the cycling NPC pool.

The *in vivo* correlates of the human cells studied in this project (early neural cells, cycling NPCs, transitional progenitors, and neurons) are likely to be neuroepithelial cells, radial glia, intermediate progenitor cells (IPCs), and early-born neurons respectively. This is based on the label transfer Stephanie performed in Păun et al. 2023²²⁰ of the differentiation model studied here onto early fetal brain transcriptomes from Braun et al. 2022²⁶⁰, and will be discussed further below.

Putting everything together, a model of HUWE1's functions during human neurogenesis emerges (Figure 49) whereby HUWE1 suppresses cell death in neuroepithelial cells through degradation of p53. This enables sufficient neuroepithelial cells to survive and undergo radial glial transition, at which point p53 suppression is no longer performed by HUWE1. Instead, when these apical radial glia express ASCL1, HUWE1-mediated degradation returns ASCL1 to baseline levels quickly – preventing its accumulation, and possibly contributing to the short periodicity of ASCL1 oscillations required for proliferation of NPCs⁹⁰. This prevents the premature differentiation of radial glia, thus expanding and preserving the progenitor pool for later-born neuronal subtypes. It would therefore be interesting to study if HUWE1 is responsible for ASCL1 protein-level oscillations on top of *ASCL1*

transcript oscillations already demonstrated in Imayoshi et al. 2013⁹⁰. In radial glia that manage to acquire sufficient ASCL1 levels (through mechanisms that will be discussed) and become transitional progenitors (likely apical IPCs), HUWE1 is then involved in completing the timely differentiation into neurons by removing residual ASCL1 alongside the transcriptional downregulation of *ASCL1* that occurs in neurons. The outcome of a prolonged ASCL1-high transitional progenitor state if HUWE1 fails to do so is currently unknown.

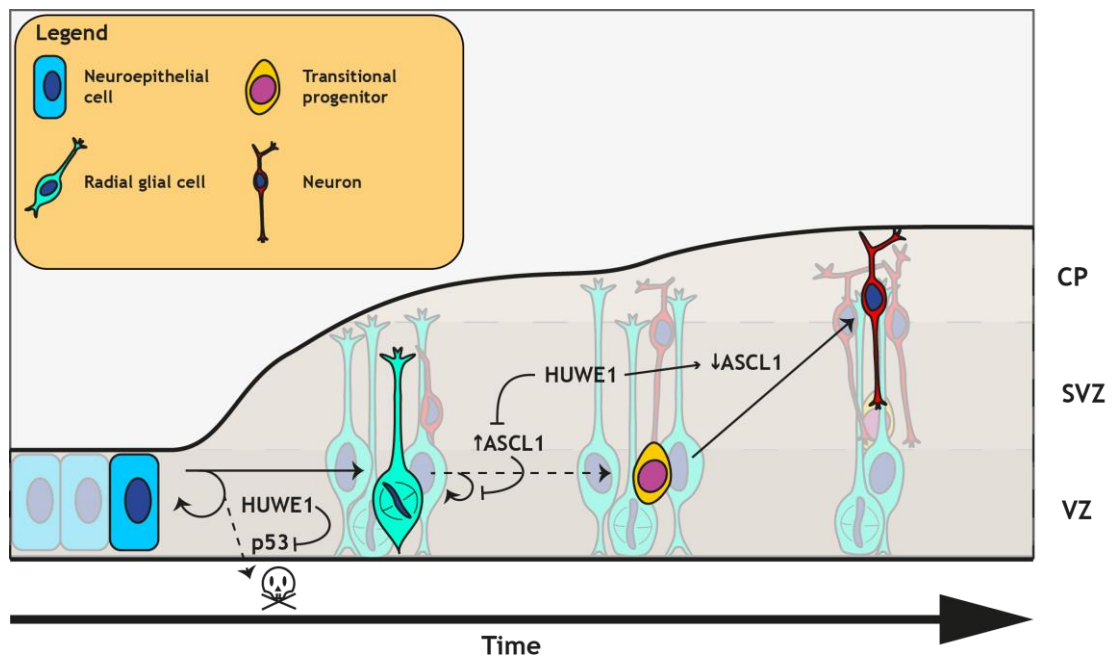


Figure 49. Proposed functions of HUWE1 during human neurogenesis. During embryonic neurogenesis, forebrain neuroepithelial cells require HUWE1 to avoid cell-death, through a p53-dependent mechanism. After radial glial transition, HUWE1 suppresses ASCL1 levels in radial glia to promote proliferation and expansion of the progenitor pool, by inhibiting entry into an ASCL1-high, ASCL1-dependent, transitional progenitor state (which may correspond to neuroblasts and intermediate progenitor cells). Completion of terminal differentiation in transitional progenitors to neurons also requires subsequent downregulation of ASCL1, which occurs transcriptionally, but is sped up by protein-level degradation through HUWE1. CP: cortical plate; SVZ: sub-ventricular zone; VZ: ventricular zone. Dashed arrows represent less-frequent events. Illustration: Yu Xuan Tan

These proposed functions of HUWE1 during human neurogenesis were inferred from a conditional knockout model (the HUWE1cKO line) that I generated. Accordingly, there are advantages and limitations to the usage of this line that could affect the interpretation of the results. Chief among these was the mosaicism (likely due to silencing of the AAVS1-integrated CAG-CreERT2) inherent in the model that gave a perfect internal control but also introduced questions on cell-autonomy. Both the

model, and cell-autonomy, will be discussed below, along with potential ways to address them in future investigations.

Additionally, while it is assumed based on mouse studies^{185,191} that HUWE1 is ubiquitinating and tagging ASCL1 (and p53 at the earlier timepoint) for degradation in my model, I did not actually perform any ubiquitination assays in my project, but rather focussed on the phenotype of HUWE1-loss in humans. Initially, a mass-spectrometry-based Lys-diGly proteomics experiment, similar to that performed in Thompson et al. 2014 for HUWE1 in BT549 breast carcinoma cells²⁷⁴, was planned to compare the “ubiquitinomes” of HUWE1-WT and HUWE1-KO neural cultures. Briefly, this works as ubiquitin is attached to lysine residues on substrates, and trypsin treatment for mass-spectrometry digests ubiquitinated proteins such that these lysines are left with characteristic di-glycine (diGly) modifications. By enriching for these diGly-modified peptides through specific antibodies before performing mass-spectrometry, all ubiquitinated proteins and their ubiquitinated sites can theoretically be identified. This can also be made quantitative by SILAC-labelling²⁷⁵, for example, of 4OHT-treated HUWE1cKO vs Veh-treated HUWE1cKO. If successful, this experiment could have revealed which diGly-modified peptides are reduced/lost in 4OHT-treated HUWE1cKOs, and hence all HUWE1 substrates in (predominantly) neural progenitor cultures. This would provide a full list of ubiquitinated candidates contributing to HUWE1-related genetic conditions beyond ASCL1. However, this experiment was not performed as it is inherently a population-level assay, and I deemed a 30-40% knockout proportion as possibly too low, such that the reduction in most diGly-modified peptides might be obscured.

8.2 Implications for the aetiology of HUWE1-related genetic disorders

By expanding on HUWE1’s scope of function during human neurogenesis, my project has also provided new avenues for exploring the aetiology of HUWE1-related genetic disorders. However, since my project was conducted in a model with a complete loss-of-function (LOF) of HUWE1, the following discussion includes

speculation on my part. Individual patient mutations are likely to be less severe, and will need to be studied to verify these hypotheses.

The first implication of my results is that p53 dysregulation is not the sole (nor likely the main) means by which HUWE1-related genetic disorders occur, despite the apparent rescue of neuronal differentiation seen in HUWE1 patient-derived cells¹⁶⁸ following p53-knockdown. Firstly, as mentioned above, the p53-driven phenotypes seen in both our models are too severe and probably greatly attenuated *in utero*. Additionally, as discussed in Results Chapter 2, patient-derived cells¹⁶² (likely including those with microduplications of *HUWE1*) can show decreased p53 levels, and would therefore not involve the p53-driven apoptotic phenotype. Lower p53 levels are also not known to result in any neurological phenotypes linked to HUWE1-related genetic disorders²²⁸. Finally, as a result of my project, we now know that involvement of p53 in HUWE1-related genetic disorders is at best temporally restricted to early neurogenesis, since NPCs do not require HUWE1 to regulate p53 levels. Consequently, p53 involvement in the disorder is likely small, temporary, and only occurring in a subset of patients with HUWE1 mutations. It would also predict that patient-derived cells with hypermorphic *HUWE1* mutations such as microduplications or p.R4063Q will not experience the cell-death phenotype seen in my project and Aprigliano et al. 2021.

Rather, a more attractive and unifying mechanism for HUWE1-related genetic disorders could involve ASCL1, since I have shown here that HUWE1 also regulates ASCL1 in human NPCs. Patients with hypomorphic mutations in *HUWE1* could have increased ASCL1 during neurogenesis, and consequently depletion of radial glia through premature differentiation, leading eventually to microcephaly and intellectual disability. Conversely, patients with hypermorphic mutations in *HUWE1*, such as those with microduplications, could have reduced ASCL1 levels throughout neurogenesis, resulting in fewer radial glia differentiating into neurons, and hence intellectual disability. Investigations into ASCL1 levels and the distribution of cell-types during differentiation of patient-derived cells, stratified by mutation type, will therefore be of great interest to confirm this hypothesis. Additionally, such investigations may require an early, transient, inhibition of p53 activity in cells with hypomorphic mutations to circumvent the expected *in vitro* cell-death phenotype.

This hypothesis would also predict the p.G4310R hypomorphic mutants rescued through permanent p53-knockdown (in Aprigliano et al. 2021¹⁶⁸) would have elevated ASCL1 levels and some degree of premature differentiation.

Should ASCL1 also be dysregulated in patient cells, another implication is that HUWE1-related genetic disorders could be neuronal subtype-specific. ASCL1 is only one of several basic helix-loop-helix (bHLH) proneural transcription factors that can drive neurogenesis (reviewed in Dennis et al. 2019⁶⁷). HUWE1's regulation of the other cortically-expressed proneural genes (such as *NEUROG1/2* or *NEUROD4*⁶) in humans remains to be investigated, but if HUWE1 exclusively regulates ASCL1 in cortical progenitors, then there would be a preferential dysregulation of differentiation into ASCL1-dependent neurons in HUWE1 patients, resulting in an imbalance of subtype proportions. Such neurons are likely to be early-born Layer I Cajal-Retzius neurons²⁷⁶ and Layer V⁷⁶ neurons, both of which have been shown to be generated following overexpression of *Ascl1* in embryonic mouse cortices. There could similarly be a dysregulation in the developing ventral forebrain, where ASCL1 expression is predominant^{19,71} and drives differentiation of radial glia into interneurons²⁵⁸, though that is beyond the scope of the current project. These are questions that might be best explored using 3D neuronal differentiation models instead to leverage their greater heterogeneity of cell-types and longer-term survival¹⁰. I would expect a 3D culture of homogenous conditional HUWE1-knockouts to preferentially differentiate into the earliest-born ASCL1-dependent neuronal subtypes at the expense of later-born upper-layer neurons, and if true, it would be interesting to see how patient cells fare.

Although my results were obtained from a mosaic model in which HUWE1 LOF cells were co-cultured with wildtype cells which could lead to questions on validity – that I raised and tried to address in Results Chapter 5 – there exists a similar mosaic situation in HUWE1-related genetic disorders that I might inadvertently have modelled. Specifically, since *HUWE1* is encoded on the X chromosome, X chromosome inactivation occurs to result in *HUWE1* mosaicism in heterozygous females. Indeed, several such individuals have been reported, all presenting with milder phenotypes of HUWE1-related genetic disorders, despite many having a minority of the mutant allele in their blood, saliva, and fibroblast samples¹⁶⁰. My

observations have demonstrated that in a similar minority-mosaic culture, specific premature differentiation of *HUWE1* LOF cells occurs, with overall unchanged differentiation due to compensation by wildtype cells. Consequently, a similar situation may be occurring in these female patients, whereby their early-born ASCL1-dependent neurons are either predominantly *HUWE1* mutants (in cases where their mutations are hypomorphic); or predominantly wildtype (in cases where mutations are hypermorphic), leaving *HUWE1* mutant progenitors to form later-born neurons. While in these cases, the overall number of neurons will probably be unaffected (due to non-cell-autonomous effects like in my model) and alleviate the symptoms, the *HUWE1* genotypes will not be evenly spread across neuronal subtypes, leading to a specific dysfunction in the affected subtype. This could be the case since *HUWE1* likely also plays a role in the functions of mature neurons¹⁶⁴: such as by forming a complex with OGT1¹⁹⁷ to promote release of GABA from presynaptic terminals¹⁹⁹; reducing glycine receptor levels²⁷⁷; or regulating axon branching²⁰⁰.

8.3 Non-cell-autonomous contribution of ASCL1 activity to HUWE1 function

On the topic of the inherent mosaicism of the model I used throughout the project potentially bringing into question the validity of my conclusions, the existing literature on Notch signalling in relation to proneural genes might serve to assuage these concerns.

Firstly, to reiterate these concerns: increased ASCL1 (like all other proneural genes) levels in a cell is expected to lead to increased extracellular Delta ligand expression in the same sender cell²⁶⁵ that then increases Notch receptor cleavage in neighbouring receiver cells, signalling these neighbours to upregulate expression of Hes genes⁹⁴ which in turn suppress expression of *ASCL1* in these neighbours^{90,278}, and thus their differentiation (in a process known as lateral inhibition). Because of the suppressed ASCL1 levels, these neighbours are also less able to express Delta ligands to trigger Notch signalling in the sender cell, allowing the sender to differentiate. This hypothetical mechanism is illustrated in Figure 50. There was evidence that this could have been occurring in my mosaic cultures – cocultured wildtype cells were being inhibited from differentiating, expressing less neuronal

markers, and proliferating more, than homogenously-cultured wildtype cells. A homeostatic situation might thus conceivably emerge in a homogenous LOF culture, whereby loss of HUWE1 actually has no effect on neural differentiation despite increased ASCL1 levels in individual cells as this might also increase lateral inhibition that further suppresses transcription of *ASCL1* in neighbours. As I demonstrated, HUWE1 acts on a post-translational level, and thus probably cannot affect ASCL1 levels in these transcriptionally-silenced neighbours. Consequently, the number of NPCs with high-ASCL1 expression, and thus terminally-differentiating, in a homogenous LOF culture may be no different from a wildtype culture. If true, this would mean HUWE1 is not necessary to suppress differentiation and expand the progenitor pool.

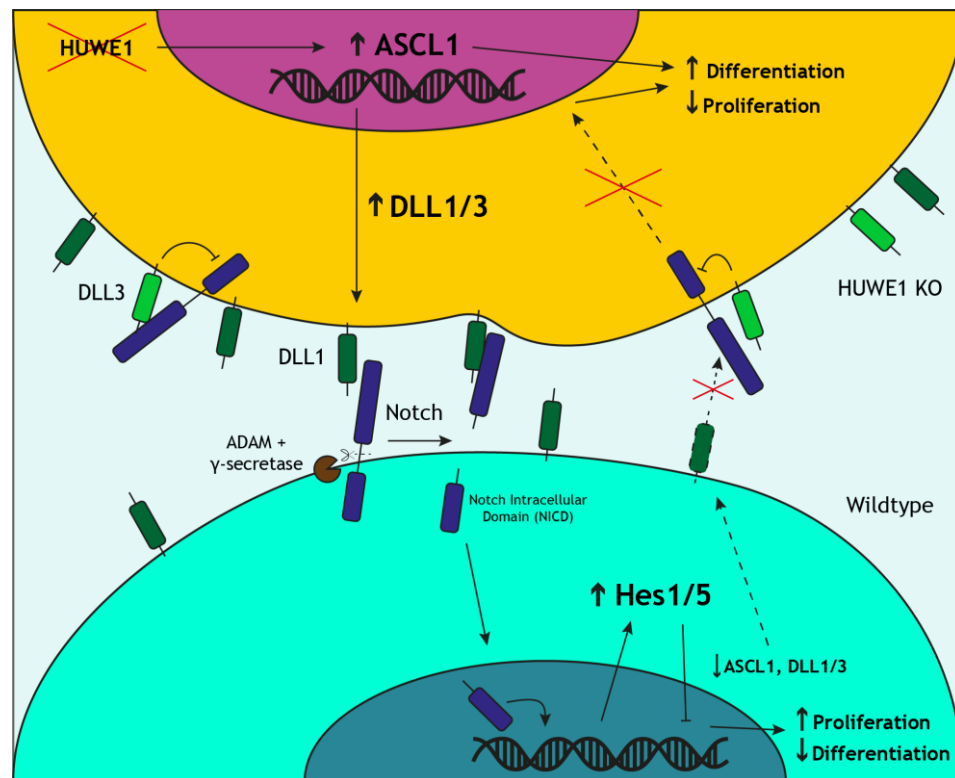


Figure 50. Increased lateral inhibition in wildtype cells may contribute to premature differentiation of HUWE1 knockouts. Loss of HUWE1 increases levels of ASCL1 in HUWE1 KOs, leading to increased expression of the Delta ligands DLL1 and DLL3, and cell-autonomous promotion of differentiation. DLL3 binds in cis to the cell's own Notch receptors and inhibits their activation⁹⁸. DLL1 binds to neighbouring cells' Notch receptors, triggering receptor cleavage by proteases to release the Notch Intracellular Domain (NICD). NICD in conjunction with other co-factors upregulates expression of the transcription factors HES1 and HES5 that in turn inhibit expression of ASCL1 while promoting proliferation at the expense of differentiation. This direction of signalling occurs from the cell with a higher ASCL1 level, and thus is more likely to occur from a HUWE1 KO to a wildtype cell. Suppression of ASCL1 results in less DLL1/3 expression, and thus less Notch signalling back to the sender cell. Consequently, there will also be less inhibition of differentiation and less proliferation in the sender cell, non-cell-autonomously. Illustration: Yu Xuan Tan.

Based on previous studies however, the likeliest outcome of a homogenous HUWE1 LOF is somewhere in-between, with the increased ASCL1 driving premature differentiation overall, but attenuated to an unknown degree by increased lateral inhibition. Firstly, a homogenous HUWE1 LOF is likely to behave more similarly to ASCL1 overexpression models than Notch-intracellular domain overexpression models, despite both likely being increased. This is because, in HUWE1 LOF:

1. Increased Notch signalling most likely occurs as a consequence of increased ASCL1 levels in HUWE1 LOF, in the mechanism described previously.
2. Though not investigated in my project, based on what we know about HUWE1 as a ubiquitin ligase, HUWE1 LOF likely increases ASCL1 levels by greatly increasing its half-life, similar to how loss of *Huwe1* results in a ~4 fold increase in ASCL1 half-life in mouse hippocampal neural stem cells¹⁸⁵. This means ASCL1 expression is more likely to take on a sustained rather than oscillatory pattern as long as its transcripts are present and translated. A sustained ASCL1 expression pattern drives neuronal differentiation, even at low levels⁹⁰.
3. Most critically, I found that ASCL1 expression was not suppressed in the wildtype cells cocultured with HUWE1-knockout cells. This shows that despite lateral inhibition likely occurring in these wildtype cells to block their differentiation, expression and translation of *ASCL1* transcripts was still occurring unaffected. Consequently, I would expect homogenous HUWE1 LOF cells to also still widely express *ASCL1* in spite of lateral inhibition since there would be less of a Notch signalling gradient between HUWE1 LOF neighbours than HUWE1 LOF-wildtype neighbours to suppress *ASCL1* transcription.

How then, might the likely increased and sustained ASCL1 in a homogenous HUWE1 LOF play out with respect to the consequently increased Notch signalling *in vivo*? Several studies have injected mRNA encoding proneural genes (such as *Xash1*²⁷⁹ (*ascl1*); *X-Ngnr1*²⁷⁹ (*neurog1*); *Xash3*²⁸⁰ (*ascl2*); *XNeuroD*²⁸⁰ (*neuroD1*); and neurogenin²⁸¹ (*neurog1*)) into xenopus and zebrafish eggs, finding that these transcripts resulted in increased expression of neuronal markers as expected. However, in all cases the ectopic neurons were found in a salt-and-pepper distribution^{279–281} as a result of lateral inhibition, with differences only in the density

of ectopic neurons, proposed to be due to the sensitivity of each of the proneural factors to lateral inhibition²⁸⁰. In light of the above points, it is likely that in humans, a homogenous loss of HUWE1's suppression of ASCL1 levels will be akin to an overexpression of ASCL1 and still result in premature differentiation attenuated partially by lateral inhibition from increased Notch signalling. Whether this truly occurs, and how sensitive it is to lateral inhibition, are potential avenues for future investigations.

8.4 Mosaicism in HUWE1 conditional knockouts during differentiation

While I expect that we can extrapolate results from the mosaicism of HUWE1cKO to draw the conclusions in Figure 49, based on the above discussion, the mosaicism itself is worth examining. HUWE1cKO contains a CAG-promoter driving expression of the hydroxytamoxifen-responsive recombinase CreERT2, integrated into the *AAVS1* locus located on chromosome 19 – widely considered to be a safe harbour site for its ability to enable stable expression of transgenes without negatively affecting the cell²⁸². During the course of my project however, I found that HUWE1cKOs underwent complete recombination at the undifferentiated hESC stage, but showed mosaic recombination, with loss of *Cre* transcripts, during differentiation into neural/ectodermal fates. This suggested silencing of the *AAVS1* locus during differentiation into an ectodermal lineage – a phenomenon that had only formally been reported during mesodermal (cardiomyocyte)²⁸³ and endodermal (hepatocyte)²⁸⁴ differentiations. One report²⁸⁵ showed that ~5% of embryoid-body-derived neural stem cells underwent silencing of *AAVS1*-integrated EGFP after 5 passages, however they did not investigate this further and concluded that *AAVS1*-driven transgene expression was retained even in differentiated cells. Consistent with these (and another²⁸⁶) reports, all our undifferentiated stem cells did not noticeably silence the *AAVS1* locus.

Consequently, the *AAVS1* locus should not be considered a safe harbour locus in humans as mosaicism of transgene expression emerges during prolonged directed differentiation protocols into any of the three germ layers. Single-cell analyses,

potentially coupled with continuous selection for non-silenced cells, will have to be carried out when working with *AAVS1*-integrated differentiation models much like in this project and Bhagwan et al. 2019²⁸³. Alternatively, the human *AAVS1* locus should only be relied on for transgene expression in the early undifferentiated state, for example to initiate differentiation by overexpression of programming factors like in the OPTi-OX¹⁴¹ system.

8.5 4-hydroxytamoxifen-specific induction of p53 in neural progenitors

Another unanticipated observation from my choice of using CreERT2 was that 4-hydroxytamoxifen (4OHT) itself could have effects on cells independent of their genotype, and dependent on cell-type. The CreERT2 system was developed as a line of research into using ligand-binding domains of hormone receptors to modulate the activity of fused proteins^{287–290}. It uses the c-terminal estrogen-binding domain²⁹¹ of the human estrogen-receptor ESR1, modified by three residue substitutions to render it unresponsive to estrogen but retain binding ability to the synthetic drug 4OHT²⁸⁹. Consequently, endogenously-produced estrogen could no longer trigger unwanted recombination events by CreERT2, allowing it to perform as intended²⁸⁹. Often, however, experiment designs neglect the reverse, and overlook controlling for the exogenously-applied 4OHT's potential effects when using it as an inducible genetic switch, despite reports of 4OHT having direct effects such as on cardiomyocyte contractility and calcium handling²⁹², and on differentiation and proliferation in developing mouse neural progenitors²³².

In my project, I found that 4OHT increased p53 levels when applied to human neural progenitors, but not when applied at the hESC stage. Fortunately, I had generated the control H1Cre line to control for direct effects of 4OHT, demonstrating that HUWE1 is unlikely to regulate p53 levels in neural progenitors as p53's levels did not increase in HUWE1-knockouts beyond those induced by 4OHT itself. Even if 4OHT might be obscuring HUWE1-knockouts' increase in p53 (perhaps by acting through the same pathway as HUWE1), the fact that H1Cre cells had more p53 following 4OHT-treatment would argue that p53 activity cannot account for HUWE1's functions

in neural progenitors since 4OHT-treated H1Cre cells differentiated and proliferated identically to Veh-treated H1Cre cells.

Why though, did p53 levels (and those of its canonical transcription target p21) increase following 4OHT addition in neural progenitors? As it turns out 4OHT, as a selective estrogen receptor modulator (SERM), is able to act as an agonist of ESR1 in some tissues, but as an antagonist in others like the brain²⁹³. Additionally, 4OHT has been demonstrated to have ESR1-independent cytotoxic effects on cells²⁹⁴, although this is not universal – proliferation and apoptosis (and p53 levels, similarly to mine) of hESCs²⁹⁵ and human vascular smooth muscle cells²⁹⁶ were unaffected by 4OHT. 4OHT should thus not be automatically considered inert in cells. Of relevance to my project, *Esr1* expression has been reported in rat neural stem cells, where application of estradiol (ESR1 agonist) led to an increase in *p21* expression²⁹⁷. This might be independent of ESR1's transcription activity (and hence whether its bound ligand is an agonist or antagonist) as ligand-bound ESR1 can bind to p53, regardless of ligand type, to prevent p53's degradation by MDM2²⁹⁸. The 4OHT-bound ESR1-p53 complex is also unable to recruit corepressors to the *CDKN1C*(p21) promoter, leading to increased *p21* expression²⁹⁹. 4OHT has also been demonstrated to promote transcription from the p53 promoter through an upregulation of c-myc by both ESR1-dependent and ESR1-independent mechanisms³⁰⁰. Finally, 4OHT has been consistently shown to increase the levels of ESR1^{299,301,302} by blocking its ubiquitination and degradation³⁰¹, and increased ESR1 levels has been shown to increase p53 levels²⁹⁸. Any combination of these mechanisms could account for the 4OHT-induced p53 I observed in my neural progenitors. Regardless, one should be wary of the appropriate controls when applying 4OHT in their models.

8.6 Increase in ASCL1 levels in transitional progenitors

One question that might arise from my project is how wildtype NPCs manage to transiently accumulate ASCL1 to sufficient levels to become transitional progenitors and subsequently neurons, considering I have also shown that HUWE1 increases in expression as NPCs differentiate, on both transcript and protein levels. This would

imply that for NPCs to differentiate, ASCL1 levels must somehow contend with a background of increasing degradation by HUWE1.

One solution, as I have shown in my collaboration with Oana Păun, is that levels of *ASCL1* transcripts also increase greatly in transitional progenitors before decreasing again in neurons. This observation was mirrored exactly on a protein-level in our flow cytometry data.

Another possibility that was not explored in this project is that ASCL1 might temporarily escape HUWE1 recognition and degradation in transitional progenitors. As I (in the case of p53), and others^{182,184,268,303}, have already shown, HUWE1 can co-exist in a cell with its substrates but be unable to degrade them. Mechanistically for example, Forget et al. 2014¹⁸⁴ and Cheng et al. 2016¹⁸⁶ showed that HUWE1 can only recognise and degrade the proneural transcription factor Atoh1 when it is phosphorylated. Similarly, ASCL1 has been shown to be phosphorylated at six serine-proline sites⁸⁵, and preventing their phosphorylation through replacement with alanine (in the form of ASCL1SA6) enhances ASCL1's ability to promote neural differentiation^{85,304}. Incidentally, ASCL1SA6 is also more stable than wildtype ASCL1, producing more protein for the same amount of mRNA^{85,305}. An intriguing possibility, now that I have shown HUWE1 degrades ASCL1 in human NPCs, is that HUWE1 preferentially degrades the phosphorylated form of ASCL1 that is present in actively-cycling progenitors⁸⁵ to suppress ASCL1 levels and maintain proliferation. Conversely, during cell-cycle exit and differentiation, dephosphorylation of ASCL1 might render it resistant to HUWE1-mediated degradation to allow it to accumulate. It would be interesting to examine this mechanism, perhaps by comparing the half-lives of wildtype ASCL1 and ASCL1SA6 in HUWE1-knockouts and HUWE1 wildtypes.

8.7 Identity of ASCL1-driven transitional progenitors and neurons

In my project, we identified, via single-cell RNAsequencing, the existence and signatures of three classes of cells in our human neural differentiations (cycling

NPCs, transitional progenitors, and neurons). By performing a label transfer onto early fetal brain transcriptomes from Braun et al. 2022²⁶⁰ (performed by Stephanie in Paun et al. 2023²²⁰) we found highly similar signatures, and surmised that our cells likely represent radial glia, intermediate progenitor cells (IPCs), and early-born neurons respectively. Given their transcriptomes (high cell-cycle exit genes, *HES6*, and *SOX4*) and RNA velocity; our short differentiations (20 Days), and the young age of the fetal brain samples (pcw 5-10); *ASCL1*-enriched transitional progenitors likely are the earliest IPCs of the VZ (apical IPCs) that will undergo a final round of self-consuming neurogenic division into give two neurons.

The appearance of neurons with GABAergic signatures in all my single-cell RNA sequencing datasets, however, was surprising. This was especially so, since the dual-SMAD inhibition used should default to a dorsal glutamatergic fate, with no GABAergic neurons, in the absence of additional patterning morphogens^{126,219}. Several possibilities could account for the unexpected detection of GABAergic neurons in my cultures:

1. This was lab-specific, for example due to contaminated consumables containing other morphogens.
2. The dual-SMAD inhibition *in vitro* differentiation protocol I used is somehow modelling ventral forebrain neurogenesis.
3. GABAergic neurons are normally formed from cortical neural progenitors in humans, and is being modelled here.
4. The GABAergic neuron signature is transient, representing instead immature neurons, and is too weak to qualify as bona fide GABAergic interneurons.

Regarding the first point, the observation that GABAergic neurons are unexpectedly obtained from a dual-SMAD inhibition-based protocol is not unique to the Guillemot lab. The Srivastava¹⁴⁴, Capetien³⁰⁶, Heine¹²⁰, Levi³⁰⁷, and Cader³⁰⁸ labs have all reported the same over several years while modelling cortical neurogenesis with variants of the dual-SMAD inhibition protocol, thus it is reproducible and unlikely to be lab-specific or supplier-specific. As for the second point, I had shown in Results Chapter 3 that my cultures express dorsal telencephalic markers and not the main ventral markers. I also argued that *ASCL1* and its transcriptional signature may not represent a ventral identity in humans due to *ASCL1*'s wide expression dorsally.

Why then did “GABAergic” neurons appear in the dataset? Another interpretation could be that these “GABAergic” neurons are derived from cortical progenitors, rather than a ventralized differentiation. This is along the lines of more recent evidence suggesting human cortical progenitors specifically are capable of giving rise to GABAergic neurons^{19,71,309}, possibly due to a wider expression of *ASCL1* dorsally than in mouse⁷¹. Furthermore, the “inhibitory neurons” I obtained are highly reminiscent of those in a recent study from the labs of Alvarez-Buylla and Nowakowski³¹, which demonstrated that individual primary human cortical progenitors are capable of generating both glutamatergic and GABAergic neurons characterised by CGE-related, but not MGE-related, signatures, such as expression of *DLX2*, *DLX5*, *PROX1*, and *CALB2* – just like the “GABAergic” neurons in my dataset.

An alternative explanation could be that the GABAergic signatures I see in my “inhibitory neurons” are transient and a reflection of general neuronal differentiation that should instead be interpreted as an immature neuronal signature. In support of this, there is also emerging evidence that cortical glutamatergic neurons share the same initial transcriptional trajectory as inhibitory neurons^{257,258,310}, which could be either because they share the same lineage¹⁹ as discussed above, or because immature neurons in general have transcriptional overlaps of “neurotransmitter-specific” markers during maturation. In support of the latter, transient expression of the GABAergic markers *GAD2*, *CALB2*, and *GAT1* (also present in my “inhibitory neurons”, not shown), has been reported in rat^{311,312}, mouse³¹³, and chick^{314,315} cortices, and may be possible in humans³¹⁶. The transient GABAergic signature has been suggested to allow for non-canonical use of GABA as a regulator of general neurogenic processes such as proliferation³¹⁷, migration, and differentiation³¹⁶ rather than as a synaptic transmitter. Similarly in mice, nearly all mature dopaminergic neurons transiently expressed the glutamatergic marker vGluT2 during maturation to allow for use of glutamate to promote axonal outgrowth, rather than as a neurotransmitter³¹⁸. In the same vein, the GABAergic signatures in my dataset could reflect transient use of GABA in cortical neural progenitors during neurogenesis. Alternatively, transient GABAergic signatures could be a transcriptional by-product of a human-specific use of *ASCL1* during early cortical neurogenesis in the

production of both GABAergic and glutamatergic cortical neurons. In support of these ideas, the “GABAergic” neurons in our Day 24 dataset were found to be most transcriptionally similar to early foetal intermediate progenitors and neurons²²⁰, suggesting they are immature and not *bona fide* GABAergic neurons.

8.8 Workarounds for addressing cell-autonomy and demonstrating causality of *ASCL1*

In Results Chapter 5, I tried unsuccessfully to obtain a model simulating a homogenous *HUWE1* LOF. Although I have argued above that this is unlikely to alter the conclusions of my mosaic-derived results, it is still an experiment that might be worth completing.

Recall that in Results Chapter 5, I tried to achieve three goals:

4. A method to acutely achieve or simulate a 100% *HUWE1*-knockout culture.
5. A method to reduce *ASCL1* levels.
6. A method to deliver *ASCL1* reduction at high efficiency into neural progenitors, or at least to label these cells.

Of these, only the third goal was achieved, through a method for electroporating NPCs that appeared to be 100% efficient in delivering a zsGreen-encoding plasmid. Ironically, this would also suggest that a Cre-encoding plasmid could have been used to induce a complete deletion of *HUWE1* in *HUWE1E81-fl*, the parental line to *HUWE1cKO* that is lacking the *AAVS1* modifications. Alternatively, if an orthogonal method of validating the *HUWE1* LOF phenotypes is still desired, in spite of the discussed possibility that their level of partial inhibition might be insufficient to obtain a phenotype, other small molecule *HUWE1* inhibitors are available such as BI8626²⁶⁸, or HU1 and HU2³¹⁹.

The other unsuccessful experiment in Results Chapter 5 was an attempt to prevent or rescue the premature differentiation of *HUWE1*-knockout cells, hypothesised to be driven by the increase in *ASCL1*. This attempt failed as neither shRNA against *ASCL1* that I tried worked. Once again, the efficient electroporation method could be

leveraged, this time to deliver CRISPR interference (CRISPRi)^{320,321} plasmids against *ASCL1*, using the gRNA validated in Olsen et al. 2021³²². Alternatively, *ASCL1* could be constitutively deleted in HUWE1cKO (or electroporated HUWE1E81-fl knockouts as described above) using the same working gRNAs and protocol that produced Siew-Lan's ASCL1KO, which we used in scRNAseq. This would allow us to see if the premature differentiation in HUWE1-knockout cells is achieved solely through ASCL1 activity.

8.9 Conclusions

In this thesis, I studied the functions of HUWE1 in the context of human neurogenesis by modelling its loss at two stages of a 2D *in vitro* neural differentiation. I first found that HUWE1 was necessary for survival of early neural cells with a possible involvement of p53 stabilization, corroborating findings from patient-derived iPSCs¹⁶⁸. I showed however that this relationship between HUWE1 and p53 was transient and lost in later stages of neural differentiation, in cells modelling radial glia. Instead, I found that HUWE1 regulates ASCL1 stability in these cells. I then showed in a collaboration²²⁰ that ASCL1 normally drives human radial glia into a transitional neuroblast-like state, prior to loss of ASCL1 and their subsequent terminal differentiation into neurons. Consequently, I showed the stabilized ASCL1 in progenitors lacking HUWE1 was temporally linked to cell-cycle exit and a premature differentiation of these cells. This premature differentiation involved the cells entering a prolonged transitional state with mixed progenitor and neuronal markers, possibly as a result of a prolonged ASCL1 presence, though they eventually could form neurons. Prior to ASCL1 expression and stabilization, loss of HUWE1 instead led to a block in progenitor differentiation, through mechanisms not studied here. Finally, I demonstrated that electroporation can efficiently deliver constructs into the neural progenitors, potentially allowing for additional questions regarding HUWE1 function to be answered.

The data presented in this thesis support a model of HUWE1 promoting survival of early neural cells (like neuroepithelia) via p53 suppression, followed by promotion of proliferation in radial glia and suppression of early neural differentiation via regulation

of ASCL1 stability – with the net result an expansion of the radial glial pool. Given that HUWE1 dysfunction is linked to both tumorigenesis and neurodevelopmental disorders (HUWE1-related genetic disorders), deeper investigation into the mechanism laid out here has great implications for human health and disease.

Reference List

1. Florio, M. & Huttner, W. B. Neural progenitors, neurogenesis and the evolution of the neocortex. *Development* **141**, 2182–94 (2014).
2. Rakic, P. Evolution of the neocortex: a perspective from developmental biology. *Nat. Rev. Neurosci.* 2009 1010 **10**, 724–735 (2009).
3. Miller, D. J., Bhaduri, A., Sestan, N. & Kriegstein, A. Shared and derived features of cellular diversity in the human cerebral cortex. *Curr. Opin. Neurobiol.* **56**, 117–124 (2019).
4. Bertrand, N., Castro, D. S. & Guillemot, F. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517–530 (2002).
5. Wilkinson, G., Dennis, D. & Schuurmans, C. Proneural genes in neocortical development. *Neuroscience* **253**, 256–273 (2013).
6. Oproescu, A. M., Han, S. & Schuurmans, C. New Insights Into the Intricacies of Proneural Gene Regulation in the Embryonic and Adult Cerebral Cortex. *Front. Mol. Neurosci.* **14**, 14 (2021).
7. Rubenstein, J. L. R. Development of the Cerebral Cortex: Implications for Neurodevelopmental Disorders. *J. Child Psychol. Psychiatry.* **52**, 339 (2011).
8. Anderson, S. & Vanderhaeghen, P. Cortical neurogenesis from pluripotent stem cells: complexity emerging from simplicity. *Curr. Opin. Neurobiol.* **27**, 151–7 (2014).
9. Pinson, A., Namba, T. & Huttner, W. B. Malformations of Human Neocortex in Development – Their Progenitor Cell Basis and Experimental Model Systems. *Front. Cell. Neurosci.* **13**, 305 (2019).
10. Paşca, S. P. The rise of three-dimensional human brain cultures. *Nature* **553**, 437–445 (2018).
11. Di Lullo, E. & Kriegstein, A. R. The use of brain organoids to investigate neural development and disease. *Nat. Rev. Neurosci.* **18**, 573–584 (2017).
12. Marín, O. & Rubenstein, J. L. R. A long, remarkable journey: Tangential migration in the telencephalon. *Nat. Rev. Neurosci.* 2001 211 **2**, 780–790 (2001).
13. Bystron, I., Blakemore, C. & Rakic, P. Development of the human cerebral

- cortex: Boulder Committee revisited. *Nat. Rev. Neurosci.* 2008 92 **9**, 110–122 (2008).
14. Götz, M. & Huttner, W. B. THE CELL BIOLOGY OF NEUROGENESIS. *Mol. CELL Biol.* **6**, (2005).
 15. Nikolopoulou, E., Galea, G. L., Rolo, A., Greene, N. D. E. & Copp, A. J. Neural tube closure: cellular, molecular and biomechanical mechanisms. *Development* **144**, 552–566 (2017).
 16. Kriegstein, A. & Alvarez-Buylla, A. The Glial Nature of Embryonic and Adult Neural Stem Cells. (2009). doi:10.1146/annurev.neuro.051508.135600
 17. Haubensak, W., Attardo, A., Denk, W. & Huttner, W. B. Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 3196–3201 (2004).
 18. Gal, J. S. *et al.* Molecular and morphological heterogeneity of neural precursors in the mouse neocortical proliferative zones. *J. Neurosci.* **26**, 1045–1056 (2006).
 19. Hansen, D. V., Lui, J. H., Parker, P. R. L. & Kriegstein, A. R. Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nat.* 2010 4647288 **464**, 554–561 (2010).
 20. Fietz, S. A. *et al.* OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. *Nat. Neurosci.* 2010 136 **13**, 690–699 (2010).
 21. Betizeau, M. *et al.* Precursor Diversity and Complexity of Lineage Relationships in the Outer Subventricular Zone of the Primate. *Neuron* **80**, 442–457 (2013).
 22. Tarabykin, V., Stoykova, A., Usman, N. & Gruss, P. Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. *Development* **128**, 1983–1993 (2001).
 23. Kalebic, N. *et al.* Human-specific ARHGAP11B induces hallmarks of neocortical expansion in developing ferret neocortex. *Elife* **7**, (2018).
 24. Florio, M. *et al.* Human-specific gene ARHGAP11B promotes basal progenitor amplification and neocortex expansion. *Science (80-.).* **347**, 1465–1470 (2015).

25. Kalebic, N. *et al.* Neocortical Expansion Due to Increased Proliferation of Basal Progenitors Is Linked to Changes in Their Morphology. *Cell Stem Cell* **24**, 535-550.e9 (2019).
26. Matsumoto, N., Tanaka, S., Horiike, T., Shinmyo, Y. & Kawasaki, H. A discrete subtype of neural progenitor crucial for cortical folding in the gyrencephalic mammalian brain. *Elife* **9**, (2020).
27. Casarosa, S., Fode, C. & Guillemot, F. Mash1 regulates neurogenesis in the ventral telencephalon. *Development* **126**, 525–534 (1999).
28. Rubin, A. N. & Kessaris, N. PROX1: A Lineage Tracer for Cortical Interneurons Originating in the Lateral/Caudal Ganglionic Eminence and Preoptic Area. *PLoS One* **8**, e77339 (2013).
29. Tao, G. *et al.* Transcription factors Sp8 and Sp9 regulate medial ganglionic eminence-derived cortical interneuron migration. *Front. Mol. Neurosci.* **12**, 75 (2019).
30. Flandin, P. *et al.* Lhx6 and Lhx8 Coordinately Induce Neuronal Expression of Shh that Controls the Generation of Interneuron Progenitors. *Neuron* **70**, 939 (2011).
31. Delgado, R. N. *et al.* Individual human cortical progenitors can produce excitatory and inhibitory neurons. *Nat.* **2021** 1–7 (2021). doi:10.1038/s41586-021-04230-7
32. Martynoga, B., Drechsel, D. & Guillemot, F. Molecular control of neurogenesis: A view from the mammalian cerebral cortex. *Cold Spring Harb. Perspect. Biol.* **4**, (2012).
33. Berg, D. A. *et al.* A Common Embryonic Origin of Stem Cells Drives Developmental and Adult Neurogenesis. *Cell* **177**, 654-668.e15 (2019).
34. Turing, A. M. The Chemical Basis of Morphogenesis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **237**, 37–72 (1952).
35. Adnani, L., Han, S., Li, S., Mattar, P. & Schuurmans, C. Mechanisms of Cortical Differentiation. *Int. Rev. Cell Mol. Biol.* **336**, 223–320 (2018).
36. Chenn, A. & Walsh, C. A. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365–369 (2002).
37. Munji, R. N., Choe, Y., Li, G., Siegenthaler, J. A. & Pleasure, S. J. Wnt

- signaling regulates neuronal differentiation of cortical intermediate progenitors. *J. Neurosci.* **31**, 1676–1687 (2011).
38. Hirabayashi, Y. *et al.* The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development* **131**, 2791–2801 (2004).
 39. Da Silva, F. *et al.* Mitotic WNT signalling orchestrates neurogenesis in the developing neocortex. *EMBO J.* **40**, e108041 (2021).
 40. Pera, E. M., Ikeda, A., Eivers, E. & De Robertis, E. M. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. *Genes Dev.* **17**, 3023–3028 (2003).
 41. Hemmati-Brivanlou, A., Kelly, O. G. & Melton, D. A. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283–295 (1994).
 42. Hébert, J. M. & Fishell, G. The genetics of early telencephalon patterning: some assembly required. *Nat. Rev. Neurosci.* **9**, 678 (2008).
 43. Li, W., Cogswell, C. A. & LoTurco, J. J. Neuronal Differentiation of Precursors in the Neocortical Ventricular Zone Is Triggered by BMP. *J. Neurosci.* **18**, 8853–8862 (1998).
 44. Gomes, W. A., Mehler, M. F. & Kessler, J. A. Transgenic overexpression of BMP4 increases astroglial and decreases oligodendroglial lineage commitment. *Dev. Biol.* **255**, 164–177 (2003).
 45. Gross, R. E. *et al.* Bone Morphogenetic Proteins Promote Astroglial Lineage Commitment by Mammalian Subventricular Zone Progenitor Cells. *Neuron* **17**, 595–606 (1996).
 46. Xie, Y. *et al.* FGF/FGFR signaling in health and disease. *Signal Transduct. Target. Ther.* **2020 51** **5**, 1–38 (2020).
 47. Yoon, K. *et al.* Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. *J. Neurosci.* **24**, 9497–9506 (2004).
 48. Sahara, S. & O’Leary, D. D. M. Fgf10 regulates transition period of cortical stem cell differentiation to radial glia controlling generation of neurons and basal progenitors. *Neuron* **63**, 48–62 (2009).

49. Rash, B. G., David Lim, H., Breunig, J. J. & Vaccarino, F. M. FGF Signaling Expands Embryonic Cortical Surface Area by Regulating Notch-Dependent Neurogenesis. *J. Neurosci.* **31**, 15604–15617 (2011).
50. Lukaszewicz, A., Savatier, P., Cortay, V., Kennedy, H. & Dehay, C. Contrasting Effects of Basic Fibroblast Growth Factor and Neurotrophin 3 on Cell Cycle Kinetics of Mouse Cortical Stem Cells. *J. Neurosci.* **22**, 6610 (2002).
51. Kuschel, S., R  ther, U. & Theil, T. A disrupted balance between Bmp/Wnt and Fgf signaling underlies the ventralization of the Gli3 mutant telencephalon. *Dev. Biol.* **260**, 484–495 (2003).
52. Seuntjens, E. *et al.* Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. *Nat. Neurosci.* **12**, 1373–1380 (2009).
53. Song, M. R. & Ghosh, A. FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. *Nat. Neurosci.* **7**, 229–235 (2004).
54. Carballo, G. B., Honorato, J. R., De Lopes, G. P. F. & Spohr, T. C. L. D. S. E. A highlight on Sonic hedgehog pathway. *Cell Commun. Signal.* **2018** *161* **16**, 1–15 (2018).
55. Tole, S., Ragsdale, C. W. & Grove, E. A. Dorsoventral patterning of the telencephalon is disrupted in the mouse mutant extra-toes(J). *Dev. Biol.* **217**, 254–265 (2000).
56. Rallu, M. *et al.* Dorsoventral patterning is established in the telencephalon of mutants lacking both Gli3 and Hedgehog signaling. *Development* **129**, 4963–4974 (2002).
57. Wang, L., Hou, S. & Han, Y. G. Hedgehog signaling promotes basal progenitor expansion and the growth and folding of the neocortex. *Nat. Neurosci.* **19**, 888–896 (2016).
58. Smith, D., Wagner, E., Koul, O., McCaffery, P. & Dr  ger, U. C. Retinoic Acid Synthesis for the Developing Telencephalon. *Cereb. Cortex* **11**, 894–905 (2001).
59. Haushalter, C., Asselin, L., Fraulob, V., Doll  , P. & Rhinn, M. Retinoic acid

- controls early neurogenesis in the developing mouse cerebral cortex. *Dev. Biol.* **430**, 129–141 (2017).
60. Siegenthaler, J. A. *et al.* Retinoic Acid from the Meninges Regulates Cortical Neuron Generation. *Cell* **139**, 597–609 (2009).
 61. Haushalter, C., Schuhbaur, B., Dolle, P. & Rhinn, M. Meningeal retinoic acid contributes to neocortical lamination and radial migration during mouse brain development. *Biol. Open* **6**, 148–160 (2017).
 62. Marklund, M. *et al.* Retinoic acid signalling specifies intermediate character in the developing telencephalon. *Development* **131**, 4323–4332 (2004).
 63. Castro, D. S. *et al.* Proneural bHLH and Brn Proteins Coregulate a Neurogenic Program through Cooperative Binding to a Conserved DNA Motif. *Dev. Cell* **11**, 831–844 (2006).
 64. Cabrera, C. V. & Alonso, M. C. Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of *Drosophila*. *EMBO J.* **10**, 2965 (1991).
 65. Aydin, B. *et al.* Proneural factors *Ascl1* and *Neurog2* contribute to neuronal subtype identities by establishing distinct chromatin landscapes. *Nat. Neurosci.* **22**, 897–908 (2019).
 66. Blomfield, I. M. *et al.* *Id4* promotes the elimination of the pro-activation factor *ascl1* to maintain quiescence of adult hippocampal stem cells. *Elife* **8**, (2019).
 67. Dennis, D. J., Han, S. & Schuurmans, C. bHLH transcription factors in neural development, disease, and reprogramming. *Brain Res.* **1705**, 48–65 (2019).
 68. Britz, O. *et al.* A Role for Proneural Genes in the Maturation of Cortical Progenitor Cells. *Cereb. Cortex* **16**, i138–i151 (2006).
 69. Kim, E. J., Battiste, J., Nakagawa, Y. & Johnson, J. E. *Ascl1* (*Mash1*) lineage cells contribute to discrete cell populations in CNS architecture. *Mol. Cell. Neurosci.* **38**, 595–606 (2008).
 70. Miyata, T., Kawaguchi, A., Okano, H. & Ogawa, M. Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* **31**, 727–741 (2001).
 71. Alzu'bi, A. & Clowry, G. J. Expression of ventral telencephalon transcription

- factors ASCL1 and DLX2 in the early fetal human cerebral cortex. *J. Anat.* **235**, 555–568 (2019).
72. Fode, C. *et al.* A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes Dev.* **14**, 67–80 (2000).
 73. Castro, D. S. *et al.* A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. *Genes Dev.* **25**, 930–45 (2011).
 74. Nieto, M., Schuurmans, C., Britz, O. & Guillemot, F. Neural bHLH Genes Control the Neuronal versus Glial Fate Decision in Cortical Progenitors. *Neuron* **29**, 401–413 (2001).
 75. Pacary, E. *et al.* Proneural Transcription Factors Regulate Different Steps of Cortical Neuron Migration through Rnd-Mediated Inhibition of RhoA Signaling. *Neuron* **69–540**, 1069 (2011).
 76. Dennis, D. J. *et al.* Neurog2 and Ascl1 together regulate a postmitotic derepression circuit to govern laminar fate specification in the murine neocortex. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E4934–E4943 (2017).
 77. Kumamoto, T. *et al.* Foxg1 coordinates the switch from nonradially to radially migrating glutamatergic subtypes in the neocortex through spatiotemporal repression. *Cell Rep.* **3**, 931–945 (2013).
 78. Toma, K., Kumamoto, T., Hanashima, C., Toma, K. & Hanashima, C. The Timing of Upper-Layer Neurogenesis Is Conferred by Sequential Derepression and Negative Feedback from Deep-Layer Neurons. *J. Neurosci.* **34**, 13259–13276 (2014).
 79. Backman, M. *et al.* Effects of canonical Wnt signaling on dorso-ventral specification of the mouse telencephalon. *Dev. Biol.* **279**, 155–168 (2005).
 80. Segklia, A. *et al.* Bmp7 regulates the survival, proliferation, and neurogenic properties of neural progenitor cells during corticogenesis in the mouse. *PLoS One* **7**, (2012).
 81. Viñals, F. *et al.* BMP-2 decreases Mash1 stability by increasing Id1 expression. *EMBO J.* **23**, 3527–3537 (2004).
 82. Abematsu, M. *et al.* Basic fibroblast growth factor endows dorsal

- telencephalic neural progenitors with the ability to differentiate into oligodendrocytes but not γ -aminobutyric acidergic neurons. *J. Neurosci. Res.* **83**, 731–743 (2006).
83. Li, S. *et al.* RAS/ERK signaling controls proneural genetic programs in cortical development and gliomagenesis. *J. Neurosci.* **34**, 2169–2190 (2014).
 84. Li, S. *et al.* GSK3 Temporally Regulates Neurogenin 2 Proneural Activity in the Neocortex. *J. Neurosci.* **32**, 7791–7805 (2012).
 85. Ali, F. R. *et al.* The phosphorylation status of Ascl1 is a key determinant of neuronal differentiation and maturation in vivo and in vitro. *Development* **141**, 2216–2224 (2014).
 86. Ali, F. *et al.* Cell cycle-regulated multi-site phosphorylation of Neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. *Development* **138**, 4267–4277 (2011).
 87. Hindley, C. *et al.* Post-translational modification of Ngn2 differentially affects transcription of distinct targets to regulate the balance between progenitor maintenance and differentiation. *Development* **139**, 1718–1723 (2012).
 88. Guillemot, F. & Hassan, B. A. Beyond proneural: emerging functions and regulations of proneural proteins. *Curr. Opin. Neurobiol.* **42**, 93–101 (2017).
 89. Hagey, D. W. & Muhr, J. Sox2 Acts in a Dose-Dependent Fashion to Regulate Proliferation of Cortical Progenitors. *Cell Rep.* **9**, 1908–1920 (2014).
 90. Imayoshi, I. *et al.* Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. *Science (80-.)*. **342**, 1203–8 (2013).
 91. Shimojo, H., Ohtsuka, T. & Kageyama, R. Oscillations in Notch Signaling Regulate Maintenance of Neural Progenitors. *Neuron* **58**, 52–64 (2008).
 92. Hatakeyama, J. *et al.* Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. *Development* **131**, 5539–5550 (2004).
 93. Hirata, H. *et al.* Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. *Science* **298**, 840–843 (2002).

94. Bray, S. J. Notch signalling in context. *Nat. Rev. Mol. Cell Biol.* 2016 1711 **17**, 722–735 (2016).
95. T, O. *et al.* Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J.* **18**, 2196–2207 (1999).
96. Alifragis, P., Poortinga, G., Parkhurst, S. M. & Delidakis, C. A network of interacting transcriptional regulators involved in *Drosophila* neural fate specification revealed by the yeast two-hybrid system. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13099–13104 (1997).
97. Mizutani, K. I., Yoon, K., Dang, L., Tokunaga, A. & Gaiano, N. Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. *Nat.* 2007 4497160 **449**, 351–355 (2007).
98. Ladi, E. *et al.* The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. *J. Cell Biol.* **170**, 983–992 (2005).
99. Borggrefe, T. *et al.* The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGF β /BMP and hypoxia pathways. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 303–313 (2016).
100. Espinosa, L., Inglés-Esteve, J., Aguilera, C. & Bigas, A. Phosphorylation by glycogen synthase kinase-3 β down-regulates Notch activity, a link for Notch and Wnt pathways. *J. Biol. Chem.* **278**, 32227–32235 (2003).
101. Stecca, B. & Ruiz I Altaba, A. A GLI1-p53 inhibitory loop controls neural stem cell and tumour cell numbers. *EMBO J.* **28**, 663–676 (2009).
102. Zavadil, J., Cermak, L., Soto-Nieves, N. & Böttinger, E. P. Integration of TGF- β /Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *EMBO J.* **23**, 1155–1165 (2004).
103. Schmid, R. S. *et al.* Neuregulin 1–erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 4251 (2003).
104. Gaiano, N., Nye, J. S. & Fishell, G. Radial Glial Identity Is Promoted by Notch1 Signaling in the Murine Forebrain. *Neuron* **26**, 395–404 (2000).
105. Kamakura, S. *et al.* Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nat. Cell Biol.* **6**, 547–554 (2004).

106. Namiyira, M. *et al.* Committed Neuronal Precursors Confer Astrocytic Potential on Residual Neural Precursor Cells. *Dev. Cell* **16**, 245–255 (2009).
107. Thomson, J. A. Embryonic stem cell lines derived from human blastocysts. *Science* (80-.). **282**, 1145–1147 (1998).
108. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131**, 861–872 (2007).
109. Carpenter, M. K. *et al.* In Vitro Expansion of a Multipotent Population of Human Neural Progenitor Cells. *Exp. Neurol.* **158**, 265–278 (1999).
110. Vescovi, A. L. *et al.* Isolation and Cloning of Multipotential Stem Cells from the Embryonic Human CNS and Establishment of Transplantable Human Neural Stem Cell Lines by Epigenetic Stimulation. *Exp. Neurol.* **156**, 71–83 (1999).
111. Zhang, S. C., Wernig, M., Duncan, I. D., Brüstle, O. & Thomson, J. A. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* 2001 1912 **19**, 1129–1133 (2001).
112. Carpenter, M. K. *et al.* Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp. Neurol.* **172**, 383–397 (2001).
113. Eiraku, M. *et al.* Self-Organized Formation of Polarized Cortical Tissues from ESCs and Its Active Manipulation by Extrinsic Signals. *Cell Stem Cell* **3**, 519–532 (2008).
114. Shi, Y., Kirwan, P. & Livesey, F. J. Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nat. Protoc.* **7**, 1836–1846 (2012).
115. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nat.* 2013 5017467 **501**, 373–379 (2013).
116. Paşca, A. M. *et al.* Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat. Methods* **12**, 671–678 (2015).
117. Qian, X. *et al.* Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. *Cell* **165**, 1238–1254 (2016).
118. Wright, L. S., Prowse, K. R., Wallace, K., Linskens, M. H. K. & Svendsen, C. N. Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in

- vitro. *Exp. Cell Res.* **312**, 2107–2120 (2006).
119. Van den Aamele, J., Tiberi, L., Vanderhaeghen, P. & Espuny-Camacho, I. Thinking out of the dish: What to learn about cortical development using pluripotent stem cells. *Trends Neurosci.* **37**, 334–342 (2014).
 120. Nadadhur, A. G. *et al.* Patterning factors during neural progenitor induction determine regional identity and differentiation potential in vitro. *Stem Cell Res.* **32**, 25–34 (2018).
 121. Harris, J., Tomassy, G. S. & Arlotta, P. Building blocks of the cerebral cortex: from development to the dish. *Wiley Interdiscip. Rev. Dev. Biol.* **4**, 529 (2015).
 122. Piccolo, S. *et al.* The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nat.* 1999 3976721 **397**, 707–710 (1999).
 123. Kretzschmar, M. & Massagué, J. SMADs: mediators and regulators of TGF- β signaling. *Curr. Opin. Genet. Dev.* **8**, 103–111 (1998).
 124. Qi, Y. *et al.* Combined small-molecule inhibition accelerates the derivation of functional cortical neurons from human pluripotent stem cells. *Nat. Biotechnol.* 2016 352 **35**, 154–163 (2017).
 125. Wilson, P. G. & Stice, S. S. Development and differentiation of neural rosettes derived from human embryonic stem cells. *Stem Cell Reviews* **2**, (2006).
 126. Shi, Y., Kirwan, P., Smith, J., Robinson, H. . & Livesey, F. J. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nat. Neurosci.* **15**, 477–86 (2012).
 127. Espuny-Camacho, I. *et al.* Pyramidal Neurons Derived from Human Pluripotent Stem Cells Integrate Efficiently into Mouse Brain Circuits In Vivo. *Neuron* **77**, 440–456 (2013).
 128. Handel, A. E. *et al.* Assessing similarity to primary tissue and cortical layer identity in induced pluripotent stem cell-derived cortical neurons through single-cell transcriptomics. *Hum. Mol. Genet.* **25**, 989–1000 (2016).
 129. Camp, J. G. *et al.* Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 15672–15677 (2015).

130. Kelava, I. & Lancaster, M. A. Stem Cell Models of Human Brain Development. *Cell Stem Cell* **18**, 736–748 (2016).
131. Yoon, S.-J. *et al.* Reliability of human cortical organoid generation. doi:10.1038/s41592-018-0255-0
132. Zhang, Z., O’Laughlin, R., Song, H. & Ming, G. li. Patterning of brain organoids derived from human pluripotent stem cells. *Curr. Opin. Neurobiol.* **74**, 102536 (2022).
133. Qian, X. *et al.* Sliced Human Cortical Organoids for Modeling Distinct Cortical Layer Formation. *Cell Stem Cell* **26**, 766-781.e9 (2020).
134. Birey, F. *et al.* Assembly of functionally integrated human forebrain spheroids. *Nature* **545**, 54–59 (2017).
135. Andersen, J. *et al.* Generation of Functional Human 3D Cortico-Motor Assembloids. *Cell* **183**, 1913-1929.e26 (2020).
136. Kim, H.-J., McMillan, E., Han, F. & Svendsen, C. N. Regionally Specified Human Neural Progenitor Cells Derived from the Mesencephalon and Forebrain Undergo Increased Neurogenesis Following Overexpression of ASCL1. *Stem Cells* **27**, 390–398 (2009).
137. Pang, Z. P. *et al.* Induction of human neuronal cells by defined transcription factors. *Nat. 2011 4767359* **476**, 220–223 (2011).
138. Wapinski, O. L. *et al.* XHierarchical mechanisms for direct reprogramming of fibroblasts to neurons. *Cell* **155**, (2013).
139. Park, N. I. *et al.* ASCL1 Reorganizes Chromatin to Direct Neuronal Fate and Suppress Tumorigenicity of Glioblastoma Stem Cells. *Cell Stem Cell* **21**, (2017).
140. Zhang, Y. *et al.* Rapid Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells. *Neuron* **78**, 785–798 (2013).
141. Pawlowski, M. *et al.* Inducible and Deterministic Forward Programming of Human Pluripotent Stem Cells into Neurons, Skeletal Myocytes, and Oligodendrocytes. *Stem Cell Reports* **8**, 803 (2017).
142. Tamburini, C. & Li, M. Understanding neurodevelopmental disorders using human pluripotent stem cell-derived neurons. *Brain Pathol.* **27**, 508–517 (2017).

143. Bershteyn, M. *et al.* Human iPSC-Derived Cerebral Organoids Model Cellular Features of Lissencephaly and Reveal Prolonged Mitosis of Outer Radial Glia. *Cell Stem Cell* **20**, 435-449.e4 (2017).
144. Adhya, D. *et al.* Atypical Neurogenesis in Induced Pluripotent Stem Cells From Autistic Individuals. *Biol. Psychiatry* **89**, 486–496 (2021).
145. Paulsen, B. *et al.* Autism genes converge on asynchronous development of shared neuron classes. *Nature* **602**, 268 (2022).
146. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
147. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
148. Chakrabarti, A. M. *et al.* Target-Specific Precision of CRISPR-Mediated Genome Editing. *Mol. Cell* **73**, 699-713.e6 (2019).
149. Chatterjee, P. *et al.* A Cas9 with PAM recognition for adenine dinucleotides. *Nat. Commun.* 2020 111 **11**, 1–6 (2020).
150. Kleinstiver, B. P. *et al.* Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* **523**, 481 (2015).
151. Schubert, M. S. *et al.* Optimized design parameters for CRISPR Cas9 and Cas12a homology-directed repair. *Sci. Reports* 2021 111 **11**, 1–15 (2021).
152. Anzalone, A. V. *et al.* Search-and-replace genome editing without double-strand breaks or donor DNA. *Nat.* 2019 5767785 **576**, 149–157 (2019).
153. Yarnall, M. T. N. *et al.* Drag-and-drop genome insertion of large sequences without double-strand DNA cleavage using CRISPR-directed integrases. *Nat. Biotechnol.* 2022 1–13 (2022). doi:10.1038/s41587-022-01527-4
154. Tian, R. *et al.* CRISPR interference-based platform for multimodal genetic screens in human iPSC-derived neurons. *Neuron* **104**, 239 (2019).
155. Tian, R. *et al.* Genome-wide CRISPRi/a screens in human neurons link lysosomal failure to ferroptosis. *Nat. Neurosci.* 2021 247 **24**, 1020–1034 (2021).
156. Black, J. B. *et al.* Master Regulators and Cofactors of Human Neuronal Cell Fate Specification Identified by CRISPR Gene Activation Screens Master Regulators and Cofactors of Human Neuronal Cell Fate Specification

- Identified by CRISPR Gene Activation Screens. (2020). doi:10.1016/j.celrep.2020.108460
157. Wang, P. *et al.* CRISPR/Cas9-mediated heterozygous knockout of the autism gene CHD8 and characterization of its transcriptional networks in neurodevelopment. *Mol. Autism* **6**, 1–18 (2015).
 158. Bell, S. *et al.* Disruption of GRIN2B Impairs Differentiation in Human Neurons. *Stem cell reports* **11**, 183–196 (2018).
 159. Bell, S. *et al.* Mutations in ACTL6B Cause Neurodevelopmental Deficits and Epilepsy and Lead to Loss of Dendrites in Human Neurons. *Am. J. Hum. Genet.* **104**, 815–834 (2019).
 160. Moortgat, S. *et al.* HUWE1 variants cause dominant X-linked intellectual disability: A clinical study of 21 patients. *Eur. J. Hum. Genet.* **26**, 64–74 (2018).
 161. Juberg, R. C. & Marsid¹², I. *A New Form of X-Linked Mental Retardation with Growth Retardation, Deafness, and Microgenitalism.* *Am J Hum Genet* **32**, (1980).
 162. Friez, M. J. *et al.* HUWE1 mutations in Juberg-Marsidi and Brooks syndromes: the results of an X-chromosome exome sequencing study. *BMJ Open* **6**, 9537 (2016).
 163. Turner, G., Gedeon, A. & Mulley, J. X-linked mental retardation with heterozygous expression and macrocephaly: Pericentromeric gene localization. *Am. J. Med. Genet.* **51**, (1994).
 164. Giles, A. C. & Grill, B. Roles of the HUWE1 ubiquitin ligase in nervous system development, function and disease. *Neural Dev.* **15**, 1–18 (2020).
 165. Santos-Rebouças, C. B. *et al.* A novel Xp11.22 duplication involving HUWE1 in a male with syndromic intellectual disability and additional neurological findings. *Eur. J. Med. Genet.* **66**, 104716 (2023).
 166. Froyen, G. *et al.* Copy-Number Gains of HUWE1 Due to Replication-and Recombination-Based Rearrangements. *Am. J. Hum. Genet.* **91**, 252–264 (2012).
 167. Hunkeler, M. *et al.* Solenoid architecture of HUWE1 contributes to ligase activity and substrate recognition. *Mol. Cell* (2021).

- doi:10.1016/J.MOLCEL.2021.06.032
168. Aprigliano, R. *et al.* Increased p53 signaling impairs neural differentiation in HUWE1-promoted intellectual disabilities. *Cell Reports Med.* 100240 (2021). doi:10.1016/j.xcrm.2021.100240
 169. Lek, M. *et al.* Analysis of protein-coding genetic variation in 60,706 humans. *Nat.* 2016 5367616 **536**, 285–291 (2016).
 170. Bosshard, M. *et al.* Impaired oxidative stress response characterizes HUWE1-promoted X-linked intellectual disability. *Sci. Rep.* **7**, 15050 (2017).
 171. Kao, S.-H., Wu, H.-T. & Wu, K.-J. Ubiquitination by HUWE1 in tumorigenesis and beyond. *J. Biomed. Sci.* **25**, 67 (2018).
 172. Uhlén, M. *et al.* Proteomics. Tissue-based map of the human proteome. *Science* (80-.). **347**, 1260419 (2015).
 173. Zhong, Q., Gao, W., Du, F. & Wang, X. Mule/ARF-BP1, a BH3-Only E3 Ubiquitin Ligase, Catalyzes the Polyubiquitination of Mcl-1 and Regulates Apoptosis. *Cell* **121**, 1085–1095 (2005).
 174. Chen, D. *et al.* ARF-BP1/Mule Is a Critical Mediator of the ARF Tumor Suppressor. *Cell* **121**, 1071–1083 (2005).
 175. Liu, Z., Oughtred, R. & Wing, S. S. Characterization of E3Histone, a novel testis ubiquitin protein ligase which ubiquitinates histones. *Mol. Cell. Biol.* **25**, 2819–31 (2005).
 176. Adhikary, S. *et al.* The Ubiquitin Ligase HectH9 Regulates Transcriptional Activation by Myc and Is Essential for Tumor Cell Proliferation. *Cell* **123**, 409–421 (2005).
 177. de Groot, R. E. A. *et al.* Huwe1-mediated ubiquitylation of dishevelled defines a negative feedback loop in the Wnt signaling pathway. *Sci. Signal.* **7**, ra26 (2014).
 178. Zhao, X. *et al.* The HECT-domain ubiquitin ligase Huwe1 controls neural differentiation and proliferation by destabilizing the N-Myc oncoprotein. *Nat. Cell Biol.* **10**, 643–653 (2008).
 179. Cox, B. J. *et al.* Phenotypic annotation of the mouse X chromosome. *Genome Res.* **20**, 1154–1164 (2010).
 180. Hao, Z. *et al.* The E3 ubiquitin ligase Mule acts through the ATM–p53 axis

- to maintain B lymphocyte homeostasis. *J. Exp. Med.* **209**, 173–186 (2012).
181. Kon, N., Zhong, J., Qiang, L., Accili, D. & Gu, W. Inactivation of arf-bp1 Induces p53 Activation and Diabetic Phenotypes in Mice. *J. Biol. Chem.* **287**, 5102–5111 (2012).
 182. Zhao, X. *et al.* The N-Myc-DLL3 Cascade Is Suppressed by the Ubiquitin Ligase Huwe1 to Inhibit Proliferation and Promote Neurogenesis in the Developing Brain. *Dev. Cell* **17**, 210–221 (2009).
 183. D'arca, D. *et al.* Huwe1 ubiquitin ligase is essential to synchronize neuronal and glial differentiation in the developing cerebellum. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 5875–5880 (2010).
 184. Forget, A. *et al.* Article Shh Signaling Protects Atoh1 from Degradation Mediated by the E3 Ubiquitin Ligase Huwe1 in Neural Precursors. *Dev. Cell* **29**, 649–661 (2014).
 185. Urbán, N. *et al.* Return to quiescence of mouse neural stem cells by degradation of a proactivation protein. *Science (80-.)*. **353**, 292–295 (2016).
 186. Cheng, Y.-F., Tong, M. & B Edge, A. S. Destabilization of Atoh1 by E3 Ubiquitin Ligase Huwe1 and Casein Kinase 1 Is Essential for Normal Sensory Hair Cell Development * Downloaded from. *J. Biol. Chem.* **291**, 21096–21109 (2016).
 187. Sander, B., Xu, W., Eilers, M., Popov, N. & Lorenz, S. A conformational switch regulates the ubiquitin ligase HUWE1. *Elife* **6**, (2017).
 188. Pandya, R. K., Partridge, J. R., Love, K. R., Schwartz, T. U. & Ploegh, H. L. A structural element within the HUWE1 HECT domain modulates self-ubiquitination and substrate ubiquitination activities. *J. Biol. Chem.* **285**, 5664–5673 (2010).
 189. Grabarczyk, D. B. *et al.* HUWE1 employs a giant substrate-binding ring to feed and regulate its HECT E3 domain. *Nat. Chem. Biol.* **2021** 1–9 (2021). doi:10.1038/s41589-021-00831-5
 190. Wang, Z. *et al.* Recognition of the iso-ADP-ribose moiety in poly(ADP-ribose) by WWE domains suggests a general mechanism for poly(ADP-ribosyl)ation-dependent ubiquitination. *Genes Dev.* **26**, 235–240 (2012).
 191. Gillotin, S., Davies, J. D. & Philpott, A. Subcellular localisation modulates

- ubiquitylation and degradation of Ascl1. *Sci. Rep.* **8**, (2018).
192. Kurokawa, M. *et al.* A network of substrates of the E3 ubiquitin ligases MDM2 and HUWE1 control apoptosis independently of p53. *Sci. Signal.* **6**, (2013).
 193. Yi, J. *et al.* DNA damage-induced activation of CUL4B targets HUWE1 for proteasomal degradation. *Nucleic Acids Res.* **43**, 4579–4590 (2015).
 194. Khoronenkova, S. V. & Dianov, G. L. USP7S-dependent inactivation of Mule regulates DNA damage signalling and repair. *Nucleic Acids Res.* **41**, 1750–1756 (2013).
 195. Zhang, X., Berger, F. G., Yang, J. & Lu, X. USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. *EMBO J.* **30**, 2177–2189 (2011).
 196. Rosa, J. L. & Barbacid, M. A giant protein that stimulates guanine nucleotide exchange on ARF1 and Rab proteins forms a cytosolic ternary complex with clathrin and Hsp70. *Oncogene* 1997 151 **15**, 1–6 (1997).
 197. Giles, A. C. *et al.* A complex containing the O-GlcNAc transferase OGT-1 and the ubiquitin ligase EEL-1 regulates GABA neuron function. *J. Biol. Chem.* (2019). doi:10.1074/jbc.RA119.007406
 198. Sluimer, J. & Distel, B. Regulating the human HECT E3 ligases. *Cell. Mol. Life Sci.* **75**, 3121–3141 (2018).
 199. Opperman, K. J. *et al.* The HECT Family Ubiquitin Ligase EEL-1 Regulates Neuronal Function and Development. *Cell Rep.* **19**, 822–835 (2017).
 200. Vandewalle, J. *et al.* Ubiquitin Ligase HUWE1 Regulates Axon Branching through the Wnt/ β -Catenin Pathway in a Drosophila Model for Intellectual Disability. *PLoS One* **8**, (2013).
 201. Khatun, R. Structural and Functional Studies of Human Ubiquitin E3 Ligase (PhD thesis). (York University, Toronto, 2017).
 202. Skarnes, W. C., Pellegrino, E. & McDonough, J. A. Improving homology-directed repair efficiency in human stem cells. *Methods* **164–165**, 18–28 (2019).
 203. Ocegüera-Yanez, F. *et al.* Engineering the AAVS1 locus for consistent and scalable transgene expression in human iPSCs and their differentiated derivatives. *Methods* **101**, 43–55 (2016).

204. Pliatsika, V. & Rigoutsos, I. 'Off-Spotter': Very fast and exhaustive enumeration of genomic lookalikes for designing CRISPR/Cas guide RNAs. *Biol. Direct* **10**, 4 (2015).
205. Wang, L. *et al.* ASCL1 is a MYCN- and LMO1-dependent member of the adrenergic neuroblastoma core regulatory circuitry. *Nat. Commun.* **10**, 1–15 (2019).
206. Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* **25**, 402–408 (2001).
207. Cicinnati, V. R. *et al.* Validation of putative reference genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR. *BMC Cancer* **8**, 350 (2008).
208. Hao, Y. *et al.* Integrated analysis of multimodal single-cell data. *Cell* **184**, 3573 (2021).
209. R Core Team. R: A Language and Environment for Statistical Computing. (2022).
210. Srivastava, A., Malik, L., Smith, T., Sudbery, I. & Patro, R. Alevin efficiently estimates accurate gene abundances from dscRNA-seq data. *Genome Biol.* **20**, (2019).
211. Germain, P. L., Robinson, M. D., Lun, A., Garcia Meixide, C. & Macnair, W. Doublet identification in single-cell sequencing data using scDbtFinder. *F1000Research* **10**, (2021).
212. Hafemeister, C. & Satija, R. Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol.* **20**, (2019).
213. Ahlmann-Eltze, C. & Huber, W. Gene expression glmGamPoi: fitting Gamma-Poisson generalized linear models on single cell count data. doi:10.1093/bioinformatics/btaa1009
214. McInnes, L., Healy, J. & Melville, J. UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. (2018). doi:10.48550/arxiv.1802.03426
215. Franzén, O., Gan, L. M. & Björkegren, J. L. M. PanglaoDB: a web server

- for exploration of mouse and human single-cell RNA sequencing data. *Database* **2019**, 46 (2019).
216. Bergen, V., Lange, M., Peidli, S., Wolf, F. A. & Theis, F. J. Generalizing RNA velocity to transient cell states through dynamical modeling. *Nat. Biotechnol.* **38**, 1408–1414 (2020).
 217. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 2012 97 **9**, 676–682 (2012).
 218. Schmidt, U., Weigert, M., Broaddus, C. & Myers, G. Cell detection with star-convex polygons. *Lect. Notes Comput. Sci. (including Subser. Lect. Notes Artif. Intell. Lect. Notes Bioinformatics)* **11071 LNCS**, 265–273 (2018).
 219. Chambers, S. M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat. Biotechnol.* **27**, 275–280 (2009).
 220. Păun, O. *et al.* Pioneer factor ASCL1 cooperates with the mSWI/SNF complex at distal regulatory elements to regulate human neural differentiation. *Genes Dev.* (2023). doi:10.1101/GAD.350269.122
 221. Karlsson, M. *et al.* A single–cell type transcriptomics map of human tissues. *Sci. Adv.* **7**, (2021).
 222. Vasconcelos, F. F. & Castro, D. S. Transcriptional control of vertebrate neurogenesis by the proneural factor ascll. *Front. Cell. Neurosci.* **8**, (2014).
 223. Toki, T. *et al.* De Novo Mutations Activating Germline TP53 in an Inherited Bone-Marrow-Failure Syndrome. *Am. J. Hum. Genet.* **103**, 440–447 (2018).
 224. Bowen, M. E. *et al.* The Spatiotemporal Pattern and Intensity of p53 Activation Dictates Phenotypic Diversity in p53-Driven Developmental Syndromes. *Dev. Cell* **50**, 212-228.e6 (2019).
 225. Sah, V. P. *et al.* A subset of p53-deficient embryos exhibit exencephaly. *Nat. Genet.* **10**, 175–180 (1995).
 226. Zhao, J. *et al.* p53 Mutant p53N236S Induces Neural Tube Defects in Female Embryos. *Int. J. Biol. Sci.* **15**, 2006–2015 (2019).
 227. Delbridge, A. R. D. *et al.* Loss of p53 Causes Stochastic Aberrant X-Chromosome Inactivation and Female-Specific Neural Tube Defects. *Cell Rep.* **27**, 442-454.e5 (2019).

228. Xiong, Y., Zhang, Y., Xiong, S. & Williams-Villalobo, A. E. A Glance of p53 Functions in Brain Development, Neural Stem Cells, and Brain Cancer. *Biology (Basel)*. **9**, 1–13 (2020).
229. Lykke-Andersen, S. & Jensen, T. H. Nonsense-mediated mRNA decay: an intricate machinery that shapes transcriptomes. *Nat. Rev. Mol. Cell Biol.* **16**, 665–677 (2015).
230. Szymczak, A. L. *et al.* Correction of multi-gene deficiency in vivo using a single ‘self-cleaving’ 2A peptide–based retroviral vector. *Nat. Biotechnol.* **22**, 589–594 (2004).
231. Pliatsika, V. & Rigoutsos, I. ‘Off-Spotter’: Very fast and exhaustive enumeration of genomic lookalikes for designing CRISPR/Cas guide RNAs. *Biol. Direct* **10**, 1–10 (2015).
232. Lee, C.-M. *et al.* Single-cell RNA-seq analysis revealed long-lasting adverse effects of tamoxifen on neurogenesis in prenatal and adult brains. *PNAS* **117**, 19578–19589 (2020).
233. Raposo, A. A. S. F. *et al.* Ascl1 coordinately regulates gene expression and the chromatin landscape during neurogenesis. *Cell Rep.* **10**, (2015).
234. Poitras, L., Ghanem, N., Hatch, G. & Ekker, M. The proneural determinant MASH1 regulates forebrain Dlx1/2 expression through the l12b intergenic enhancer. *Development* **134**, (2007).
235. Olsen, T. K. & Baryawno, N. Introduction to Single-Cell RNA Sequencing. *Curr. Protoc. Mol. Biol.* **122**, e57 (2018).
236. Hwang, B., Lee, J. H. & Bang, D. Single-cell RNA sequencing technologies and bioinformatics pipelines. *Exp. Mol. Med.* **50**, 1–14 (2018).
237. Luecken, M. D. & Theis, F. J. Current best practices in single-cell RNA-seq analysis: a tutorial. *Mol. Syst. Biol.* **15**, e8746 (2019).
238. Paulsen, B. *et al.* Autism genes converge on asynchronous development of shared neuron classes. *Nat.* **602**, 268–273 (2022).
239. Harris, L. *et al.* Coordinated changes in cellular behavior ensure the lifelong maintenance of the hippocampal stem cell population. *Stem Cell* **28**, 863–876.e6 (2021).
240. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating

- single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* **36**, 411 (2018).
241. Bae, S. K., Bessho, Y., Hojo, M. & Kageyama, R. The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. *Development* **127**, 2933–2943 (2000).
 242. Gratton, M.-O. *et al.* Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. *Mol. Cell. Biol.* **23**, 6922–6935 (2003).
 243. Fior, R. & Henrique, D. A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. *Dev. Biol.* **281**, 318–333 (2005).
 244. Bergsland, M., Werme, M., Malewicz, M., Perlmann, T. & Muhr, J. The establishment of neuronal properties is controlled by Sox4 and Sox11. *Genes Dev.* **20**, 3475 (2006).
 245. El-Ghissassi, F. *et al.* BTG2(TIS21/PC3) induces neuronal differentiation and prevents apoptosis of terminally differentiated PC12 cells. *Oncogene* **21**, 6772–6778 (2002).
 246. Andersen, J. *et al.* A Transcriptional Mechanism Integrating Inputs from Extracellular Signals to Activate Hippocampal Stem Cells. *Neuron* **83**, 1085 (2014).
 247. Kelsom, C. & Lu, W. Development and specification of GABAergic cortical interneurons. *Cell Biosci.* **2013 31** **3**, 1–19 (2013).
 248. Bergsland, M., Werme, M., Malewicz, M., Perlmann, T. & Muhr, J. The establishment of neuronal properties is controlled by Sox4 and Sox11. *Genes Dev.* **20**, 3475–3486 (2006).
 249. Mo, Z. & Zecevic, N. Is Pax6 Critical for Neurogenesis in the Human Fetal Brain? *Cereb. Cortex* **18**, 1455–1465 (2008).
 250. Zuccotti, A., Magueresse, C. Le, Chen, M., Neitz, A. & Monyer, H. The transcription factor Fezf2 directs the differentiation of neural stem cells in the subventricular zone toward a cortical phenotype. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 10726–10731 (2014).
 251. Ma, T. *et al.* Subcortical origins of human and monkey neocortical

- interneurons. *Nat. Neurosci.* 2013 1611 **16**, 1588–1597 (2013).
252. Torii, M. A. *et al.* Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. *Development* **126**, 443–456 (1999).
 253. Nasif, S. *et al.* Islet 1 specifies the identity of hypothalamic melanocortin neurons and is critical for normal food intake and adiposity in adulthood. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E1861–E1870 (2015).
 254. Qiu, P. Embracing the dropouts in single-cell RNA-seq analysis. *Nat. Commun.* 2020 111 **11**, 1–9 (2020).
 255. Westoby, J., Artemov, P., Hemberg, M. & Ferguson-Smith, A. Obstacles to detecting isoforms using full-length scRNA-seq data. *Genome Biol.* **21**, 1–19 (2020).
 256. van Bruggen, D. *et al.* Developmental landscape of human forebrain at a single-cell level identifies early waves of oligodendrogenesis. *Dev. Cell* **57**, 1421-1436.e5 (2022).
 257. Nowakowski, T. J. *et al.* Spatiotemporal gene expression trajectories reveal developmental hierarchies of the human cortex. *Science* **358**, 1318 (2017).
 258. Ma, L. *et al.* Developmental programming and lineage branching of early human telencephalon. *EMBO J.* **40**, e107277 (2021).
 259. Riemondy, K. A. *et al.* clustifyr: an R package for automated single-cell RNA sequencing cluster classification. *F1000Research* **9**, (2020).
 260. Braun, E. *et al.* Comprehensive cell atlas of the first-trimester developing human brain. *bioRxiv* 2022.10.24.513487 (2022). doi:10.1101/2022.10.24.513487
 261. Goto, K. *et al.* G-protein-coupled receptor signaling through Gpr176, Gz, and RGS16 tunes time in the center of the circadian clock. *Endocr. J.* **64**, 571–579 (2017).
 262. Duan, C. & Allard, J. B. Insulin-Like Growth Factor Binding Protein-5 in Physiology and Disease. *Front. Endocrinol. (Lausanne)*. **11**, 100 (2020).
 263. Hansen, D. V., Lui, J. H., Parker, P. R. L. & Kriegstein, A. R. Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature* **464**, 554–561 (2010).

264. Johnston, L. How to Identify Problems with Flow Cytometry Panel Design – Bad Data Part 2 | Cytometry and Antibody Technology. *The University of Chicago, Cytometry and Antibody Technology Blog* (2020). Available at: <https://voices.uchicago.edu/ucflow/2020/11/19/how-to-identify-problems-with-panel-design-bad-data-part-2/>. (Accessed: 7th February 2023)
265. Henke, R. M., Meredith, D. M., Borromeo, M. D., Savage, T. K. & Johnson, J. E. Ascl1 and Neurog2 Form Novel Complexes and Regulate Delta-like3 (Dll3) Expression in the Neural Tube. *Dev. Biol.* **328**, 529 (2009).
266. Vossenkamper, A. & Warnes, G. A Flow Cytometric Immunophenotyping Approach to the Detection of Regulated Cell Death Processes. *J. Immunol. Sci.* **2**, 6–12 (2018).
267. Soares, D. S., Homem, C. C. F. & Castro, D. S. Function of Proneural Genes Ascl1 and Asense in Neurogenesis: How Similar Are They? *Front. Cell Dev. Biol.* **10**, 228 (2022).
268. Peter, S. *et al.* Tumor cell-specific inhibition of MYC function using small molecule inhibitors of the HUWE1 ubiquitin ligase. *EMBO Mol. Med.* **6**, 1525–41 (2014).
269. Cho, H. I. *et al.* BRPF3-HUWE1-mediated regulation of MYST2 is required for differentiation and cell-cycle progression in embryonic stem cells. *Cell Death Differ.* **27**, 3273–3288 (2020).
270. Khoronenkova, S. V. & Dianov, G. L. USP7S-dependent inactivation of Mule regulates DNA damage signalling and repair. *Nucleic Acids Res.* **41**, 1750 (2013).
271. Nolden, L. *et al.* Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase. *Nat. Methods* 2006 36 **3**, 461–467 (2006).
272. Moffat, J. *et al.* A Lentiviral RNAi Library for Human and Mouse Genes Applied to an Arrayed Viral High-Content Screen. *Cell* **124**, 1283–1298 (2006).
273. Yang, X. *et al.* Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev. Biol.* **269**, 81–94 (2004).
274. Thompson, J. W. *et al.* Quantitative Lys- ϵ -Gly-Gly (diGly) proteomics

- coupled with inducible RNAi reveals ubiquitin-mediated proteolysis of DNA damage-inducible transcript 4 (DDIT4) by the E3 ligase HUWE1. *J. Biol. Chem.* **289**, 28942–55 (2014).
275. Fulzele, A. & Bennett, E. J. Ubiquitin diGLY proteomics as an approach to identify and quantify the ubiquitin-modified proteome. *Methods Mol. Biol.* **1844**, 363–384 (2018).
 276. Dixit, R. *et al.* Ascl1 Participates in Cajal–Retzius Cell Development in the Neocortex. *Cereb. Cortex* **21**, 2599–2611 (2011).
 277. Zhang, Z. Y. *et al.* Ubiquitination and inhibition of glycine receptor by HUWE1 in spinal cord dorsal horn. *Neuropharmacology* **148**, 358–365 (2019).
 278. Sueda, R., Imayoshi, I., Harima, Y. & Kageyama, R. High Hes1 expression and resultant Ascl1 suppression regulate quiescent vs. active neural stem cells in the adult mouse brain. *Genes Dev.* **33**, 511–523 (2019).
 279. Talikka, M., Perez, S. E. & Zimmerman, K. Distinct patterns of downstream target activation are specified by the helix-loop-helix domain of proneural basic helix-loop-helix transcription factors. *Dev. Biol.* **247**, 137–148 (2002).
 280. Chitnis, A. & Kintner, C. Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in *Xenopus* embryos. *Development* **122**, 2295–2301 (1996).
 281. Kim, C. H. *et al.* Overexpression of neurogenin induces ectopic expression of HuC in zebrafish. *Neurosci. Lett.* **239**, (1997).
 282. Pavani, G. & Amendola, M. Targeted Gene Delivery: Where to Land. *Front. Genome Ed.* **2**, 36 (2021).
 283. Bhagwan, J. R. *et al.* Variable expression and silencing of CRISPR-Cas9 targeted transgenes identifies the AAVS1 locus as not an entirely safe harbour. *F1000Research* **8**, (2019).
 284. Ordovás, L. *et al.* Efficient Recombinase-Mediated Cassette Exchange in hPSCs to Study the Hepatocyte Lineage Reveals AAVS1 Locus-Mediated Transgene Inhibition. *Stem Cell Reports* **5**, 918 (2015).
 285. Tay, F. C. *et al.* Targeted transgene insertion into the AAVS1 locus driven by baculoviral vector-mediated zinc finger nuclease expression in human-

- induced pluripotent stem cells. *J. Gene Med.* **15**, 384–395 (2013).
286. Smith, J. R. *et al.* Robust, Persistent Transgene Expression in Human Embryonic Stem Cells Is Achieved with AAVS1-Targeted Integration. *Stem Cells* **26**, 496–504 (2008).
 287. Picard, D. Regulation of protein function through expression of chimaeric proteins. *Curr. Opin. Biotechnol.* **5**, 511–515 (1994).
 288. Eilers, M., Picard, D., Yamamoto, K. R. & Bishop, J. M. Chimaeras of Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* **340**, 66–68 (1989).
 289. Feil, R., Rgen Wagner, J., Metzger, D. & Chambon, P. Regulation of Cre Recombinase Activity by Mutated Estrogen Receptor Ligand-Binding Domains. *Biochem. Biophys. Res. Commun.* **237**, 752–757 (1997).
 290. Feil, R. *et al.* Ligand-activated site-specific recombination in mice. *Genetics* **93**, 10887–10890 (1996).
 291. Kumar, V., Green, S., Staub, A. & Chambon, P. Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor. *EMBO J.* **5**, 2231–2236 (1986).
 292. Asp, M. L., Martindale, J. J. & Metzger, J. M. Direct, Differential Effects of Tamoxifen, 4-Hydroxytamoxifen, and Raloxifene on Cardiac Myocyte Contractility and Calcium Handling. *PLoS One* **8**, e78768 (2013).
 293. Lewis, J. S. & Jordan, V. C. Selective estrogen receptor modulators (SERMs): Mechanisms of anticarcinogenesis and drug resistance. *Mutat. Res.* **591**, 247–263 (2005).
 294. Kohli, L. *et al.* 4-hydroxytamoxifen induces autophagic death through K-Ras degradation. *Cancer Res.* **73**, 4395–4405 (2013).
 295. Sumi, T., Tsuneyoshi, N., Nakatsuji, N. & Suemori, H. Apoptosis and differentiation of human embryonic stem cells induced by sustained activation of c-Myc. *Oncogene* 2007 2638 **26**, 5564–5576 (2007).
 296. Bennett, M. R., Littlewood, T. D., Schwartz, S. M. & Weissberg, P. L. Increased Sensitivity of Human Vascular Smooth Muscle Cells From Atherosclerotic Plaques to p53-Mediated Apoptosis. *Circ. Res.* **81**, 591–599 (1997).

297. Brännvall, K., Korhonen, L. & Lindholm, D. Estrogen-Receptor-Dependent Regulation of Neural Stem Cell Proliferation and Differentiation. *Mol. Cell. Neurosci.* **21**, 512–520 (2002).
298. Liu, G., Schwartz, J. A. & Brooks, S. C. Estrogen Receptor Protects p53 from Deactivation by Human Double Minute-2 1. *CANCER Res.* **60**, 1810–1814 (2000).
299. Konduri, S. D. *et al.* Mechanisms of estrogen receptor antagonism toward p53 and its implications in breast cancer therapeutic response and stem cell regulation. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15081–15086 (2010).
300. Hurd, C., Dinda, S., Khattree, N. & Moudgil, V. K. Estrogen-dependent and independent activation of the P1 promoter of the p53 gene in transiently transfected breast cancer cells. *Oncogene* **18**, 1067–1072 (1999).
301. Wijayarathne, A. L. & McDonnell, D. P. The Human Estrogen Receptor- α is a Ubiquitinated Protein Whose Stability is Affected Differentially by Agonists, Antagonists, and Selective Estrogen Receptor Modulators. *J. Biol. Chem.* **276**, 35684–35692 (2001).
302. Swetzig, W. M., Wang, J. & Das, G. M. Estrogen receptor alpha (ER α /ESR1) mediates the p53-independent overexpression of MDM4/MDMX and MDM2 in human breast cancer. *Oncotarget* **7**, 16049–16069 (2016).
303. King, B. *et al.* The ubiquitin ligase Huwe1 regulates the maintenance and lymphoid commitment of hematopoietic stem cells. *Nat. Immunol.* **17**, 1312–1321 (2016).
304. Ghazale, H. *et al.* Ascl1 phospho-site mutations enhance neuronal conversion of adult cortical astrocytes in vivo. *Front. Neurosci.* **16**, 1353 (2022).
305. Azzarelli, R. *et al.* ASCL1 phosphorylation and ID2 upregulation are roadblocks to glioblastoma stem cell differentiation. *Sci. Rep.* **12**, (2022).
306. Pauly, M. G. *et al.* Adherent vs. Free-floating neural induction by dual SMAD inhibition for neurosphere cultures derived from human induced pluripotent stem cells. *Front. Cell Dev. Biol.* **6**, 3 (2018).
307. Yao, Z. *et al.* A Single-Cell Roadmap of Lineage Bifurcation in Human ESC

- Models of Embryonic Brain Development. *Cell Stem Cell* **20**, 120–134 (2017).
308. Handel, A. E. *et al.* Assessing similarity to primary tissue and cortical layer identity in induced pluripotent stem cell-derived cortical neurons through single-cell transcriptomics. *Hum. Mol. Genet.* **25**, 989–1000 (2016).
 309. Petanjek, Z., Kostović, I. & Esclapez, M. Primate-specific origins and migration of cortical GABAergic neurons. *Front. Neuroanat.* **3**, 26 (2009).
 310. Telley, L. & Jabaudon, D. A mixed model of neuronal diversity. *Nat. 2021* 5557697 **555**, 452–454 (2018).
 311. Fonseca, M., Del Río, J. A., Martínez, A., Gómez, S. & Soriano, E. Development of calretinin immunoreactivity in the neocortex of the rat. *J. Comp. Neurol.* **361**, 177–192 (1995).
 312. Yan, X. X., Cariaga, W. A. & Ribak, C. E. Immunoreactivity for GABA plasma membrane transporter, GAT-1, in the developing rat cerebral cortex: transient presence in the somata of neocortical and hippocampal neurons. *Dev. Brain Res.* **99**, 1–19 (1997).
 313. Del Rio, J. A., Soriano, E. & Ferrer, I. Development of GABA-immunoreactivity in the neocortex of the mouse. *J. Comp. Neurol.* **326**, 501–526 (1992).
 314. Lundgren, P., Mattsson, M. olof, Johansson, L., Ottersen, O. P. & Sellström, Å. Morphological and GABA-immunoreactive development of the embryonic chick telencephalon. *Int. J. Dev. Neurosci.* **13**, 463–472 (1995).
 315. Lundgren, P., Johansson, L., Englund, C., Sellström, Å. & Mattsson, M. O. Expression pattern of glutamate decarboxylase (GAD) in the developing cortex of the embryonic chick brain. *Int. J. Dev. Neurosci.* **15**, 127–137 (1997).
 316. Verney, C. Phenotypic expression of monoamines and GABA in the early development of human telencephalon, transient or not transient. *J. Chem. Neuroanat.* **26**, 283–292 (2003).
 317. LoTurco, J. J., Owens, D. F., Heath, M. J. S., Davis, M. B. E. & Kriegstein, A. R. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron* **15**, 1287–1298 (1995).

- 318. Kouwenhoven, W. M. *et al.* VGluT2 Expression in Dopamine Neurons Contributes to Postlesional Striatal Reinnervation. *J. Neurosci.* **40**, 8262–8275 (2020).
- 319. Zhang, W. *et al.* System-wide modulation of HECT E3 ligases with selective ubiquitin variant probes. *Mol. Cell* **62**, 121 (2016).
- 320. Larson, M. H. *et al.* CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc.* **2013 811** **8**, 2180–2196 (2013).
- 321. Huebsch, N. *et al.* CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. *Cell Stem Cell* **18**, 541–553 (2016).
- 322. Olsen, R. R. *et al.* ASCL1 represses a SOX9+ neural crest stem-like state in small cell lung cancer. *Genes Dev.* **38**, 1–23 (2021).