Elucidating the molecular pathology of *DEPDC5* mutation induced mTOR-hyperactivation associated with epilepsy and autism spectrum disorder

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Mattson Jones

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Dekan: Prof. Dr. Sven Klimpel

1. Gutachterin: Prof. Dr. Jasmin Hefendehl

2. Gutachter: PD. Dr. Andreas Chiocchetti

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IV. List of acronyms used in this study

Acronym	Definition
ADHD	attention-deficit hyperactivity disorder
AMY	amygdala
ASD	autism spectrum disorder
BLAST	Basic Local Alignment Search Tool
CAMKII	calcium/calmodulin-dependent protein kinase 2
Cas9	CRISPR associated protein 9
CBC	cerebellar cortex
cDNA	complementary DNA
CNV	copy number variations
CPM	counts per million reads
CRISPR	clustered regularly interspaced short palindromic repeats
DA	dopaminergic
DEE	developmental and epileptic encephalopathies
DFC	dorsal frontal cortex
DMEM	Dulbecco's modified eagle medium

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DSM-5	diagnostic and statistical manual of mental disorders
E+(number)	embryonic day
EEG	electroencephalography
EIMFS	epilepsy of infancy with migrating focal seizures
ER	endoplasmic reticulum
ESC	embryonic stem cell
FCD	focal cortical dysplasia
FCD1	focal cortical dysplasia type 1
FCD2	focal cortical dysplasia type 2
FDA	food and drug administration
fdr	false discovery rate
FFEVF	familial focal epilepsy with variable foci
FGF	fibroblast growth factor
Flox/flox	flanking/flanked by LoxP
fMRI	functional magnetic resonance imaging
GEF	guanine exchange factor
GEFS+	genetic epilepsy with febrile seizures plus
PCA	principal components analysis
GO	gene ontology
GW	gestational week
hclust	hierarchical clustering
HIP	hippocampus
hiPSC	human induced pluripotent stem cell
hNPC	human neural progenitor cell
ICD-10	international classification of diseases 10th revision
ICD-11	international classification of diseases 11th revision
ID	intellectual disability
IDT	integrated DNA technologies
ILAE	international league against epilepsy
IP	intermediate progenitor
iPSC	induced pluripotent stem cell
KCl	potassium chloride
KO	knockout
MAE	myoclonic-atonic epilepsy
MCD	malformations of cortical development
MD	mediodorsal nucleus of the thalamus
MDS	multidimensional scaling
MFC	medial frontal cortex
mGluR	metabotropic glutamate receptor
mTOR	mechanistic target of rapamycin
NCBI	national center for biotechnology information
NE	neuroepithelial
NF	neurofibromatosis
NF1	neurofibromatosis type 1
NF2	neurofibromatosis type 2
NMD	nonsense mediated decay
NMDA	N-methyl-D-aspartate
NPC	neural progenitor cell
NSC	neural stem cell
NTC	non-targeting control
OFC	orbitofrontal cortex
ON	overnight
OR	odds ratio
PAM	protospacer adjacent motif
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
PCA	principal component analysis
PCR	polymerase chain reaction
phNPC	primary human neural progenitor cell
PO-L	poly-L-lysine
PVDF	polyvinylidene difluoride
QC	quality control
RA	retinoic acid
RG	radial glial
RIN	RNA integrity number
RNA	ribonucleic acid
RT	room temperature
RT-qPCR	reverse transcription quantitative PCR
SCZ	schizophrenia
SEGA	subependymal giant cell astrocytoma
sgRNA	single guide RNA
STEM	science-technology-engineering-math
STR	striatum
SUDEP	sudden unexpected death in epilepsy
SVZ	subventricular zone
TALEN	transcription activator-like effector nucleases
TSC	tuberous sclerosis complex

VZ	ventricular zone
WGCNA	weighted gene co-expression network analysis
WT	wildtype

V. List of genes/proteins

Gene/Protein	Full Gene/Protein name
4E-BP1	*eukaryotic translation initiation factor 4E-binding protein 1*
ACSBG1	*acyl-CoA synthetase bubblegum family member 1*
ADNP	*activity dependent neuroprotector homeobox*
ADNP2	*ADNP homeobox 2*
AKT3	*AKT serine/threonine kinase 3*
AMPK	*5'AMP-activated protein kinase*
AQP4	*aquaporin 4*
ASCL1	*achaete-scute family bHLH transcription factor 1*
ATF4	*activating transcription factor 4*
ATP6V0E1	*ATPase H+ transporting V0 subunit e1*
ATP6V0E2	*ATPase H+ transporting V0 subunit e2*
ATP6V1F	*ATPase H+ transporting V1 subunit F*
AURKB	*aurora kinase B*
BBC3	*BCL2 binding component 3*
BCL11A	*BAF chromatin remodeling complex subunit BCL11A*
BDNF	*brain derived neurotrophic factor*
BMP7	*bone morphogenetic protein 7*
BP1	*distal-less homeobox 4*
BRD7	*bromodomain containing 7*
CAMKII	*calcium/calmodulin-dependent protein kinase 2*
Cas9	*CRISPR-associated protein 9*
CASTOR1	*cytosolic arginine sensor for mTORC1 subunit 1*
CCDC8	*coiled-coil domain containing 8*
CD151	*CD151 molecule (Raph blood group)*
CDKN1A	*cyclin dependent kinase inhibitor 1A*
CENPE	*centromere protein E*
CENPF	*centromere protein F*
CHAT	*choline O-acetyltransferase*
CHD2	*chromodomain helicase DNA binding protein 2*
CHD5	*chromodomain helicase DNA binding protein 5*
CHD7	*chromodomain helicase DNA binding protein 7*
CHD8	*chromodomain helicase DNA binding protein 8*
CHRNA4	*cholinergic receptor nicotinic alpha 4 subunit*
COL4A2	*collagen type IV alpha 2 chain*
COL6A1	*collagen type VI alpha 1 chain*
COL6A2	*collagen type VI alpha 2 chain*
COL9A3	*collagen type IX alpha 3 chain*
COMT	*catechol-O-methyltransferase*
COX6C	*cytochrome c oxidase subunit 6C*
CPS1	*carbamoyl-phosphate synthase 1*
CUX2	*cut like homeobox 2*
DCX	*doublecortin*
DDIT4	*DNA damage inducible transcript 4*
DEPDC5	*DEP domain containing 5, GATOR1 subcomplex subunit*
DEPTOR	*DEP domain containing mTOR interacting protein*
DHCR7	*7-dehydrocholesterol reductase*
DRD2	*dopamine receptor D2*
EFNA5	*ephrin A5*
EFNB2	*ephrin B2*
eIF4B	*eukaryotic translation initiation factor 4B*
eIF4E	*eukaryotic translation initiation factor 4E*
eIF4F	*eukaryotic initiation factor 4F*
EMX1	*empty spiracles homeobox 1*
ERBB4	*erb-b2 receptor tyrosine kinase 4*
ERK	*extracellular signal-regulated kinase or extracellular receptor kinase*
ESCO2	*establishment of sister chromatid cohesion N-acetyltransferase 2*
FGF1	*fibroblast growth factor 1*
FKB39	*FK506-binding protein of 39 kDa*
FKBP10	*FKBP prolyl isomerase 10*
FKBP12	*FK506 binding protein 12*
FLCN-FNIP	*folliculin/folliculin-interacting protein 1*
FMR1	*FMRP translational regulator 1*
FOXJ1	*forkhead box J1*
FOXP2	*forkhead box P2*
FZD2	*frizzled class receptor 2*
GABBR2	*gamma-aminobutyric acid type B receptor subunit 2*

GABRA	*gamma-Aminobutyric acid type A receptor subunit alpha*
GAD1	*glutamate decarboxylase 1*
GALNT17	*polypeptide N-acetylgalactosaminyltransferase 17*
GAT1	*solute carrier family 6 member 1*
GATM	*glycine amidinotransferase*
GATOR1	*gap activity toward rags 1*
GATOR2	*gap activity toward rags 2*
GFAP	*glial fibrillary acidic protein*
GLAST	*L-Glutamate/L-Aspartate Transporter*
GNAO1	*G protein subunit alpha o1*
GNG2	*G protein subunit gamma 2*
GRIA2	*glutamate ionotropic receptor AMPA type subunit 2*
GRIA4	*glutamate ionotropic receptor AMPA type subunit 4*
GRIK1	*glutamate ionotropic receptor kainate type subunit 1*
GRIN2A	*glutamate ionotropic receptor NMDA type subunit 2A*
GRIN2B	*glutamate ionotropic receptor NMDA type subunit 2B*
GSK-3	*glycogen synthase kinase 3*
GUCY1B1	*guanylate cyclase 1 soluble subunit beta 1*
HAUS1	*HAUS augmin like complex subunit 1*
HIF1α	*hypoxia-inducible factor 1-alpha*
HK2	*hexokinase 2*
HTR2B	*5-hydroxytryptamine receptor 2B*
IRS1	*insulin receptor substrate 1*
KCNAB1	*potassium voltage-gated channel subfamily A regulatory beta subunit 1*
KCNN2	*potassium calcium-activated channel subfamily N member 2*
KCNQ2	*potassium voltage-gated channel subfamily Q member 2*
KCNQ3	*potassium voltage-gated channel subfamily Q member 3*
Ki67	*marker of proliferation Ki-67*
KLF4	*kruppel like factor 4*
KNSTRN	*kinetochore localized astrin (SPAG5) binding protein*
KPTN	*kaptin, actin binding protein*
LAMA4	*laminin subunit alpha 4*
LAMA5	*laminin subunit alpha 5*
LAMTOR	*late endosomal/lysosomal adaptor, MAPK and MTOR activator 1*
LRP10	*LDL receptor related protein 10*
MAGI1	*membrane associated guanylate kinase, WW and PDZ domain containing 1*
MAP2	*microtubule associated protein 2*
MAPK	*mitogen-activated protein kinases*
MCL1	*MCL1 apoptosis regulator, BCL2 family member*
MCM6	*minichromosome maintenance complex component 6*
MDM2	*MDM2 proto-oncogene*
MEG3	*maternally expressed 3*
MGLL	*monoglyceride lipase*
MKI67	*marker of proliferation Ki-67*
mLST8	*mTOR associated protein, LST8 homolog*
mSin1	*mitogen-activated protein kinase 2 associated protein 1*
mTOR	*mammalian target of rapamycin*
mTOR2	*mammalian target of rapamycin complex 2*
mTORc1	*mammalian target of rapamycin complex 1*
MYC	*MYC proto-oncogene, bHLH transcription factor*
NES	*nestin*
NeuN	*Fox-3, Rbfox3, or Hexaribonucleotide Binding Protein-3*
NF1	*neurofibromin 1*
NGFR	*nerve growth factor receptor*
NOTCH1	*notch receptor 1*
NPRL2	*NPR2 like, GATOR1 complex subunit*
NPRL3	*NPR3 like, GATOR1 complex subunit*
NPY	*neuropeptide Y*
NR4A1	*nuclear receptor subfamily 4 group A member 1*
NR4A3	*nuclear receptor subfamily 4 group A member 3*
NRXN1	*neurexin 1*
NRXN3	*neurexin 3*
NT-3	*neurotrophin-3*
NUCKS1	*nuclear casein kinase and cyclin dependent kinase substrate 1*
NUSAP1	*nucleolar and spindle associated protein 1*
OCT-4	*octamer-binding transcription factor 4*
OLIG2	*oligodendrocyte transcription factor 2*
P53	*tumor protein p53*
PAX6	*paired box 6*
PDGFRA	*platelet derived growth factor receptor alpha*
PDLIM1	*PDZ and LIM domain 1*
PI3K	*phosphatidylinositol-4,5-bisphosphate 3-kinase*
PKA	*protein kinase A*
PKC	*protein kinase C*
PKG	*protein kinase G*

PRAS40	*proline-rich AKT substrate of 40kDa*
PRC1	*protein regulator of cytokinesis 1*
pS6	*phosphorylated S6 ribosomal protein*
PSD95	*discs large MAGUK scaffold protein 4*
PSMD7	*proteasome 26S subunit, non-ATPase 7*
PSRC1	*proline and serine rich coiled-coil 1*
PTEN	*phosphatase and tensin homolog*
PTGDS	*prostaglandin D2 synthase*
PTK2B	*protein tyrosine kinase 2 beta*
PTN	*pleiotrophin*
PTTG1	*PTTG1 regulator of sister chromatid separation, securin*
PYGB	*glycogen phosphorylase B*
RagA	*Ras-related GTP-binding protein A*
RagC	*Ras-related GTP-binding protein C*
RagD	*Ras-related GTP-binding protein D*
RAPTOR	*regulatory associated protein of MTOR complex 1*
RAPTOR	*Regulatory Protein Associated with mTOR*
REDD1	*DNA damage inducible transcript 4*
Reelin	*large secreted extracellular matrix glycoprotein*
RFX4	*regulatory factor X4*
RHEB	*Ras homolog, mTORC1 binding*
RICTOR	*RPTOR independent companion of MTOR complex 2*
RPS6	*ribosomal protein S6*
RPS6KA2	*ribosomal protein S6 kinase A2*
RRM1	*ribonucleotide reductase catalytic subunit M1*
S100	*S100 calcium binding protein A1*
S6K1	*p70S6 Kinase 1 OR S6 kinase 1*
SAMTOR	*S-adenosylmethionine sensor upstream of mTORC1*
SCD	*stearoyl-CoA desaturase*
SCD5	*stearoyl-CoA desaturase 5*
SCN1A	*sodium voltage-gated channel alpha subunit 1*
SCN2A	*sodium voltage-gated channel alpha subunit 2*
SCN3A	*sodium voltage-gated channel alpha subunit 3*
SERPINE1	*serpin family E member 1*
SERPINE2	*serpin family E member 2*
SERT1	*Sertoli cell protein 1*
SESN2	*sestrin 2*
SGK1	*serum and glucocorticoid-regulated kinase 1*
SLC12A4	*solute carrier family 12 member 4*
SLC17A7	*solute carrier family 17 member 7*
SLC1A3	*solute carrier family 1 member 3*
SLC25A39	*solute carrier family 25 member 39*
SLC38A10	*solute carrier family 38 member 10*
SLC38A9	*solute carrier family 38 member 9*
SLC3A2	*solute carrier family 3 member 2*
SLC7A5	*solute carrier family 7 member 5*
SORCS2	*sortilin related VPS10 domain containing receptor 2*
SOX2	*SRY-box transcription factor 2*
SREBP	*sterol responsive element binding protein*
ST6GAL1	*ST6 beta-galactoside alpha-2,6-sialyltransferase 1*
ST6GALNAC5	*ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5*
STAT4	*signal transducer and activator of transcription 4*
STMN1	*stathmin 1*
STMN2	*stathmin 2*
STRADA	*STE20 related adaptor alpha*
STX1B	*syntaxin 1B*
SYP	*synaptophysin*
SZT2	*SZT2 subunit of KICSTOR complex*
TACC3	*transforming acidic coiled-coil containing protein 3*
TBC1D7	*TBC1 domain family member 7*
TF	*transferrin*
TFEB	*transcription factor EB*
TH	*tyrosine hydroxylase*
THBS1	*thrombospondin 1*
THBS4	*thrombospondin 4*
TMEM119	*transmembrane protein 119*
TMEM260	*transmembrane protein 260*
TNC	*tenascin C*
TNFRSF10B	*TNF receptor superfamily member 10b*
TOP2A	*DNA topoisomerase II alpha*
TPX2	*TPX2 microtubule nucleation factor*
TRIP12	*thyroid hormone receptor interactor 12*
TRIP6	*thyroid hormone receptor interactor 6*
TSC1	*TSC complex subunit 1*
TSC2	*TSC complex subunit 2*

TTC21B	*tetratricopeptide repeat domain 21B*
TUBB3	*tubulin beta 3 class III*
TUJ1	*class III beta-tubulin*
UBE2C	*ubiquitin conjugating enzyme E2 C*
UBE2T	*ubiquitin conjugating enzyme E2 T*
UBE3A	*ubiquitin protein ligase E3A*
ULK1	*unc-51 like autophagy activating kinase 1*
USP16	*ubiquitin specific peptidase 16*
USP7	*ubiquitin specific peptidase 7*
WNT2B	*Wnt family member 2B*
ZEB2	*zinc finger E-box binding homeobox 2*

VI. Ehrenwörtliche Erklärung

Hiermit erkläre ich, Mattson Jones, geboren am 16.06.1987, dass ich die im Fachbereich Biowissenschaften an der Johann Wolfgang Goethe-Universität in Frankfurt am Main eingereichte Dissertation mit dem Titel „**Elucidating the molecular pathology of DEPDC5 mutation induced mTOR-hyperactivation associated with epilepsy and autism spectrum disorder**.“ in der Klinik für Psychiatrie, Psychosomatik und Psychotherapie des Kindes- und Jugendalters unter Betreuung und Anleitung von Dr. Andreas G. Chiocchetti und Dr. Jasmin Hefendehl selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Ort, Datum Mattson Jones

VII. Zusammenfassung

Hintergrund

Epilepsie ist eine neurologische Erkrankung, der eine abnorme Hirnaktivität zugrunde liegt, welche Krampfanfälle und weitgreifende pathologische Probleme verursacht, sodass es zu Schwierigkeiten kommen kann, normale Aufgaben zu erfüllen und Betroffene in ihrer Lebensführung eingeschränkt sind. Epilepsie ist durch generalisierte (im gesamten Gehirn) oder fokale Anfälle (in einem einzelnen Gehirnbereich) gekennzeichnet (Devinsky et al. 2018). Derzeit wird bei 50 Millionen Menschen weltweit eine Epilepsie diagnostiziert (Epilepsie 2019). Neben Krebs, Infektionskrankheiten oder traumatischen Hirnverletzungen als Ursache für Epilepsie, haben einige Menschen eine genetische Prädisposition bzw. Mutationen, die Epilepsie auslösen können. Mehr als 977 Gene wurden bisher mit Epilepsie in Verbindung gebracht, und in genetischen Studien werden immer wieder neue Varianten bzw. Gene entdeckt (Wang et al. 2017).

Häufig treten genetische Anomalien bei Epilepsie in Verbindung mit anderen Krankheiten auf. Insbesondere bei der Autismus-Spektrum-Störung (ASS) tritt Epilepsie in 8-21,5% der Fälle als Komorbidität auf und umgekehrt zeigen auch 5-40% der Personen mit Epilepsie eine komorbide ASS-Diagnose (Besag et al. 2018). ASS ist durch Beeinträchtigungen in der sozialen Kommunikation und repetitive Verhaltensweisen gekennzeichnet (DSM-5; American Psychiatric Association, 2013). Derzeit ist weltweit 1 von 44 Personen betroffen (Maenner et al. 2021). In großen genetischen Studien wurden ebenso mehrere Hundert Risikogene identifiziert, die der Ätiologie von ASS zugrunde liegen können, welche stark mit Epilepsiegenen überlappen (De Rubeis et al. 2014; ILAE, 2018; Iossifov et al. 2014; Wang et al. 2017).

In den letzten zehn Jahren gab es vermehrt Hinweise auf einen Zusammenhang zwischen Epilepsie und ASS mit dem DEP-domain-containing-5 (DEPDC5)-Protein, wobei Personen, die Mutationen in diesem Gen aufweisen, häufig eine Komorbidität mit ASS und Epilepsie zeigen. DEPDC5 wurde als Aminosäuresensor des mTOR-Signalwegs identifiziert, der zelluläre Signale zur Aufrechterhaltung von Wachstum und Homöostase reguliert. mTOR selbst ist eine Kinase, die an der Proteintranslation, Ribosomenbiogenese, Lipidsynthese, aeroben Glykolyse, Nukleotidsynthese, Autophagie und Lysosomenbiogenese beteiligt ist. Der Funktionsverlust von DEPDC5 führt hierbei zu einer Hyperaktivität von mTOR und damit zu einem Ungleichgewicht der Aufrechterhaltung zellulärer Signale. Der komplette

Verlust von mTOR oder DEPDC5 im Tiermodell führt häufig zu einem vorzeitigen Tod, was seine Notwendigkeit für eine normale Zellfunktion unterstreicht. Ziel dieser Studie war es, die Auswirkungen des Verlusts von DEPDC5 zu verstehen und die assoziierten Genexpressionsmuster sowie morphologischen Auswirkungen im humanen Zellmodell zu charakterisieren, um so den Wirkungsmechanismus besser zu verstehen.

Methoden

Humane neurale Vorläuferzellen (hNPCs), die aus fötalem Kortex gewonnen wurden, wurden charakterisiert und anschließend einen Monat lang zu Neuronen differenziert. Mittels CRISPR-Cas9-gesteuertem Gen-Editing wurde *DEPDC5* ausgeschaltet (knock-out/KO), indem im Exon 2 ein frühzeitiges Stopp-Codon erzeugt wurde. Die hNPCs mit DEPDC5-Funktionsverlust wurden anschließend auf eine erhöhte mTOR-Aktivität untersucht. Um die zugrundeliegenden Veränderungen in der Genexpression zu verstehen, wurde eine Transkriptomanalyse durchgeführt und auf mögliche Veränderungen in den Signalwegen im Vergleich zu den Kontrollen untersucht. Ein mTOR-Inhibitor, Rapamycin, wurde ebenfalls eingesetzt, um die durch die erhöhte mTOR-Aktivität verursachten Effekte umzukehren.

Die differenziell exprimierten Gene wurden mit Hilfe von bioinformatischen Tools und der “Gene Ontology”-Datenbank sowie des KEGG-Pfad-Tools untersucht um biologische Prozesse zu identifizieren, die durch den Verlust von DEPDC5 gestört werden. Eine weitere Analyse wurde mit Hilfe der "gewichteten Gen-Koexpressions-Netzwerkanalyse" (WGCNA) durchgeführt, um Gene auf der Grundlage ihrer Koexpression zu gruppieren und so biologisch ko-regulierte Module zu identifizieren, mit welchen DEPDC5 assoziiert ist. Durch den Vergleich mit veröffentlichten großen ASS- und Epilepsie-Gendatensätzen wurde zudem versucht zu verstehen, ob der DEPDC5-KO besonders assoziierte Krankheitsgene beeinflusst, oder ob dessen Genexpressionsmuster spezifisch ist.

Schließlich wurde untersucht, ob differenziell regulierte Gene mit Gennetzwerken assoziiert sind, welche die Gehirnentwicklung steuern, um so zu verstehen wann und wo während der Gehirnentwicklung die betroffenen Gene aktiv sind. Mit Hilfe der Laser Scanning Mikroskopie wurden die verwendeten Zelllinien auf morphologische Veränderungen hinsichtlich Zellgröße, Dendritenlänge, Anzahl der Dendriten und Anzahl der neuronalen Subtypen untersucht.

Ergebnisse

Es konnte gezeigt werden, dass hNPCs radiale Glia-Marker exprimieren und in der Lage sind, nach 1 Monat in Tuj1-positive Neuronen zu differenzieren. Sanger-Sequenzierung konnte bestätigen, dass die Zellen Insertionen oder Deletionen im Exon 2 von *DEPDC5* aufweisen, sodass ein frühzeitiges Stopp-Codon entstand. Die Western-Blot-Analyse konnte zudem kein DEPDC5-Protein nachweisen, sodass von einem vollständigen KO von DEPDC5 auszugehen ist. Es zeigte sich zudem, durch die erhöhte Phosphorylierung des S6-Proteins, eines nachgeschalteten Effektors von mTOR, dass die Zellen mTOR-hyperaktiviert waren. Diese Hyperaktivität konnte auch durch den Einsatz von Rapamycin rückgängig gemacht werden, sodass die Spezifität für mTOR bestätigt wurde.

Die Transkriptomanalyse identifizierte insgesamt 738 Gene, die durch den Verlust von DEPDC5 sowohl in der Proliferations- als auch in der Differenzierungsphase dereguliert waren. Die Zugabe von Rapamycin stellte die Gesamtexpression für alle außer 148 Gene wieder her. Drei dieser 148 Gene waren sowohl in der Proliferations- als auch der Differenzierungsphase dereguliert, nämlich NPY, SERPINE1 und STMN2 (Gennamen siehe Abkürzungsverzeichnis). Analyse der KEGG-Signalwege zeigte, dass deregulierte Gene mit Prozessen assoziiert sind, die Zellzyklus/P53-bezogene Prozesse oder Stoffwechsel und Lysosom/Proteindegradation regulieren. Abgleich mit der Gene-Ontology-Datenbank zeigte sowohl in Proliferations- als auch in Differenzierungsdatensätzen eine Assoziation mit allgemeiner Systementwicklung und der Entwicklung anatomischer Strukturen. Die WCGN-Analyse ergab, dass DEPDC5 ein Gennetzwerk moduliert, welches spezifisch im Zusammenhang mit dem endoplasmatischen Retikulum und der Ribosomenbiogenese steht.

Die Analyse der ASS- und Epilepsie-Risikogene zeigte keine signifikante Assoziation mit Genen, die durch DEPDC5-KOs dereguliert sind. Deskriptiv waren 18 ASS- oder Epilepsie-Gene während der Proliferation und 51 während der Differenzierung differentiell exprimiert. Bei der Analyse von Gendatensätzen für neurologische Erkrankungen konnte eine signifikante Überlappung mit Genen gezeigt werden, die mit TSC1/2 Defekten assoziiert sind (Grabole et al. 2016, Martin et al. 2017). Interessanterweise wurden dieser Assoziationen durch Rapamycingabe zwar gemildert aber nicht vollstädnig aufgehoben.

Zudem wurde eine signifikante Assoziation mit einem entwicklungsspezifischen Gennetzwerk gefunden, welches bei ASS-Patienten dereguliert ist (Voineagu et al. 2011). Hierbei konnte die Rapamycin-

Hemmung von mTOR diese Assoziation wieder aufheben. Schließlich zeigte die Analyse von publizierten Transkriptomdaten der Gehirnentwicklung (Kang et al. 2011), dass die DEPDC5-KO-Gene mit der frühen Entwicklung des Hippocampus, der Amygdala und des Kortex im Zusammenhang stehen. Diese assoziierten Gene sind mit den bereits identifizierten Mechanismen "Zellzyklusregulation" und "synaptische Übertragung" assoziiert.

Die morphologische Analyse ergab eine allgemeine Zunahme von MAP2-positiven Neuronen, die durch Rapamycin-Gabe auf ein Wildtyp-Niveau zurückgebracht werden konnte. DEPDC5-KO-Neuronen wiesen zudem eine verringerte Axonlänge und vergrößerte dendritische Äste auf, die aber nicht durch Rapamycin abgeschwächt werden konnten. Die morphologische Analyse zeigte eine Verringerung der Zellgröße während der Proliferation und Differenzierung, die durch die Anwendung von Rapamycin ebenso nicht rückgängig gemacht werden konnte.

Diskussion

Es zeigte sich, dass hNPCs charakteristischerweise frühen Entwicklungsstadien des fötalen Gehirns ähneln und als Modell für die Analyse der Entwicklung neurologischer Störungen geeignet sind. DEPDC5 wurde in diesen Zellen erfolgreich ausgeknockt, was zur erwarteten Hyperaktivität von mTOR führte (Klofas et al. 2020). Diese Hyperaktivität ist größtenteils, aber nicht ausschließlich, ursächlich für die DEPDC5-assoziierten deregulierten Prozesse, die bisher in menschlichen Neuronen nicht gezeigt worden waren. Diese Deregulierung der nachgeschalteten mTOR-Prozesse deutet auf einen möglichen Mechanismus hin, der zu Entwicklungsanomalien in der neuronalen Verzweigung und Axonbildung führt. Interessanterweise stehen diesem Befund aber eine Reihe von Genen und morphologischen Änderungen gegenüber, die nicht auf die mTOR Hyperaktivierung zurückzuführen sind.

In ähnlicher Weise kehrt Rapamycin bei Patienten den neurologischen Phänotyp, der durch den Verlust von DEPDC5 entsteht, nicht vollständig um. Die zentralsten nicht wiederherstellbaren Gene NPY, SERPINE2 und STMN2 könnten einen Hinweis auf die Pathomechanismen geben. Diese sind aber bisher nicht als Risikogene für ASD oder Epilepsie bekannt. Ein weiterer Mechanismus, der die neurobiologischen Anomalien bei ASS oder Epilepsie erklären könnte, ist die beobachtete schnellere Differenzierung der Neuronen. Dieser Befund deckt sich mit früheren Studien, die zeigten, dass eine verstärkte Proliferation und Differenzierung bei mTOR-Erkrankungen häufig ist (Magri et al. 2011). Eine aktuelle Hypothese zur Entstehung von ASS legt nahe, dass Proliferations- und

Differenzierungsmechanismen zu ASS-Phänotypen führen können. Unsere Studie deutet auch darauf hin, dass der richtige Zeitpunkt der Differenzierung für eine normale Entwicklung entscheidend ist und durch *DEPD5C*-Mutationen gestört sein könnte.

Im Gegensatz zu früheren Studien, die zeigten, dass mTOR-Hyperaktivität häufig durch vergrößerte Zellen gekennzeichnet ist, wurde hier beobachtet, dass die Soma-Größen in DEPDC5-KOs verringert waren. Die Ursache für diesen Befund kennen wir aktuell nicht. Die Unterschiede könnten auf Einschränkungen des Zelltyps (hNPCS) selbst oder auf einen von mTOR-unabhängigen Mechanismus zurückzuführen sein. Die Gabe von Rapamycin kehrte den Phänotyp nicht um, was darauf hindeutet, dass andere Mechanismen die Veränderungen der Zellgröße beeinflussen könnten.

Diese detaillierte Analyse der Genexpression, die durch den Verlust von DEPDC5 während zweier Entwicklungsstadien verursacht wird zeigt also, dass verschiedene mTOR assoziierte aber auch mTOR unabhängige Prozesse, zu einer schnelleren Differenzierung und veränderten Morphologie bei Verlust von DEPDC5 führen. Diese Veränderungen verdeutlichen die Rolle von DEPDC5 und mTOR bei der Entstehung neurologischer Phänotypen und geben Hinweise auf mögliche künftige Angriffspunkte für Medikamente.

1. Abstract

Patients harboring mutations in the gene *DEPDC5* often display variations of neurological diseases including epilepsy, autism spectrum disorders (ASD) and other neuro-architectural alterations. DEPDC5 protein has been identified as an amino acid sensor responsible for negatively regulating the mechanistic target of rapamycin (mTOR), a central regulator in cell growth and cell homeostasis. Often, mutations of the DEPDC5 protein result in mTOR hyperactivity leading to abnormal neuronal phenotypes and the generation of excitatory/inhibitory imbalances in animal models. Complete knockout (KO) of *DEPDC5* results in death shortly after birth, while inhibition of mTOR activity recovers postnatal death (Marsan et al. 2016). However, heterozygous *DEPDC5*-KOs in animals have been variable in their disease phenotypes during adulthood indicating developmental differences between subspecies and early development mechanisms which could be impactful on the outcome of the diseases.

To understand the mechanisms underlying *DEPDC5* mutations during early development, a novel primary human neural progenitor cell line extracted from fetal tissue was characterized during proliferation and differentiation. CRISPR-Cas9 induced mutations of the *DEPDC5* gene resulted in hyperphosphorylation of mTOR signaling processes and rapid expansion of the neuronal population during differentiation. Analysis of transcriptome data identified deregulation amongst p53 signaling, ribosome biogenesis, nucleotide and lipid synthesis as well as protein degradation pathways due to loss of DEPDC5. Disease gene datasets identified a correlation between Tuberous Sclerosis mutations as being more closely associated with *DEPDC5* mutations while also finding overlap with some ASD and epilepsy genes. By using the mTOR inhibitor rapamycin, a substantial amount of the deregulated gene network was recovered while also reversing rapid neuronal differentiation caused by loss of DEPDC5. Though we saw increased dendritic arborization and subsequent decreases in dendrite lengths and soma sizes, rapamycin failed to recover these effects suggesting mTOR independent processes produced by *DEPDC5*-KO. This study provides new insights on the relationship between mutations in *DEPDC5* and the functional, genomic and deregulatory networks it intertwines in humans and highlights that the *DEPDC5* associated pathomechanisms are not fully related to mTOR hyperactivation, but include independent processes. This also sheds light on the question why rapamycin treatment only partially restores *DEPDC5* related phenotypes and gives insight on treatments for DEPDC5 patients.

2. Introduction

2.1 Preamble

The focus of this dissertation is on the gene and protein *Dep domain containing 5* (*DEPDC5*), which has been shown to relate to the amino acid sensing component of the mechanistic target of rapamycin (mTOR) pathway. Mutations thereof have been identified in patient and family studies as well as in large genetic cohorts for multiple pathological diagnoses. Predominantly, *DEPDC5* mutations have been related to epilepsy but patients often have comorbidities with other diseases, not necessarily restricted to the brain. Therefore, it is rather difficult to address different neurodevelopmental, neuropsychiatric and epileptic diseases as separate disorders as the clinical diagnoses and overall phenotypes are variable. More and more genetic studies have identified converging pathways, genes and variants or duplications/deletions of large genetic sections, resulting in varying disease phenotypes with some being more extreme than others. Therefore, this introduction aims at painting a clear picture of the cross-reactivity of such networks with a focus on brain disease phenotypes. As *DEPDC5* mutations often result in epilepsy, a focus will be given to its role in epileptic phenotypes but also in the comorbid ASD disorders. Current research findings link the two disorders together by the mechanisms and genes shared between the diseases. Lastly, this introduction will conclude with the developmental model chosen for this thesis. Our understanding of the underlying mechanisms is paramount to developing treatment modalities for patients.

2.2 Epilepsy

2.2.1 Classification, diagnosis, symptoms and treatment

It is estimated that around 50 million people worldwide are diagnosed with epilepsy making it one of the most common neurological diseases (GBD Epilepsy Collaborators 2019). Epileptic seizures occur due to the excessive or synchronous neural activity in the brain where they can originate focally (60%), generalized (40%) or as focal seizures which become generalized (Devinsky et al. 2018). Focal seizures originate in one or more areas within the brain while generalized seizures are widespread throughout both hemispheres. Confirmation and identification of the type of epilepsy is based upon neuroimaging and electroencephalography (EEG) findings. Epileptic phenotypes can be variable in the quantity of symptoms but often appear as behavioral changes, loss of awareness, stiffening, jerking, a burnt rubber smell or déjà vu (Devinsky et al. 2018). Although epilepsy related genes play a role in the disease, it can also

manifest with certain cancers, infectious diseases, traumatic brain injury or autoimmune diseases (Devinsky et al. 2018).

Epilepsy diagnosis is based on the International Classification of Diseases 10th revision (ICD-10) where the variable seizure types and epilepsy syndromes are further classified based on the Commission on Classification and Terminology of the International League Against Epilepsy 2017 (ILAE). Such distinctive clinical diagnoses help to characterize patterns of clinical and electrical features allowing the identification of severity along with treatment options, especially when comorbidities are present. Incidences of epilepsy development occur often in infancy and early childhood or in individuals over the age of 50-60 (Fiest et al. 2017). Although worldwide men are affected by epilepsies more often than women, research suggests that this may be due to socioeconomic reasons or differences in diagnostic criteria rather than actual prevalence as countries with higher gross domestic product show no differences (Fiest et al. 2017). Individuals suffering from epilepsies have increased premature mortality risks which are the highest for children during their first year of life (Thurman et al. 2017).

More than 20 anti-seizure medications have been approved by the United States Food and Drug Administration (FDA) and European Medicines Agency, yet for around one-third of patients medication remains ineffective. Drug refractory epilepsy is defined as the failure of anti-epileptic drugs to maintain a seizure-free state for one year or more after testing at least two appropriate drugs (Kwan et al. 2010). Surgery has proven to be the most helpful, however, very few are eligible for such surgeries due to the surgical site and type of surgery required. This forces patients to seek other alternatives such as dietary therapies, neurostimulation devices or clinical trials of new drugs in development (Wiebe et al. 2001).

2.2.2 Genetics of Epilepsy

Often patients with epileptic phenotypes can be classified based on underlying genetic syndromes. However, although 977 epilepsy-associated genes have been identified, only 84 of these are specifically related to epilepsy while the other genes categorize as neurodevelopmental, systemic or unverified genes (Wang et al. 2017). Furthermore, epilepsy specific genes are associated with a variety of different subtypes of epilepsies. Therefore, it becomes difficult to identify the specific underlying mechanisms; however, genes often relate to signal transduction mechanisms, synaptic plasticity and alterations of molecular pathways (Wang et al. 2017).

The recent development of sequencing technologies has allowed investigations into unaccounted risk genes (Jallon and Latour 2005). In a large genome-wide mega-analysis, 15,212 epilepsy cases were analyzed against controls, which identified 16 specific genetic-risk loci mapping to 146 genes. Ion channels (*SCN3A, GABRA2, KCNAB1, GRIK1)*, transcription factors (*ZEB2, STAT4, BCL11A*), as well as genes involved in histone modification (*BRD7*), synaptic transmission (*STX1B*) and forebrain development (*TTC21B*) were identified as being significantly associated with epilepsy (ILAE, 2018; For gene names see List of genes/proteins). Other studies with smaller cohorts also pointed to variants encoding the inhibitory synapse related GABAA receptor (*GABAAR*) subunits and an enrichment of epileptic encephalopathy genes (Epi4K consort. 2017; May et al. 2018).

Other genetic contributions from twin and clinical studies have identified focal epilepsy associated genes in a acetylcholine receptor (*CHRNA4)*, voltage-gated potassium channels *(KCNQ2, KCNQ3),* a voltage-gated sodium channel *(SCN2A)*, along with pathological variants identified in the amino acid sensing part of the mTOR pathway (*DEPDC5, NPRL2, NPRL3;* Steinlein et al. 1995; Dibbens et al. 2013). Although, these genes are characteristic of particular types of focal epilepsy (e.g. autosomal dominant sleep-related hypermotor epilepsy, self-limited familial neonatal epilepsy, familial focal epilepsy with variable foci (FFEVF) and non-lesional focal epilepsy) they often overlap other focal epilepsies with malformations of cortical development (MCD; Scheffer et al. 2014). Thus, many epilepsy disease genes are heterogeneous in their disease phenotypes and can result in neuroarchitectural defects.

Gene studies often identify overlap between syndromes and neurological disorders with variable severity (Wang et al. 2016). For several epilepsy associated mutations, comorbid syndromes have been described, e.g., *SCN1A* mutations often result in Dravet Syndrome while *TSC1/2* mutations result in tuberous sclerosis. Patients of these syndromes often show intellectual disability (ID) and developmental impairment or ASD with other comorbid health problems. *TSC1/2* mutations and *DEPDC5* mutations are both involved in similar pathway mechanisms and patients often show similar disease phenotypes such as epilepsy and ASD.

2.2.3 Mechanisms of Epilepsy

Seizures are clinical manifestations of abnormal and excessive hypersynchronous discharge from a population of neurons while epilepsy is the property of continuous seizures resulting from unprovoked stimuli (Bromfield et al. 2005). Epileptic seizures can be induced through either the activation of synapses

and voltage-gated excitatory conductance or by the inverse blocking of inhibitory mechanisms (Staley et al. 2015). Thus, an imbalance between excitatory and inhibitory cells of the brain causes the subsequent epileptiform seizure and mutational changes in inhibitory or excitatory transmitters are often detected (Scharfman et al. 2007; Carvill et al. 2014; Macdonald and Kang 2012). Similarly, often ion channel genes are implicated resulting in ion gradient imbalances leading to similar excitatory/inhibitory imbalances (Wang et al. 2017).

This imbalance is hypothesized as emerging amongst any of the proteins associated with the tripartite synapse along with any of the genes responsible for maintaining normal synaptic function. For instance, presynaptic neurotransmitter vesicles require the coordination of docking, priming, fusion and replenishment mechanisms for proper excitation of the post-synapse. Reduction or errors regarding such a process may not permit post-synaptic neurons to achieve excitability and would prevent excitatory transmission (Meier et al. 2014). On the post-synapse, receptor membrane trafficking, neurotransmitter reuptake and receptor desensitization are also subject to precisely controlled mechanisms. Underlying these processes are proteins involved in post-translational modifications and mechanisms necessary for guiding transporters or receptors to membrane organelles. Imbalances due to functional loss in these systems can lead to epileptic phenotypes. Mutations in transport or modification proteins have also led to the underlying excitatory/inhibitory imbalance causing epilepsy. Thus, epilepsy genes are not only isolated to synaptic proteins but also pathways for transport, receptor binding, cell adhesion, signal transduction, membrane trafficking, cytoskeleton and nucleic acid binding (Wang et al. 2017).

Interestingly, risk genes such as *DEPDC5* or *TSC1/2* (i.e. members of the mTOR pathway) have been implicated in regulating cell growth and cell homeostasis mechanisms. Loss of either of these genes results in a variety of epileptic disorders, neuroarchitectural abnormalities and comorbidities with other psychiatric disorders. Thus, understanding how gene mutations result in such dysfunction helps uncover possible treatments or drugs to reverse the underlying disease phenotypes.

2.3 Autism Spectrum Disorder

Due to the evidence of comorbidities like ASD with genes associated with epilepsy including *DEPDC5*, addressing the developmental overlap can provide possible explanations on disease progression and treatment. An analysis of ASD genes and the molecular mechanisms are elaborated further.

2.3.1 Classification, diagnosis, symptoms, treatment

The most recent diagnostic systems, the International Classification of Diseases 11th Revision (ICD-11; World Health Organization 2019) and Diagnostic and Statistical Manual of Mental Disorders (DSM-5; American Psychiatric Association 2013), classify ASD as an umbrella term covering the heterogeneity of disorders manifesting as impairments in social communication and interaction as well as repetitive behaviors, sensory anomalies including heterogeneous variability in ID (American Psychiatric Association 2013; Lord et al. 2020). Diagnosis is often obtained prior to three years of age where difficulties in communication can be identified, and impairments in parent and child interactions can be observed. Individuals diagnosed with ASD frequently exhibit other neurological and psychiatric co-morbidities such as attention-deficit hyperactivity disorder (ADHD), anxiety, depression and epilepsy suggesting shared genetic influences (Ronald and Hoeskstra 2011). Epilepsy in ASD is estimated to be 8-21.5% while patients with epilepsy having ASD comorbidity vary between 5-40% (Besag et al. 2018).

The prevalence of autism is higher in males than females at an estimated 4:1 ratio with about 1 in 44 being children and 1 in 100 as adults (Brugha et al. 2016; Maenner et al. 2021). There seems to be a rise in the number of autism cases from 67 in 10,000 cases in 2000 to 146 in 10,000 in 2012. However, debates on whether this is actually due to a rise in ASD cases, an artifact due to better diagnostic procedures, the merging of autism as a spectrum diagnosis or the addition of previously undiagnosed teens/adults are still ongoing (Maenner et al. 2021; Lord et al. 2020).

Environmental risk factors for autism have been related to later parental age, birth trauma, maternal obesity, valproate use during pregnancy, among others (Modabbernia et al. 2017). Birth-related associations point toward assisted reproductive technologies, prolonged labor or delivery by cesarean section, including underlying molecular mechanisms such as hypoxic damage, inflammation or stress. Yet, despite these environmental risks, the complex underlying mechanisms are commonly related to genetic (40-90%) as well as epigenetic mechanisms (Rylaarsdam and Gamboa et al. 2019; Modabbernia et al. 2017). EEG and functional magnetic resonance imaging (fMRI) studies have identified differences in brain function in autistic individuals which are suggestive of alterations in synaptic signaling pathways during development similar to epilepsy disease mechanisms (Johnson et al. 2015).

Treatment of ASD is usually confined to minimizing challenges interfering with daily functioning or improving quality of life and currently no medication is available to treat the ASD phenotype. Current

medication use helps to address behavioral, developmental, educational, social or psychological issues ASD patients may have. Only two drugs, risperidone and aripiprazole, are approved by the FDA which aim to treat irritability associated with ASD (DeFilippis et al. 2016). Other medications are used to treat other comorbidities such as depression, aggression, hyperactivity or inattention (DeFilippis et al. 2016).

2.3.2 Genetics of Autism Spectrum Disorder

Heritability of autism in twin and family studies is estimated to be around 40-90% where currently more than 100 genes and genomic regions have been identified to be associated with autism. Many of them are related to neuronal development converging on the formation and function of neuronal synapses similar to epilepsy studies (Gaugler et al. 2014; Modabbernia et al. 2017; Sanders et al. 2015; Satterstrom et al. 2019). Larger genetic studies have helped identify 83 high confidence genes (false discovery rate <0.1) although it is estimated that there may be 450-1000 autism related genes (Yuen et al. 2015; 2017; Geschwind and State 2015). Genetic variations ranging from small nucleotide changes to large copy number variations (CNVs, i.e., deletions or duplications of chromosomal regions) can lead to autism with variable phenotypic differences (Levy et al. 2011; Sanders et al. 2012). Estimations between 10-20% predict *de novo* point mutations or CNVs provoking ASD with an increasing likelihood if patients have comorbidities with ID, have seizures or multiple unaffected siblings (Iossifov et al. 2014; De Rubeis et al. 2014). *De novo* CNVs carry a significant risk of 5-15% for autism while the general population harbors 1-2% (Geschwind and State, 2015).

Around 10% of ASD mutations can be linked to genetic syndromes with variable neurological comorbidities such as tuberous sclerosis (*TSC1/2*; ~1% of ASD cases), fragile X syndrome (*FMR1;* 1-2%), NF1 syndrome (*NF1*; <1%) and PTEN hamartoma syndrome (*PTEN* ~1%), among others (Vasic et al. 2021; Devlin et al. 2012; For gene names see List of genes/proteins). Many of these mutations point toward signaling pathway irregularities, chromatin modification and gene expression changes (*PTEN*), or synaptic protein deregulations (fragile X syndrome) which result in autism and often epilepsy (O'Roak et al. 2012; Gilman et al. 2011; Satterstrom et al. 2019). As *DEPDC5* is implicated in the mTOR pathway, studies also show its significance in the development of ASD. The comprehensive ASD risk gene database Simons Foundation Autism Research Initiative (SFARI) lists *DEPDC5* as "syndromic" and is linked to increased risk of developing ASD comorbid with epilepsy (https://gene.sfari.org/; Abrahams et al.

2013). *DEPDC5* epilepsy patients have 9% comorbidity with ASD showing gene overlap between the two diseases (Baldassari et al 2019).

Spatio-temporal convergence of ASD risk genes against post-mortem cortical tissues during development found that the genes were expressed in the mid-fetal prefrontal and primary motor-somatosensory cortices (Willsey et al. 2013). Similarly, Parikshak and colleagues used whole-transcriptome data to identify co-expression networks of autism genes in the developing brain where the most significant overlap occurred in the prenatal and early postnatal synaptic development within the cortical plate indicating how alterations in synaptic development during early fetal stages contributes to ASD (Parikshak et al. 2013). Similarly, another study implemented weighted gene correlation network analysis (WCGNA) using ASD post-mortem tissue and found two significant gene expression modules correlating to glia and neuron alterations in the cortex (Voinegu et al. 2011). These implicated genes corresponded to alterations in frontal and temporal cortex development for ASD patients. Thus, genes necessary for early and late cortex development are altered in ASD patients, and often overlap with disease genes for epilepsy such as *DEPDC5*.

2.3.3 Mechanisms of ASD

Identification of ASD genes has elucidated multiple related pathways resulting in the disorder. These pathways identify neurogenesis and migration mechanisms, cytoskeletal arrangements, synaptogenesis and plasticity and transcription and translational pathways (Basilico et al. 2020). Synaptic function, chromatin remodeling, neuronal development and signaling pathway mechanisms have also been identified as the most altered in autistic individuals in both child development and adults (Bourgeron, 2015; Tang et al. 2014). Because of the significance of these pathways in brain development, researchers hypothesize that many of these mechanisms are related to sensitive and critical time periods related to complex behavior and cognition development (Meredith et al. 2015).

Direct mutations in cytoskeletal proteins or synaptic development result in altered brain architecture. Although variable in their function, these mutations change neuronal migration as well as function and can cause delayed or enhanced differentiation of cells in the brain promoting excitatory/inhibitory imbalances. Often ASD risk genes are found in receptors, transporters or trafficking machinery extending to the tripartite synapse (Clement et al. 2012; Hashimoto et al. 2008; Yashuhara 2010; Fatemi et al. 2002; Collins et al. 2006). Mutations in these genes result in impaired synaptic plasticity and immature spine

formation leading to impaired sociability and hyperactivity in ASD mice models (Tatsukawa et al. 2019). Alterations in proteins involved in proper neuronal development and brain function are likely the main drivers of the disease phenotypes. However, similar phenotypes have been observed in transcription or protein complexes regulating synapse function.

Similarly, genes involved with protein synthesis and degradation have also been implicated in ASD disease outcomes. Gene mutations in mTOR pathway regulators, such as *PTEN, TSC1/2 or DEPDC5*, have resulted in ASD phenotypes with similar behavior and synaptic deficits as transcription regulator mutations. A change in mTOR pathway function alters protein synthesis mechanisms leading to increased axons, changes in axon guidance mechanisms and overall cellular stress (Han and Sahin et al. 2011). Similar alterations have also been seen related to protein degradation mechanisms where mutations have altered synapse function along with behavioral abnormalities. Corrections of mutations early have ameliorated ASD phenotypic outcomes while having smaller effects in adults, reinforcing time-sensitive mechanisms during development (Tsai et al. 2018).

Though the ASD phenotype is characterized by behavioral and neuronal abnormalities most likely from gene mutations during early developmental stages, multiple genes in a variety of pathways can result in the same phenotype. One of the most recent disease genes related to ASD and epilepsy is *DEPDC5*, a regulator in the mTOR pathway.

2.4 MTOR pathway

DEPDC5 has been recently discovered as a disease gene in ASD and epilepsy. It functions as a modulator of the amino acid sensing part of the mTOR pathway which functions in cell growth and mechanisms related to maintaining normal cellular function and homeostasis (Figure 1). The mTOR pathway was first experimentally characterized in 1994 when biochemical studies in mammals identified its function through the drug rapamycin which inhibited cell growth and proliferation signal transduction pathways (Chung et al. 1992, Sabatini et al. 1994, Brown et al.1994).

Similar to *DEPDC5*, rapamycin functions as a negative inhibitor of the mTOR pathway. Rapamycin has been extensively utilized in the laboratory setting as having a positive reversal of increased mTOR expression as seen in mTOR disease phenotypes related to animal and cellular models (Figure 1). It has also undergone several clinical trials targeting cancer, COVID-19, epilepsy, ASD and other psychiatric

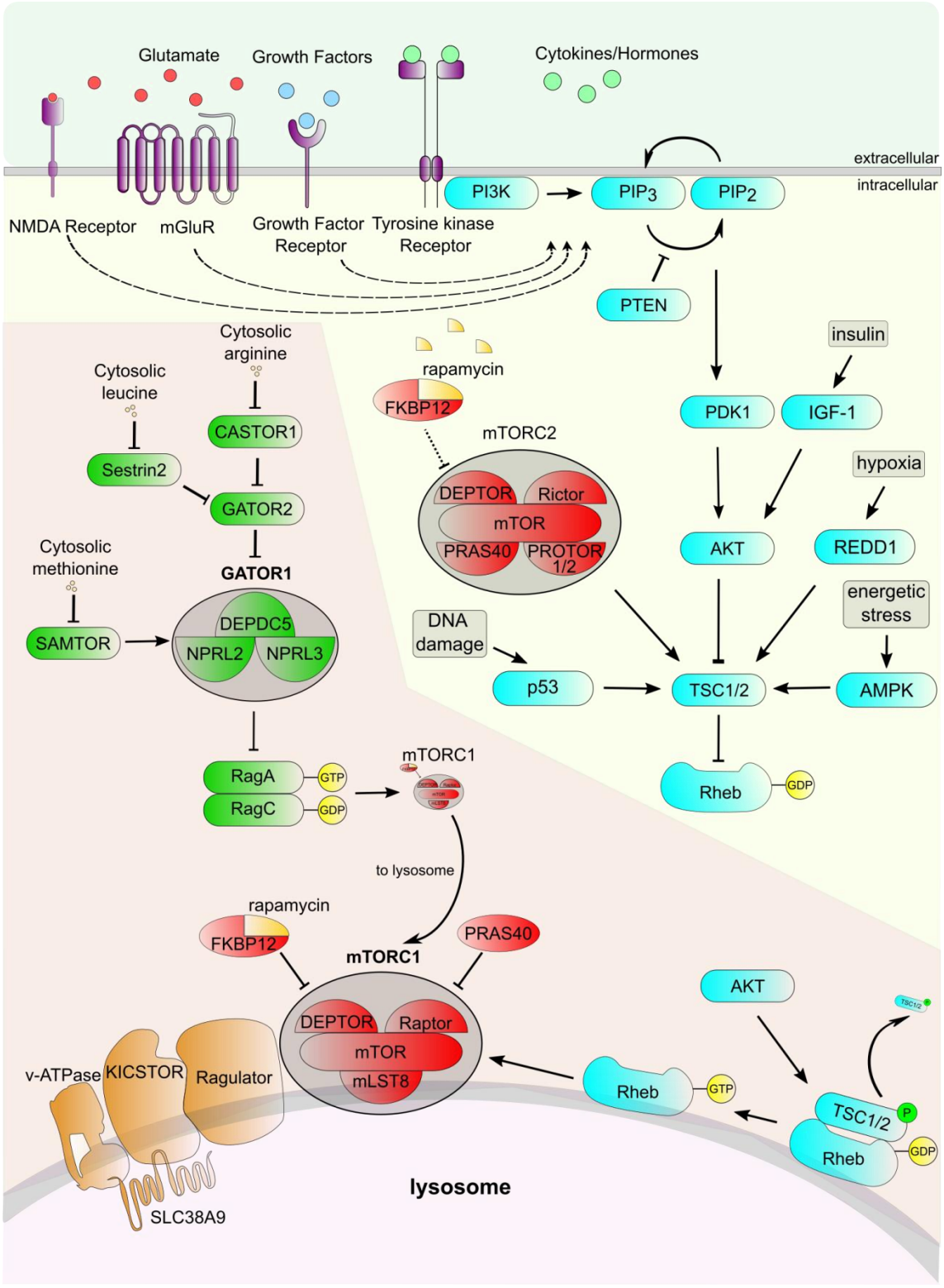


Figure 1 Schematic overview of the mTOR pathway.
mTORC1 receives signals upstream through a variety of extracellular signals with concurrent binding to receptors on the plasma membrane (purple). One checkpoint funnels into the activation of the PI3K/AKT pathway causing a phosphorylation cascade where AKT activates the TSC1/2 complex and causes disassociation from Rheb on the lysosomal surface. The other checkpoint occurs through the amino acid sensing pathway colliding on GATOR1 GTPase hydrolyzation of RhebA/C triggering mTOR relocation to the lysosome and subsequent activation. Both pathways allow binding of mTORC1 to the lysosome where it can become activated. (adapted and expanded from Lui and Sabatini 2017).

diseases as a possible therapeutic modality to mitigate or reverse diseases (Hu et al. 20021; Griffith and Wong 2019; Selvarani et al. 2021). Though still under investigation as a therapeutic drug, rapamycin has allowed the discovery of what is now termed the PI3K-AKT-mTOR pathway, an elaborate and highly regulated pathway responsible for cellular function. The related genes addressed in the following paragraphs can be visualized in Figure 1.

2.4.1 mTORC1

mTOR is a serine/threonine protein subunit that forms the much larger mTORC1 and mTORC2 protein complexes. mTORC1 is the central hub complex necessary for controlling and maintaining the homeostasis of nutrients and growth factors within the cell indirectly control metabolic homeostasis, lysosome biogenesis, lipid and nucleotide synthesis, glycolysis, mitochondrial biogenesis, protein synthesis, autophagy and proliferation (Figure 2; Hara et al. 2002; Kim et al. 2002, 2003). mTORC1 is a protein containing the subunits mTOR, Regulatory Protein Associated with mTOR (RAPTOR), FKBP Prolyl isomerase 1A (FKBP12), mTOR associated protein, LST8 homolog (mLST8), DEP domain containing mTOR interacting protein (DEPTOR), proline-rich AKT substrate of 40kDa (PRAS40) which function as a kinase (see Figure 1).

2.4.1.1 Downstream of mTORC1

Functional mTORC1 undergoes dimerization between two mTORC1 complexes where the RAPTOR and mTOR subunits contact one another and allow the mTOR kinase domain to be exposed to substrate phosphorylation (Aylett et al. 2016, Baretic et al. 2016). Interestingly, studies have shown that the mTOR kinase catalytic cleft narrows upon rapamycin binding to the mLST8 subunit thus inhibiting mTOR protein phosphorylation (Feldman et al. 2009; Kang et al. 2013; Thoreen et al. 2009).

mTORC1 activation causes substrate phosphorylation of the p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP) complexes which are involved in the activation of protein synthesis (Figure 2). Both effectors are involved in 5’ cap-dependent mRNA translation through the eIF4F complex necessary for the protection, export and translation of mRNA transcripts (Gingras et al. 1999; Holz et al. 2005). However, activation of S6K1 phosphorylates other downstream substrates necessary for ribosome biogenesis (Biever et al. 2015). Phosphorylation sites S235/236 and S240/244 on the S6K1 effector, ribosomal protein S6 (RPS6), are often used as indicators of protein synthesis and mTOR activation and were also analyzed in this study (Biever et al. 2015). S6K1 has also been shown to activate transcription factors

necessary for pyrimidine synthesis, fatty acid/cholesterol biosynthesis and p53 related mechanisms thus relating mTOR function to other mechanisms within cell function (Lui et al.2020).

mTORC1 also plays the role of activating the mitochondrial tetrahydrofolate cycle necessary for purine synthesis through activation of activating transcription factor 4 (ATF4; Ben-Sahra et al. 2016; Robitaille et al. 2013). This can occur simultaneously with activations of other transcription factors such as Hypoxia-inducible factor 1-alpha (HIF1α) or sterol responsive element binding protein (SREBP) forcing a switch from oxidative phosphorylation to glycolysis and causing NADPH production or lipid synthesis respectively (Duvel et al. 2010; Porstmann et al. 2008). mTORC1 can also suppress autophagy and lysosome biogenesis through phosphorylation of the downstream effectors unc-51 like autophagy activating kinase 1 (ULK1) and transcription factor EB (TFEB; Kim et al. 2011, Settembre et al. 2012). Thus, mTORC1 is involved in the activation of multiple downstream effectors necessary for maintaining cell growth and repressing autophagy and degradation mechanisms. Imbalances of these pathways have also been implicated in epilepsy and ASD suggesting changes in mTOR function could imbalance some or all of these pathways (Lamming and Sabatini 2013, Valvezan et al. 2017, Ajith et al. 2021, Tamiji and Crawford 2010, Zhou et al. 2013, Zhao et al. 2015)

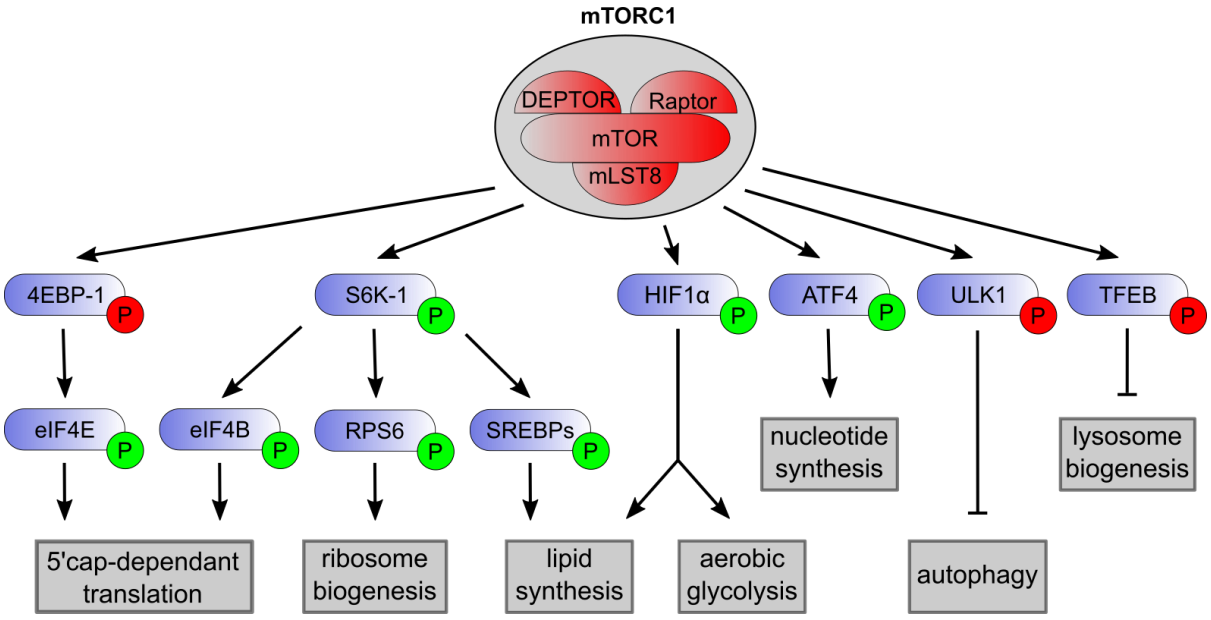


Figure 2 mTORC1 causes the activation of multiple downstream pathways
mTORc1 functions as a kinase leading to phosphorylation events which cause activation (green) or repression (red) of signaling cascades necessary for cell growth and homeostasis within cells. (adapted and expanded from Lui and Sabatini 2020)

2.4.1.2 Upstream of mTORC1

Two separate mechanisms which converge on mTORC1 are the “amino acid sensing” branch and the PI3K/AKT/mTOR branch. DEPDC5 is a subunit of the GATOR1 complex and resides in the “amino acid sensing” branch while other mentioned mTOR modulators, such as TSC1/2, reside in the PI3K/AKT/mTOR branch. Mutations in either pathway lead to epilepsy and ASD phenotypes due to mTOR deregulation. Thus, both are key to understanding mTOR function (Figure 1).

2.4.1.2.1 Amino acid sensing branch of the mTOR Pathway

Coordination of amino acid levels are controlled through amino acid transporters residing at the membrane of organelles and the plasma membrane. Alterations in the quantities of such transporters, such as the amino acid and non-amino acid substrate transporter, SLC7A5, can alter the functionality of mTOR pathway and have even been linked to ASD phenotypes (Tarlungeanu et al. 2016). Similarly, altered branched chain amino acid catabolism can change available cytosolic amino acid concentrations and has also resulted in neurological abnormalities (Tarlungeanu et al. 2016). Therefore, multiple metabolic processes regulate mTORC1 through the levels of intracellular amino acids and disruptions can result in epilepsy or ASD.

Amino acids can also be gated behind sensors such as SESN2 and CASTOR1 which detect cytosolic levels of leucine and arginine respectively (For gene names see List of genes/proteins). When present, SESN2 and CASTOR1 can operate as negative regulators of the GATOR2 complex which functions to mainly inhibit the GATOR1 complex (DEPDC5, NPRL2, NPRL3) and allow the functionality of mTOR signaling (Chantranupong et al. 2014, 2016). GATOR1 can also be inhibited by SAMTOR, a detector of the methionine metabolite S-adenosylmethionine (Gu et al. 2017). Therefore intracellular amino acid sensors can inhibit GATOR1 activity, where inadequate supply causes downstream inactivation of mTOR.

GATOR1 is composed of the subunits DEPDC5, NPRL2 and NPRL3 where DEPDC5 mainly acts as a scaffolding protein for NPRL2 and NPRL3 to bind to RagA thus allowing NPRL2 catalyzation of RagA GTP hydrolysis (Bar-Peled et al. 2013; Shen et al. 2017). More recently, the identification of FK506 binding protein 39 (*fkb39*) has been shown to regulate *nprl3* activity in Drosophila, indicating another mechanism for which GATOR1 can be regulated (Zhou et al. 2021). Yet, despite the central role *NPRL2* (6%) and *NPRL3* (11%) plays in providing function to GATOR1, predominant genetic data has identified *DEPDC5* (83%) as more commonly mutated in disease related phenotypes (Baldassari et al. 2019).

Due to the discoveries of the past 20 years and the continued expansion of information on the regulatory networks and their interconnectedness, the discoveries of the amino acid sensing of the mTOR pathway are some of the most detailed to date (Figure 1). Directly upstream of mTORC1 in the amino acid branch lies the small GTPase protein Rheb which can directly bind and activate mTORC1 located at the lysosomal membrane (Long et al. 2005). Though normally cytosolic, this recruitment of mTORC1 to the lysosome is facilitated by the Rag GTPases which directly bind to the Ragulator complex composed of 5 LAMTOR subunits (Figure 1; Sancak et al. 2010, Bar-Peled et al. 2012 For gene names see List of genes/proteins). Two of these subunits, LAMTOR 2/3, couple with the Rag GTPases and function as a guanine-exchange factor (GEF) for RagA/B. mTORC1 can also be regulated in the lysosomal membrane by v-ATPases (vacuolar H+-ATPase) and SLC38A9. V-ATPases are able to sense both intra-lysosomal and cytosolic amino acid concentrations and control mTORC1 activity while SLC38A9 can detect lysosomal arginine concentrations and relay this information to modulate mTOR activity (Wang et al. 2015; Zoncu et al. 2011). However, cytosolic arginine and leucine concentrations are mainly controlled by separate regulatory mechanisms provided by the GATOR1 complex and its direct inhibition of mTOR.

Much like TSC2 in the growth factor pathway, GATOR1 acts as the main roadblock for the detection of sufficient cytosolic amino acid levels for mTOR activity. GATOR1 is tethered to the lysosomal surface through the KICSTOR complex where it acts as a GAP for the Rag A/B complex and prevents mTORC1 recruitment to the lysosome (Wolfson et al. 2017). Concordantly, the folliculin/folliculin-interacting protein 1 (FLCN-FNIP) complex functions as a GAP for RagC/D while residing on the lysosomal surface, which is also dependent on GATOR1 (Petit et al. 2013; Tsun et al. 2013).

Intrinsically GTP dissociates from GDP, although very slowly. These Rag GTPases both exist in an inactive GDP-bound form and an active GTP-bound state where this inactive state can be enhanced through increasing the rate of hydrolysis with highly g-protein specific GAPs. Working in opposition are the guanine exchange factor (GEF) proteins which bind to GTPases, sever the GDP molecules, protect them from unfolding and reactivate GEFs through GTP reattachment. By default, GTP is much higher than GDP in the cytosol at 10:1 indicating that GEF activity is essential in halting and signaling when nutrients are not available (Bos et al. 2007). These inactive GDP-bound forms can be cycled for activated G-proteins to quickly transfer phosphate groups and increase enzymatic activity. In the presence of amino acids, the Rag heterodimer RagA/C is in an active state where RagA is attached to GTP and RagC is bound to

GDP. This allows mTORC1 to bind to RagA in the cytosol, become recruited to the lysosomal membrane and become activated by Rheb. However, when amino acids are diminished, the GTP and GDP states change phosphorylation states on the RagA/C heterodimer binding, thus preventing the tethering to mTORC1 and subsequently inactivating mTOR activity.

2.4.1.2.2 PI3K/AKT/mTOR pathway

The other outlet leading to mTOR activation is the PI3K/AKT/mTOR branch. Extracellular signals bind to receptor tyrosine kinases (RTK), metabotropic glutamate receptors (mGluRs), or growth factor receptors (GFR) to activate PI3K and cause phospholipid signaling through PIP2 and PIP3. This signaling can be modulated through PTEN, a syndromic and epilepsy associated gene responsible for mTOR inhibition. Activation of other effectors (e.g. PDK1, mTORC2) eventually leads to the phosphorylation and activation of AKT. AKT activation can promote cell survival, proliferation and growth through other downstream processes along with operating as an indirect inhibitor of mTORC1 itself through TSC2. This process can also be inhibited through S6K1 activation which causes a negative feedback loop by repressing insulin receptor substrate 1 (IRS1), a positive upstream activator of PI3K/AKT (Hsu et al. 2011, Harrington et al. 2004). Therefore, mTOR activation usually corresponds with weakened AKT phosphorylation.

One of the most common and most studied inhibitors of the mTOR pathway is TSC which has been linked to tuberous sclerosis and is often comorbid with epilepsy or ASD (Figure 1). Composed of three subunits TSC1, TSC2 and TBC1D7, the TSC complex works as a GTPase activating protein (GAP) for the mTOR activating protein Rheb (Dibble et al. 2012, Inoki et al. 2003). Multiple growth factor pathways converge on the TSC complex including the insulin-related PI3K/AKT and the MAPK/ERK pathway which is triggered through growth factor receptor signaling. Thus, inhibition of mTORC1 occur through multiple pathways which converge on the TSC complex and activate its inhibitory function.

Energy insufficiencies can also cause mTOR inhibition through TSC2 and Raptor phosphorylation through 5'AMP-activated protein kinase (AMPK) regulation causing downstream regulation of cell growth and autophagy (Mihaylova and Shaw 2011). AMPK also plays partial roles as a regulatory mechanism of the DNA damage response pathway and hypoxia where feedback mechanisms cause AMPK to become activated and initiate autophagosome production. Lack of oxygen can also cause Regulated in DNA damage and development 1 (REDD1) to become activated and directly mitigates mTORC1 activity as well through TSC2. REDD1 has also demonstrated the modulation of mTORC1 in a suppressed state during

serum deprivation and thus preventing cell death (Dennis et al. 2013). Cellular stress causes upregulation in p53 DNA damage response pathway which in turn can also modulate mTORC1 activity by inhibition and subsequent regulation of downstream targets involved in autophagy through AMPK and TSC1/2 activation (Feng et al. 2005).

Often growth factor alterations have been attributed to neurological disease phenotypes leading to downstream signaling changes in neurons and implicating mTOR activity to such disease. Brain-derived neurotrophic factor (BDNF) can cause activation of mTOR and downstream protein production in isolated dendrites (Takei et al. 2004). Blocking of microtubules or actin filaments in neuronal growth cones blocks netrin-1 protein synthesis and both play a role in the mTORC1-eIF4E-BP1 translation initiation pathway for cue-induced axonal protein synthesis (Piper et al. 2015). The ASD associated extracellular matrix protein Reelin can also bind to RTK in the plasma membrane where successive activation of mTOR can cause changes in branching and growth of dendrites in hippocampal neurons (Jossin and Goffinet 2020). Research also shows that NMDA (N-methyl-D-aspartate) receptors can activate the mTOR-S6K pathway in shafts located in dendrites suggesting localization of fast dendritic translation and dendrite development (Cammalleri et al. 2003). Thus upstream regulators of mTORC1 can modulate its function and activate downstream processes leading to neuronal alterations causing epilepsy and ASD. Another upstream regulator is mTORC2.

2.4.1.3 mTORC2

mTORC2 is also composed of the mTOR subunit along with Rapamycin-insensitive companion of mTOR (Rictor), Protein observed with rictor-1/2 (Protor1/2) and mitogen-activated protein kinase 2 associated protein 1 (mSin1) to form a separate complex upstream of mTORC1. mTORC2 has an inhibitory function on the mTOR pathway and has also been implicated in cytoskeleton organization (Frias et al. 2006; Jacinto et al. 2004; Pearce et al. 2007; Peterson et al. 2009; Sarbassov et al. 2004). Less research is known about mTORC2 however recent studies have uncovered some of its mechanisms.

While mTORC1 has rather broad implications in terms of homeostasis and regulation within cells, mTORC2 specifically focuses on growth and metabolic mechanisms. mTORC2-specific phosphorylation of the AGC protein kinases PKA, PKG and PKC were shown to activate cytoskeletal remodeling and cell migration while another AGC protein kinase, SGK1, could be activated to regulate ion transport and cell survival (Gan et al. 2012; Jacinto et al. 2004; Sarbassov et al.2004; Thommanetz et al. 2013). It also has

the function of activating the central effector of the insulin/PI3K signaling pathway, AKT (Figure 1). Endoplasmic reticulum (ER) stress can also cause GSK-3β to phosphorylate mTORC2 through Rictor and prevent AKT binding (Chen et al. 2011). Thus, though mTORC2 can indirectly regulate mTORC1 through AKT phosphorylation and TSC2 inhibition, mTORC1 can also regulate mTORC2 through S6K1 inhibition of AKT activation showing the complexity and the many feedback mechanisms for mTOR pathway regulation.

A key aspect of mTORC2 is that the effects of rapamycin treatment are severely diminished when acutely administered but become more effective with long term treatment (Jacinto et al. 2004). This is thought to be because mTORC2 complexes are unable to be created due to the constant rapamycin-FKBP12-mTOR inhibitory complexes and thus cause an effect due to inadequate supply of mTOR. Therefore rapamycin can possibly regulate mTORC1 and mTORC2 both resulting in inhibition.

2.4.1.4 GATOR Diseases

GATOR1 mutations have been repetitively identified as being mutated in patients with a variety of epileptic phenotypes (Baldassari et al. 2019). Though sometimes *de novo*, often they are inherited. Linkage studies identified the region of chromosome 22q12 as the location where the epilepsy disease phenotype likely stems from and ultimately where *DEPDC5* is genetically encoded (Berkovic et al. 2004, Klein et al. 2012, Morales-Corraliza et al. 2010). Follow-up studies later confirmed mutation in *DEPDC5*, but also *NPRL3* and *NPRL2* mutations, as being predominantly associated with focal epilepsies connected to MCD (Baulac et al. 2015; Dibbens et al. 2013; Ishida et al. 2013;Jansen et al. 2015).

The largest genetic study of GATOR1 mutations identified 183 unrelated individuals with 140 variants of which 85% were in *DEPDC5*, 11% in *NPRL3* and 6% in *NPRL2* (Baldessari et al. 2019). Of these disease-associated genes, 67% were predicted to have loss-of-function and 27% were missense variants. Penetrance of *DEPDC5* mutations is estimated to be at 66% from investigations with seven families with FFEVF although some individuals were not affected by the disease despite harboring the mutation (Dibbens et al. 2013). Yet individuals with afflictions do have variable age of onset, epilepsy severity or location and often have comorbid conditions such as ASD, ID or other psychiatric disorders (Baldassari et al. 2019; Dibbens et al. 2013; Ricos et al. 2016; Scheffer et al. 2014). *NPRL2* and *NPRL3* were also identified as genetic components responsible for a variety of focal epilepsies (Korenke et al. 2016; Ricos

et al. 2016). This confirms that mutations in all the subunits of the GATOR1 complex can lead to neurological disease.

In few cases, ASD has manifested due to *DEPDC5* mutations without epilepsy (Dibbens et al. 2013; Burger et al. 2017). In an Australian cohort, a mutation in tyrosine 7 (c.21C>G) in *DEPDC5* exon 2 resulted in family members with epilepsy, ASD or both (Dibbens et al. 2013). This particular mutation is interesting as it is multifaceted and results in variable phenotypes related to *DEPDC5* loss. Exon 2 is also present in almost all isoforms indicating that it is an essential exon for *DEPDC5* function (ensembl.org).

Though epilepsy is often the main phenotype observed amongst patients with GATOR1 mutations, neuroarchitectural abnormalities are common as well. GATOR1 mutants often result in focal cortical dysplasias (FCD) which are sub-diagnosed as FCD1, focal lesions with dyslamination of the neocortex, or FCD2 composed of cortical dyslamination and dysmorphic neurons and/or balloon cells (Najm et al. 2018). In a study of 80 patients with epilepsy and FCD, mTOR pathway related variants were identified solely in FCD2 cases with characteristic balloon cell morphology in p-S6 positive cells (Baldassari et al. 2019). Such phenotypes are also fairly common with *TSC1/2* patients which result in mTOR hyperactivity similar to GATOR1 mutations. Therefore, mTOR hyperactivity often results in an epileptic phenotype but is comorbid with a variety of neuropsychological and neuroarchitectural abnormalities.

2.4.2 Functional Studies of mTOR pathway

2.4.2.1 mTOR Animal models

Because mTOR signaling is such a key component in monitoring the levels of oxygen, glucose, amino acids, energy and insulin within the cell, deregulation of upstream partners of mTOR or changes in mTOR functionality can cause detrimental process changes within the cell's behavior. Multiple experimental studies have followed up the possible mechanisms for disease with animal and cell models to help explain the effects implicated by mTOR mutations. Pathological variants of mTOR pathway related genes that are involved with MCDs and epilepsies have been identified in *PI3K, PTEN, AKT3, TSC1, TSC2, RHEB, mTOR, STRADA, DEPDC5, NPRL2, NPRL3, KPTN, SZT2, and TBC1D7* (Kim and Lee 2019, Nguyen and Bordey 2021; For gene names see List of genes/proteins). Developmental time points of somatic mutation occurrence within these genes elucidate the severity of affected brain regions and cortical malformations, where mutations later in development result in more focal localization within the brain while earlier somatic mutations are broad (D'Gama et al. 2017).

At E14-E15 in mice, cortical pyramidal neurons are generated from radial glia in the ventricular zone and propagate to the L2/3 zones (Kast and Levitt 2019; For detailed description see 2.5.1). Using in-utero electroporation at E14-15, KO mutations in *TSC2, NPRL3* or *DEPDC5* result in neuronal migration defects in the somatosensory cortex and medial prefrontal cortex in animal studies (Iffland et al. 2020; Ribierre et al. 2018; Tsai et al. 2014). However, no migration effects could be seen after this developmental time window suggesting key time points of disease generation during gestation (Onori et al. 2020). In a mouse mTORC1 subunit conditional KO, $Raptor^{fl/fl;nestin\text{-}Cre}$, progeny died shortly after birth and exhibited microcephaly and decreased neurogenesis (Cloetta et al. 2013). Similarly, another hyperactive *Raptor* conditional KO under the Emx1-promotor also had decreased brain size due to cortical atrophy related to progenitor death in the subventricular zone (SVZ; Kassai et al. 2014). In addition, another mouse model with a mutated mTORC2 subunit *Rictor* cause decreases in Purkinje neurons soma sizes, increases in dendrites, and decreases in dendritic length and volume (Thomanetz et al. 2013). Hyperactivated mTOR signaling has often caused an enlargement of neuronal soma size amongst animal models which could further be reduced with treatment with rapamycin in both pre- and post-natal animals (Dawson et al. 2020; Park et al. 2018; Zhang et al. 2019; Zhong et al. 2021). KOs of *TSC1* in DA neurons also show increased dendritic branching, increased soma sizes, increased dendritic length and reduced intrinsic excitability (Kosillo et al. 2019). Studies with *PI3K*, *PTEN*, *RHEB* and *DEPDC5* also showed increased dendritic thickness and complexity (Chen et al. 2015; Dawson et al. 2020; Lin et al. 2016; Ribierre et al. 2018; Zhong et al. 2021).

The mislocalizations of neurons are suggested to be due to the 4E-BP1 pathway where experiments constitutively activated 4E-BP1 repression of eIF4F and simultaneously hyperactivated mTOR via a *RHEB-S16H* mutant causing proper neuronal locational transitions (Lin et al. 2016). Neuronal cell size enlargement was also reversed in a hyperactive mTOR mutant L2427P with the 4E-BP1 pathway through eIF4E knockdown (Kim et al. 2019). The S6K1/2 pathway was also shown to play a role in reversing enlarged neuronal soma size when knocked down in a hyperactive mTOR variant but not enough to cause abnormal neuron localization or soma growth when expressed as wildtype (WT; Kassai et al. 2014; Pelorosso et al. 2019). S6K1/2 KOs also showed a decrease in body size, cell size and higher rates of protein synthesis (Ruvinsky et al. 2005, Koehl et al. 2021). Enhancements of 4E-BP1 or reducing S6K1/2 activity prevented axon overgrowth suggesting that both pathways are involved in abnormal axonal function (Gong et al. 2015). Therefore, the 4E-BP1 pathway seems to cause the main morphological and

transitional pathway for the neuronal defects while both pathways are responsible for the axonal and dendritic defects (Choi et al. 2008; Morita and Sobue 2009; Li et al. 2008).

2.4.2.2 DEPDC5 animal models

To date there have been a total of 8 different models of *DEPDC5* KOs in zebrafish, rat and mouse models all with variable results (see Table 1). *DEPDC5* knockdown in a zebrafish model displayed hyperkinesia, circular swimming and increased neuronal activity while also exhibiting reduced motor activity, increased seizure susceptibility and premature death after 2-3 weeks post-fertilization (de Calbiac et al. 2018). In a rat model, loss of DEPDC5 results in S6 phosphorylation indicating hyperactivation of mTOR leading to embryonic death around E14.5 (Marsan et al.2016).

Multiple mouse models have also been developed to study *DEPDC5*-KOs in animals. A conditional knock-out using $DEPDC5^{fl/fl;Syn1\text{-}Cre}$ strain displayed brain enlargement and dysmorphism of neurons accompanied by increased seizure susceptibility (Yuskatis et al. 2018). Another *DEPDC5*-KO model utilizing Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR associated protein 9 (CRISPR-Cas9) resulted in pups with reduced body size, altered cranial structure and abnormal cardiovascular development (Hughes et al. 2017). Such cardiac abnormalities were also seen with an *NPRL3*-KO mouse model (Kowalczyk et al. 2012).

In all models, complete KO of *DEPDC5* resulted in mTOR hyperactivity and premature death which was attenuated through the use of rapamycin. Spontaneous seizures and cortical structural abnormalities were only present in double-KO mice and were absent in heterozygous *DEPDC5* models. To circumvent this problem, a mouse and rat model were developed using in utero electroporation using CRISPR-Cas9 technology. At E13, electroporated rats developed enlarged soma, abnormal EEG recordings and spontaneous seizures (Hu et al. 2018). Electroporated mouse models at E14.5 developed similar features along with migration abnormalities and even sudden unexpected death in epilepsy (SUDEP) phenotype. Conditional KO models under the Emx-1 promoter in mice of *NPRL2*, *NPRL3* and *DEPDC5* produced enlarged neuronal cells and spontaneous seizures (Ishida et al. 2021). When rapamycin was administered, seizure occurrence decreased but neuronal cell size did not recover to normality. Thus, loss of DEPDC5 results in premature death, alterations in organ development, altered cell sizes and increased seizure susceptibility in mice.

Investigations into the cause of *DEPDC5* mutations relating to disease have suggested inhibitory/excitatory imbalances generated during development. RNA interference of *DEPDC5* found increased excitatory synaptic transmission and increased intrinsic excitability in pyramidal neurons extracted from mice indicating excitability/inhibitory imbalances in the brain (De Fusco et al. 2020). This was also demonstrated through another *DEPDC5* KO model in zebrafish where GABAergic neurons showed a decrease in neurofilaments and punctae on dendrites suggesting the main imbalance arising from the developing GABAergic network (Swaninathan et al. 2018). This suggests that during brain maturation, *DEPDC5* mutants cause dysfunctional cellular networks leading to excitatory/inhibitory imbalances.

Table 1 Current research models. on DEPDC5

DEPDC5 Animal and Cell Models					
Publication	**DEPDC5 Model**	**Increased mTOR activity**	**Morphological changes**	**Electrophysiological changes**	**Behavioral Changes**
Marsan et al. 2016	Rat complete KO	Increased p-S6 in -/- but not in +/-	Dysmorphic neurons	Altered excitability	N/A
Hughes et al. 2017	Mouse complete KO	Increased p-S6 in -/- but not in +/-	Reduced body size, abnormal cranial structure, Normal neuron morphology in +/-	N/A	N/A
Yuskaitis et al. 2018	Mouse conditional KO (Syn1-Cre)	Increased p-S6 in -/-	Dysmorphic neurons, macrocephaly, Increased astrogliosis	Lower seizure threshold	Increased hind-limb clasping
Hu et al. 2018	CRISPR-KO in utero mouse	p-S6 increased	N/A	N/A	Spontaneous seizures
De Fusco et al. 2020	Mouse +/- KO + siRNA	p-S6 increased only with siRNA	Increased dendritic arborization	Increased excitability, more excitatory synapses	
Klofas et al. 2020	Mouse DEPDC5 conditional KO (Emx1-Cre)	p-S6 increased	Macrocephaly	Impaired GABAergic post-synaptic input	Spontaneous seizures
Ishida et al. 2021	Mouse DEPDC5 conditional KO (Emx1-Cre)	p-S6 increased	Decreased neuronal density, increased cortical thickness	N/A	Spontaneous seizures
De Calbaic et al. 2018	Zebrafish DEPDC5 knockdown	N/A	N/A	Increased neuronal activity	Circular swimming Coiling and twitching
Swaminathan et al. 2018	Zebrafish complete KO	p-S6 increased in +/- and -/-	Decreased body length	Increased neuronal activity Decrease in GABAergic signaling	Seizures Coiling Decreased swimming distance
Iffland et al. 2018	N2a mouse neuroblastoma cells and mouse NPCs	p-S6 increased	Increased filopodia	N/A	N/A
Iffland et al. 2020	N2a mouse neuroblastoma cells	p-S6 increased	increased cell size	N/A	N/A
Klofas et al. 2020	**iPSCs from patient DEPDC5 mutations**	**p-S6 increased**	**Dysmorphic NPCs and neurons**	**N/A**	**N/A**

Rat/mouse models (gray), zebrafish models (purple) and mouse as well as human *in-vitro* models (green). The only human model (Klofas et al. 2020) is highlighted in bold. +/+: homozygous, +/-: heterozygous, KO: knockout.

2.4.3 mTOR Cell Models

2.4.3.1 TSC2 and PTEN human cell models

Although substantial studies have been performed and summarized in the investigation of tuberous sclerosis regarding *TSC1/2* mutations and *PTEN* hamartoma syndrome, a summary of human cell models is addressed here as it is pertinent to this thesis (Blair et al. 2020; Vasic et al. 2021; Wong et al. 2020). Differentiation experiments of human induced pluripotent stem cell (hiPSC; see 2.5.4) models of *TSC1* or *TSC2* mutation carriers show a decrease in neurons when compared to their respective controls which could be related to such aspects as an increase in astroglia, delayed neuronal differentiation or increased neuronal death (Winden et al. 2019; Zucco et al. 2018). Transcriptome analysis also confirmed increased protein synthesis, metabolic activity, gliogenesis and inflammatory responses with reduced neuronal differentiation markers (Grabole et al. 2016). *TSC2* KO models also found abnormal axon extension and an increase in GABAergic signaling at inhibitory synapses suggesting neuronal morphological and signaling aberrations playing a role in the neuronal network (Alsaqati et al. 2020, Catlett et al. 2021). *TSC2* organoid models (see 2.5.5) developed from hiPSC TSC2 patients with drug-resistant epilepsy also show mTOR hyperactivity, increased proliferation and the development of cortical tubers (Eichmuller et al. 2020). Similarly, with *PTEN* mutations in hiPSCs, an increase in neural progenitors and delayed neuronal differentiation are recorded as the main phenotypes in two different models (Lee et al. 2016; Li et al 2017). Interestingly, when various *PTEN* mutations were analyzed, more severe *PTEN* mutations show a decreased protein half-life and an increase in polyubiquitination suggesting the mTOR downstream processes cause the more severe phenotypes (Wong et al. 2020). *PTEN* KO cerebral organoids generated from human embryonic stem cells (ESCs; see 2.5.5) developed with higher surface folding when compared with *PTEN* KO mouse organoids. This finding suggests clear species differences in development and addresses why animal models may not exhibit the full developmental or ASD/epilepsy phenotypes as seen in humans (Li et al. 2017).

2.4.3.2 DEPDC5 cell models

Currently there are very few cell models characterizing phenotypes generated from *DEPDC5* mutations and only one study so far utilizing human cells (see Table 1). A study analyzing knockdown effects of *DEPDC5* and *NPRL3* using mouse neural progenitor cells and Neuro2a cells derived from human neuroblastoma, showed that knockdown prevented mTOR colocalization to the lysosome under amino acid starved conditions (Iffland et al. 2018). The cells also exhibited increases in cell size along with

neural process outgrowth in filopodia pointing towards morphological changes associated with decreased *DEPDC5* and *NPRL3* expression. More recently a human cell model using iPSCs from patients with *DEPDC5* mutations has been published (Klofas et al. 2020). Here, the authors identified increased mTOR hyperactivity through p-S6 phosphorylation, increased soma sizes and increased rate of cell proliferation when compared with non-isogenic controls. Such alterations could also be mitigated with rapamycin treatment.

Most *DEPDC5* models have characterized later stage developmental abnormalities and have not addressed what drives these defects. Only one transcriptome analysis has been performed in adult whole zebrafish brains and only one human cell model has been generated (Klofas et al. 2020; Swaminathan et al. 2018). To address these issues, we investigated human cell models which could fulfill the necessary criteria to understand what drives *DEPDC5* loss and cause abnormality in early development and how this could contribute to disease phenotypes in humans.

2.5 Human Cell Models

2.5.1 Early Brain Development

Neural progenitor cells (NPCs) are a subset of cells, which have already become fate restricted and are no longer pluripotent. They can only become neural specific cell types and thus have proceeded farther down a developmental path unlike ESC or iPSCs. The earliest stage neural stem cell is a neuroepithelial cell.

Neuroepithelial cells (NEs) reside in the ventricular zone (VZ) of the developing brain where they proliferate and undergo symmetrical division. These cells can then differentiate into radial glial (RG) cells, expressing astrocyte markers such as GFAP, GLAST and TNC. These neural progenitor cells can then be distributed into apical progenitors expressing PAX6 in the VZ or extend slightly outward into basal progenitors expressing PAX6 and TBR2 in the SVZ. Apical radial glial (RG) cells differ in that they can undergo symmetric or asymmetric division. They can produce a progenitor cells, differentiated intermediate progenitors (IP) or neurons which migrate to the basal lamina of the developing cortex (Noctor et al. 2004). IPs are committed to neuronal differentiation although they can go through several rounds of symmetric division before doing so. Similarly, RGs do retain the ability to differentiate into glial cells including astrocytes although this usually occurs in the later stages of embryonic neurogenesis

(Malatesta et al. 2000, 2003). Adult neurogenesis takes place in the SVZ or the subgranular zone of the hippocampal dentate gyrus (Galiakberova and Dashinimaev et al. 2020).

Neurogenesis in humans starts at gestational week (GW) 5 and concludes around GW20 (Bzstron et al. 2008). Mice, on the other hand, have a much shorter neuronal development time window between embryonic days (E) 11 to E19 (Caviness 1982). Neurons existing in the cerebral cortex are derived from neural progenitors and are usually classified as spiny excitatory neurons or smooth inhibitory neurons (Costa and Müller 2015). Excitatory cortical neurons are generated in the VZ and extend outward into the cortical regions of the brain where inner regions are first established and outer regions form over time (Ohtaka-Maruyama and Okado et al. 2015). TBR2 positive neural progenitor cells residing in the SVZ can contribute neurons to all layers of the cortex (Vasisstha et al. 2014). Emx1 positive progenitors are specific to the dorsal telencephalon and can only differentiate into glutamatergic neurons in cortical layers, except for GABAergic cortical neurons (Gorski et al. 2002). From E12 in mice, Cux2 is also expressed in subsets of progenitor cells in the VZ and SVZ and later are expressed in layer II-IV (Nieto et al. 2004; Franco et al. 2012). The generation of upper layer neurons produces a feedback signal from the post-mitotic neurons to progenitors through NT-3 signaling (Parthasarathy et al. 2014). Many post-mitotic neocortical neurons express NT-3 as well (Seuntjens et al. 2009). Thus, neural progenitors originate in the SVZ and through asymmetric or symmetric division differentiate into intermediate and neuronal cells extending into the developing cortex. Alterations in such mechanisms lead to neurological disease.

2.5.2 Modeling neurodevelopmental disease

Understanding human development is key to understanding the underlying mechanisms resulting in neurological disease in humans. Significant progress has been made through the use of animal models in mimicking disease phenotypes observed in humans. However, differences during development or genetics between species may shroud underlying differences. Therefore, modeling human development provides the best way to understand the genetic effects related to a human disease.

In the brain, neural progenitor cells have always been of pertinent interest when trying to link the complex relationships resulting in developmental diseases. For example, of those affected with psychiatric disorders, it has been estimated that 75% appear between childhood and early adulthood (mid-20s) while the brain is undergoing significant remodeling (Kessler et al. 2005). ASD disease genes are often expressed during early development which suggests that this developmental time window is key to

developing the disease (Meredith et al. 2015; Parikshak et al. 2013). Studies also indicate significant structural changes within the cortex relating to neuropsychiatric disorders (Opel et al. 2020). For example, autistic patients have shown increases in cortical surface area in infancy (van Erp et al. 2018, Hazlett et al. 2017). Thus, modeling how the brain develops in humans is imperative to understanding the effects of mutations and their overall treatment.

Currently, human cell models provide an excellent tool for understanding diseases in humans. The main models available are embryonic stem cells, induced pluripotent stem cells, organoid models and primary human neural progenitors.

2.5.3 Embryonic Stem Cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of preimplantation embryos (Zakrewski et al. 2019). Through differentiation they can any other cell of the body which is triggered by induction of specific growth factors and thus can help understand normal developmental processes. However, ethical concerns about using somatic cells from donors can be difficult in some countries. In addition, multiple cell lines have different genomes, which may have disease mutations requiring sequencing and checking of chromosomal abnormalities. For disease modeling, the cells still need to undergo genetic manipulation to cause gene disruption and subsequent generation of disease phenotypes. An alternative to these limitations are using the recent advances in iPSC technology.

2.5.4 Induced Pluripotent Cells

Human induced pluripotent stem cells (hiPSCs) are reprogrammed somatic cells which have the potential to differentiate into all the germ layers and subsequent derived cell types similar to ESCs. These cells carry the same DNA as the individuals they are extracted from and allow researchers to characterize disease phenotypes associated with mutational abnormalities *in vitro*. They can also be manipulated as well through genetic editing. iPSCs mostly are generated from PBMCs extracted from whole blood samples or from fibroblasts received via skin punches. Patient specific cells are cultured and subsequently treated with a combination of transcription factors (OCT4, SOX2, KLF4, MYC) to reprogram the cells to iPSCs (Takahashi et al. 2007). From then on, the cells can be differentiated into other cells of the body using similar differentiation techniques as ESCs.

Some limitations are that often the reprogramming uses retroviruses which integrate randomly in the genome and can express genes related to cancer. Reprogramming of certain somatic cells can also have less than 0.02% rate of becoming stem cells with the majority being only partially reprogrammed and pluripotent. There tends to be variability between cell lines based upon human genetic variations resulting in differences in gene expression and epigenetic mechanisms (Val Cervo et al. 2021). ESCs and iPSCs are often grown in 2D culture but can also model a 3D architecture with the advancement of organoid models.

2.5.5 Organoids

Organoids are the generation of fate restricted cells composing a 3-dimensional network similar to normal organ structures of the body. Often, they are generated through hiPSCs or ESCs. Through propagation of differentiation and growth factors, the cells collectively generate an organ-like network composed of multiple cell types mimicking normal developmental processes and allow the study of normal or abnormal development within an organ-like structure. Although they can be generated through other animals, often they are used to understand human development and altered structures which may arise through mutational loss of genes of interest. Cerebral organoids allow the development of brain-like structures with heterogeneous cell populations mimicking brain development. However, they also have heterogeneity between differentiations and are limited in their level of maturity and function often mimicking earlier development (Hofer and Lutolf et al. 2021). As they do not have efficient nutrient supply throughout the organ (i.e. vascularization does not occur), many cells also die due to lack of nutrients reaching the center cells. Therefore, they are limited in size and complexity.

2.5.6 Primary Human Neural Progenitor Cells

Cells extracted from postmortem brains provide an unbiased depiction of developmental gene expression and regional characteristics within human gestational periods. Several depositories have allowed the extensive study of a variety of cells and tissues extracted from post-mortem patients. In the brain, transcriptomic data has been collected from neurons and astrocytes along with whole intact brains to provide contextual evidence on gene expression during development (Hawrylycz 2012, Kang et al. 2011, Lewis et al. 2010). The consistency of data remains robust and specific to the donor without sample preparation or differences between laboratories meaning that compounding data will allow a more accurate representation of human development (Danielsson et al. 2015). However, such methods do have

disadvantages. Early developmental tissues can conflict with ethical guidelines established by countries or their states resulting in non-uniformity among the scientific community similar to ESCs (Matthews and Morali 2020). There is also a concern of utilizing the same biological material multiple times from the same donor over multiple time points which may impact the integrity of the specimen.

Regardless, studies have shown that post-mortem tissue obtained from early developmental stages is more characteristically similar to *in vivo* development in comparison to differentiated iPSCs or ESCs (Stein et al. 2014). Extracted primary human neural progenitor cells (phNPCs) were shown to have expression patterns similar to *in vivo* gene expression and network architecture when differentiated into neurons along with having similar developmental timescales (Konopka et al. 2009, 2012; Stein et al. 2014). Transcripts were also shown to express differently than other *in vitro* models including human iPSCs. Experiments also found that phNPCs were able to successfully differentiate and exhibit electrophysiological characteristics similar to neurons of the brain (Piper et al. 2000).

Although many previous studies utilized extracted neural progenitors by growing them in 3-dimensional neurospheres, risks of the lack of nutrients reaching the center progenitor cells or induced cellular trauma from harsh passage conditions were predicted to cause spontaneous differentiation and disruption of tight junction formation (Svendsen et al. 1998). Therefore, growing the cells in a 2D format has provided secure regulation over confluency and cell passaging and can be executed with minimal exposure to surface detachment enzymes. phNPCs are often extracted between GW 7-21 by taking different sections of the SVZ or cortex of the developing fetus to isolate specific cell types to understand development. Such extractions have been shown to contain many NPC and IPC populations in both humans and rodents with proliferative capacity (Svendsen et al. 1998, Weiss et al. 1996; Palmer et al. 1997). Thus, because of their similarity to human brain development and their similarity in gene expression to developing embryos, primary human neural progenitor cells were selected as a model for understanding the mechanisms related to neurodevelopmental disorders caused by loss of DEPDC5.

2.6 Summary and aims

Neurodevelopmental disorders and epilepsy, although resulting in clearly specific and distinguished phenotypes, are proving to be more and more genetically similar elucidating the overlap in comorbidities. Reinforcement of such data has been widely recognized due to large genetic studies targeting epilepsy, autism and schizophrenia (Satterstrom et al. 2019; ILAE Consortium, 2018). *DEPDC5* mutations have been identified from studies of focal epilepsies and often patients have comorbidities with ASD, IDs and focal cortical dysplasia (Baldassari et al. 2018). Such phenotypes appear relatively early in life pointing toward epileptogenesis early in childhood. Similarly, ASD often develops early in age. *DEPDC5* has been identified as a key regulator of the mTOR pathway, which in turn plays an important role in controlling and maintaining the homeostasis of nutrients and growth factors within the cell. Through downstream mechanisms, mTOR can indirectly control metabolic homeostasis, lysosome biogenesis, lipid and nucleotide synthesis, glycolysis, mitochondrial biogenesis, protein synthesis, autophagy and proliferation (Hara et al. 2002; Kim et al. 2002, 2003). *DEPDC5* functions as an inhibitory complex necessary for the sensing of amino acids and directly blocks mTOR if amino acid levels are insufficient. Thus, mutations in *DEPDC5* avert mTOR inhibition resulting in a hyperactive phenotype characteristic of cell size alteration, proliferation, dendritic arborization and synaptic defects in neurons (Yuskatis et al. 2018).

Although the effects of *DEPDC5* mutations in animal models have been widely characterized, only one study has investigated *DEPDC5* in human cells using a non-isogenic model (Klofas et al. 2020). Therefore, validation of the phenotypes characterized in human cells has yet to be performed, as there could be differences due to iPSC reprogramming and the genetic background of the samples. Homozygous knockouts in animal models have mainly resulted in early pre- and post-natal death (Marsan et al. 2016; Hughes et al. 2017). However, the reasons are unclear and need to uncover through gene expression data. Lastly, transcriptomics has only been investigated in a zebrafish model and not in a human model. Differences in expression of genes have been hypothesized to be key in normal development in humans and this can be dramatically different between species (Varki et al. 2008; Zhao and Bhattacharyya 2018). Therefore, we set out to confirm and investigate the effects of DEPDC5 in a primary human cell model during two early stages of development adapting an isogenic study design with the aim to characterize the gene expression signatures and morphological differences to further understand the impact *DEPDC5* mutations on normal development.

The aims of the study were to:

1. Generate an isogenic *DEPDC5*-KO model in primary human neural progenitor cells including the confirmation at DNA, mRNA and protein level
2. Confirm mTOR hyperactivity in *DEPDC5*-KO progenitor cells and analyze changes in mTOR downstream processes at protein level
3. Characterize differences of progenitors and differentiated cultures at transcriptomic and morphological levels comparing knockout cell lines to isogenic controls
4. Investigate if any differences can be attenuated with the mTOR inhibitor rapamycin

With respect to the transcriptomic analyses comparing *DEPDC5*-KO progenitors and neurons to respective controls with and without rapamycin administration, the following sub-aims were defined:

a) Identify the effects of *DEPDC5*-KO
b) Delineate the rescue-capacity of rapamycin
c) Describe the systems-wide gene interaction network of *DEPDC5*-KO
d) Compare affected genes with disease-specific genetic datasets to observe similarities in gene expression
e) Investigate the role of the key *DEPDC5*-KO genes during neural development of the brain using a spatial-temporal brain dataset (Kang et al. 2011)

3. Material and Methods

3.1 Cell culture

3.1.1 Growth conditions of D62 neural progenitors

We utilized extracted phNPCs from the developing neocortex at GW 14 from previously established biobanks and protocols (La Torre-Ubieta et al. 2018; Stein et al. 2014; Rosen et al. 2011; Wexler et al. 2011; Svendsen et al. 1998). The cells were kindly provided by the Geschwind lab (UCLA, Los Angeles USA), and experiments were positively reviewed by the IRB ethics board at the UCLA.

The cells were isolated and grown as neurospheres and thereafter transferred to cell culture plates where they were grown as a 2D culture (Stein et al. 2014). Cell culture plates were coated with 5µg/mL poly-L-ornithine (PO; 0.01% solution; Sigma) and incubated for 1-2 hours at 37°C. The solution was aspirated and plates re-coated with 5 µg/mL fibronectin (F; Sigma) over night (ON) at 37°C. Fibronectin solution was aspirated and plates dried under a sterile cell culture hood for 3 hours. Dried PO-F plates were wrapped in parafilm (Bemis Company Inc.) and stored at 4°C for no more than 1 month.

hNPCs were expanded on PO-F coated cell culture plates and maintained in proliferation media consisting of Neurobasal A w/o Glutamine (Gibco) supplemented with 1X Antimycotic-Antibiotic (Sigma), 1X Glutamaxx (100X, Gibco), 10% Knockout serum replacement (Gibco), 1µg/mL Heparin (AppliChem), 20pg/µL EGF (Peprotech) and 20pg/µL FGF (Peprotech). As EGF/FGF concentrations would deplete through cell metabolism, medium was changed every 2-3 days by refreshing half of the total volume supplemented with the double amount of epidermal growth factor (EGF) and fibroblast growth factor (FGF; final concentrations: 20pg/µl) to ensure replenishment and stability. hNPCs reaching 80-90% confluency were split 1:2 by first washing with Dulbecco's phosphate-buffered saline (DPBS; Gibco), to remove debris and dead material, and then detached with Accutase (Sigma) by incubating for 1-2 minutes (min) at 37°C. Afterwards, the cells were fully detached by pipetting using Proliferation medium to inactivate the Accutase and then collected and centrifuged at 300xg for 3 min. The cell pellet was resuspended in fresh Proliferation medium supplied with EGF/FGF. hNPCs were always kept at a confluency higher than 50% as lower confluency significantly slowed growth rates and isolated cells separated from colonies spontaneously differentiate or die.

3.1.2 Cryopreservation

Expanded hNPC cultures were collected as described above and pellets were resuspended in freezing medium consisting of Proliferation medium without growth factors and with 10% dimethyl sulfoxide (DMSO) in shatter proof cryovials (Avanto). Cryovials were then transferred to -80°C in a Mr. Frosty container containing isopropanol (Sigma-Aldrich) to ensure a gradual freezing process of -1°C/minute. After a minimum of 18 hours, cells were transferred to liquid nitrogen storage tanks until further use.

3.1.3 Differentiation of hNPCs

Cells were attached to coverslips coated with poly-L-ornithine and laminin. Coverslips were washed 48 hours in a 93% nitric acid solution (Avantor) at room temperature (RT) on an orbital shaker. Nitric acid was removed followed by three washes in ddH_20 and air dried after washing with 100% ethanol overnight (ON) under a sterile workbench. Nitric acid washed coverslips were collected and stored at RT until further use. Acid-washed coverslips were precoated with 5µg/mL poly-L-ornithine for a minimum of 1 hour at 37°C. After aspiration, the coverslips were then coated with 10µg/mL laminin (Corning) and incubated overnight at 37°C. Poly-L-ornithine and laminin (PO-L) coated coverslips were then immediately used or stored at 4°C for no more than 1 week.

For differentiation, hNPCs were seeded with a density of $2.5x10^4$cells/cm^2 on PO-L coated coverslips in differentiation medium consisting of Neurobasal A w/o Glutamine (Gibco) with 1X Antibiotic-Antimycotic (Sigma), 1X B27 serum supplement with Vitamin A (50X; Gibco), 1X Glutamaxx, 20µM Bucladesine (Caymen Chemical), 10mM Potassium Chloride (KCl), 500ng/mL Retinoic Acid (RA; Sigma), 10pg/µL BDNF (Immunotools), and 10pg/µL NT-3 (Immunotools). Cells were differentiated for 4 weeks with 2-3 half media changes per week with doubling of Bucladesine, RA, BDNF and NT-3 to achieve the above-mentioned final concentrations.

3.1.4 Transduction of hNPCS

Utilization of polybrene to enhance transduction resulted in cell death and therefore the cells were transduced with pure virus (see 3.2.2). Lentiviral integrations follow a Poisson distribution of randomly occurring independent events where the numbers of infected cells are predicted to have a single integration at 30% (Kustikova et al. 2003). Therefore, we aimed for a transduction efficiency of 30% to ensure single integrations and to reduce the likelihood of double integrations. The lentiviral titer was estimated by generating a linear curve using a modified lentiCRISPRv2 construct where the puromycin

resistance cassette was replaced by a red-fluorescent-protein (RFP) construct (lentiCRISPRv2_RFP_NTC; a kind gift from Frank Schnütgen). After virus production, D62 hNPCs were transduced with $3.6x10^8$ viral-particles/µL lentiCRISPRv2_NTC_RFP at increasing volumes (0.001µL, 0.005µL, 0.01µL, 0.05µL, 0.1µL, 0.5µL, 1µL). After 1 day of incubation, cells were counted with the exclusion of dead and doublet cells using a BD FACS Celesta (Experimental immunology:AG Ullrich). Quantities of sgRNA transduction virus were calculated using the amount of virus needed for 30% transduction efficiency.

D62 cells were transduced with lentiCRISPRv2 constructs (containing the sequence for Cas9 as well as the sgRNAs for NTC, Dep2.1 and Dep2.2, respectively) for 18 hours, followed by full media changes. After another 72 hours, transduced cells were selected with 1µg/mL puromycin (Sigma) for 5-7 days. Surviving cells were then pooled together across multiple dishes for each resulting cell line to ensure confluency above 70% following selection resulting in bulk cultures.

3.2 CRISPR-Cas9 design

To investigate the impact of the knockout of *DEPDC5* regarding hNPC development, CRISPR-Cas9 was used to induce mutations leading to premature stop codons (Ran et al. 2013). CRISPR-Cas9 utilizes a single guide RNA (sgRNA) and a leading 3 base pair protospacer adjacent motif (PAM) which targets a specific position of DNA. The Cas9 molecule induces a double stranded break causing the error prone DNA repair machinery to often result in base pair insertion or deletions resulting in premature stop codons and truncated protein. The mutation (c.21C>G) changes a tyrosine in exon 2 of *DEPDC5* and was identified in an Australian family exhibiting ASD, epilepsy or in combination (Dibbens et al. 2013). Exon 2 was shown to be included for all transcripts of *DEPDC5* based upon transcript analysis using the Ensembl Genome Browser (www.ensembl.org). It should be mentioned that *DEPDC5* exon 1 is a pseudoexon and sometimes exon 2 is referred to as exon 1. However, for consistency with other literature we chose to adhere with exon 2. Therefore, sgRNAs were chosen to target exon 2 of the *DEPDC5* gene (hg19).

Two *DEPDC5*-targeting sgRNAs and one non-targeting control (NTC) were designed using a CRISPR design tool (currently discontinued; https://zlab.bio/guide-design-resources). sgRNA targets were verified for minimal off-target effects, lack of neuronal related genes and lack of overlapping exon regions using the CRISPR-Cas9 online predictor CCTop (cctop.cos.uni-heidelberg.de:8043; Stemmer et al. 2015).

3.2.1 LentiCRISPRv2 plasmid design and cloning

It has been reported that transfections methods using hNPCs result in relatively low transfection rates (5-20%) and tend to be quite variable depending on method, age of fetal material or the length of time the cells are *in vitro* (Dieterlen et al. 2009, Karra and Dahm 2010). Preliminary experiments yielded very low transfection efficiencies (~10%; data not shown) therefore lentivirus was used utilizing the widely published lentiCRISPRv2 plasmid (Plasmid #52961; Addgene; Ran et al. 2013). sgRNA oligomers were designed as described previously (see 3.2, Table 2). A guanine was added at the 5' end as it increases U6 promotor efficiency (https://www.addgene.org/crispr/zhang/).

Ordered oligos (Sigma) were phosphorylated and annealed using 10µM forward and reverse primers, 1X T4 PNK ligation buffer (NEB: New England Biologicals), 1mM ATP (NEB) and 0.5U/µL T4 PNK (NEB). After annealing at 37°C for 1hr, 95°C for 5 min and ramping down to 25°C at 5°C/min, resulting oligo duplexes were diluted 1:250. sgRNAs were annealed into plasmids using 5ng/µL lentiCRISPRv2, 4nM annealed oligo duplex, 1x FastDigest Buffer (Fermentas), 10mM DTT (Applichem), 10mM ATP (NEB), 0.05 FastDigest BsmBI, 0.5U/µL T4 DNA ligase (Thermo). The ligation reaction was conducted in a thermocycler (SensoQuest Labcycler) 37°C for 5 min and 23°C for 5 min for 6 cycles and stored at 4°C. Unwanted recombination products were removed using 11µL ligation reaction, 1X PlasmidSafe buffer (1X), 10mM ATP (1mM) and 0.6 U/µL PlasmidSafe exonuclease (Lucigen) at 37°C for 30 min.

Plasmids were transformed into 50µL Stbl3 E. coli cells (NEB) by heat shock. After 30 min on ice, the cell/plasmid mix was transformed for 30 s at 42°C and cooled for 5 min on ice. 950µL prewarmed NEB 10-beta/Stable Outgrowth Medium was added and cells were incubated at 30°C in a bacteria incubator at 225rpm. Transformed Stbl3 bacteria were placed onto warmed LB agar plates (30°C) with 100ng/mL ampicillin and incubated overnight at 37°C. Individual colonies were picked and expanded in LB medium for 24 hours and plasmids were extracted (Nucleobond Xtra Midi Kit; Macherey-Nagel) and validated with Sanger-sequencing (Eurofins) using forward primers targeting the U6 promotor (Table 2). Successful insertion of sgRNAs into lentiCRISPRv2 resulted in the constructs NTC (lentiCRISPRv2_NTC), Dep2.1 (lentiCRISPRv2_Dep2.1) and Dep2.2 (lentiCRISPRv2_Dep2.2).

Table 2 lentiCRISPRv2 oligo inserts and lentiCRISPRv2 validation primers

sgRNA oligos for lentiCRISPRv2		
Target	**Forward Primer**	**Reverse Primer**
DEPDC5_Ex2.1	CACCGCAAACTCGTCATCCACAAGA	AAACTCTTGTGGATGACGAGTTTGC
DEPDC5_Ex2.2	CACCGTGCAAGATGAGAACAACAA	AAACTTGTTGTTCTCATCTTGCAC
NTC	CACCGTTCCGGGCTAACAAGTCCT	AAACAGGACTTGTTAGCCCGGAAC
lentiCRISPRv2 sgRNA validation primers		
Target	**Forward Primer**	**Reverse Primer**
Human U6 Fwd	GAGGGCCTATTTCCCATGATTCC	

3.2.2 Lentivirus Production/Transduction

For lentiviral production, HEK293T cells (ATCC) were expanded in 10% Fetal Bovine Serum (Gibco), 1mM Sodium Pyruvate (100mM; Gibco), 1X Pen/Strep (10,000 units/mL Penicillin, 10,000 µg/mL Streptomycin; Gibco) in Dulbecco's Modified Eagle Medium (DMEM with Glutamine, 25mM HEPES, 4.5g/L Glucose; Gibco). Subsequently, cells were transfected by calcium chloride transfection composed of 2X Hank's Balanced Salt Solution (Bioworld), 2.5M $CaCl_2$ (VWR International) and 25mM Chloroquine (Bioworld) using transfer plasmids (lentiCRISPRv2 with the respective inserts) and helper plasmids: pMDLg/pRRE (Plasmid: #12251; Addgene), pRSV/Rev (Plasmid: #12253; Addgene) and pMD2.G (#12259; Addgene). Cell culture media containing viral particles were subsequently collected every 24 hours for three days and concentrated via ultracentrifugation at 50,000xg for 1 hour at 4°C (Avanti JXN-30; Beckman Coulter) three days after transfection. Viral titer was calculated by qPCR Titration Kit according to manufacturer's instructions (abm).

3.3 DNA methods

3.3.1 Primer design

qPCR primers were designed using Roche Assay Design Center (currently discontinued; https://qpcr.probefinder.com) while normal primers were designed using the IDT PrimerQuest Tool (https://eu.idtdna.com/pages/tools/primerquest). The specificity was rechecked using the National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Criteria for possible hairpins were checked (QC cutoff $-2 \leq \Delta G \leq 2$; $Tm < 40°C$) as well as for homo- and heterodimers (cutoff $\Delta G \geq -8$) using the Integrated DNA Technologies (IDT) Oligo Analyzer tool (https://eu.idtdna.com/calc/analyzer).

3.3.2 DNA extraction

DNA was extracted from cell pellets using Tissue and Cell lysis extraction buffer (MasterPure DNA Purification Kit, Lucigen) + 67ng/µl Proteinase K (Applichem) at 65°C for 15 min with vortexes. The lysate was treated with 17ng/µl RNase at 37°C for 30 min and then cooled for 5 min on ice. Protein was then removed using MPC Protein Precipitation Reagent and removed through centrifugation at 10,000xg for 10 min at 4°C. DNA was isolated with isopropanol, centrifuged and then concentrated with 70% ethanol with another centrifugation. Extracted DNA was resuspended in TE Buffer and concentration measured with a nanophotometer.

3.3.3 DNA sequencing

Confirmation of DNA sequences for CRISPR-Cas9 *DEPDC5*-KOs utilized the generation of polymerase chain reaction (PCR) fragments from exon2 validation primers (Table 3) and Sanger sequencing (Eurofins). Plasmid ligation constructs (see 3.2.1) were directly sequenced from the plasmid using the U6 promotor (Table 2). For PCR, 20ng DNA was mixed with 200nM forward and reverse primer pairs, 1X Bioxyme Taq Polymerase (Biozym) and 200µM dNTPs (Biozym). After an initial denaturation step of 95°C for 1 minute, DNA was amplified at 95°C for 15 seconds (s), 60°C for 15 s and 72°C for 30 s for a total of 40 reaction cycles in a thermocycler (SensoQuest Labcycler). Amplified and purified PCR fragments were sent for Sanger sequencing (Eurofins) using forward or reverse primers designed outside sgRNA target regions for DEPDC5 exon 2. Plasmids were sequenced with the U6 forward primer. Resulting ab1 files were analyzed using Benchling (Benchling.com).

Table 3 DEPDC5-exon2 validation primers

DEPDC5 exon2 validation primers		
Target	**Forward Primer**	**Reverse Primer**
DEPDC5 exon2	CCCTTAGTTCCTGGATTCTGTG	TAGTCTGTTTAGTCGCCTGTTTAG

3.4 Protein

3.4.1 Gel Electrophoresis/Western Blot

2-3x10^6 cells were lysed with modified RIPA buffer: 50mM Tris/HCl, pH8, 0.1% NP-40, 0.5% Na-deoxycholate, 150mM NaCl, 2mM $MgCl_2$, 0.1% SDS, 40µL/mL Proteinase Inhibitor Cocktail, 1mM Sodium Orthovanadate, 100mM NaF, 2mM DTT (all Sigma), 1:1000 Pierce Universal Nuclease (Thermo Fisher). Cells were vortexed every 5 min for 30 min on ice and subsequently centrifuged for 30 min at 17,000xg at 4ºC. Lysate was collected and concentrations were measured using Pierce BCA Protein Assay Kit (Life

Technologies) according to manufacturer's recommendation; measurements were done on a Multiskan FC 96 well nanophotometer (Implen) at 570nm. 50µg of protein was loaded onto a 10% SDS gel [resolving gel: 10% acrylamide/bis-acrylamide (37.5:1; Serva), 0.375M Tris pH 8.8, 0.1% SDS (both AppliChem), 0.05% APS, 0.1% TEMED (both Carl Roth); stacking gel: 4% acrylamide/bis-acrylamide (37.5:1; Serva), 0.125M tris pH8.8, 0.1% SDS (both AppliChem), 0.1% APS, 0.2% TEMED (both Carl Roth)]. To collect samples in the stacking gel, the gels were run at 50V for 15 min and at 150V until complete separation in 1X running buffer (25mM Tris, 192mM glycine, 0.1% SDS (AppliChem).

Gels were semi-dry blotted (Trans-Blot Semi-Dry Transfer Cell; Bio-rad) on a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL) for 80 min at 1mA/cm^2 in Blotting Buffer (48mM Tris, 39mM glycine, 20% methanol). Blotted membranes and transferred gels were subjected to Ponceau S-Solution (AppliChem) or PageBlue Staining Solution (Thermo Scientific) respectively and visualized using FluorChem Q Imaging System (Protein Simple). Ponceau solution was removed with 3 washes of 1XPBS (Thermo Fisher) + 0.1% Tween-20 (PBST; AppliChem;) and blots were incubated for 1 hour in blocking buffer (iBind Flex Solution; Invitrogen) on an orbital shaker at RT. Blots were then incubated with primary antibody in blocking solution overnight at 4ºC (Table 4) followed by 3 washes at 3 min with 1XPBS+0.1%Tween-20 (PBST). Blots were then incubated with secondary antibodies coupled to horseradish peroxidase for 1 hour at RT followed by 3 washes (Table 4). Visualization was performed using a 30 s incubation of Trident Femto Western Blot ECL Solution (Genetex) yielding a chemiluminescent signal and images were taken using a FluorChem Q Imaging System (Protein Simple) after 1, 5 and 10 min. Blots were then stripped between stains using 100mM 2-mercaptoethanol (Carl Roth), 2% SDS, 62.5mM tris pH 6.7 (AppliChem) for 30 min at 50ºC on a roller, washed 3 times with PBST, and reblocked and immunostained as described above.

Table 4 Western Blot antibodies

Western Blot Primary Antibodies			
Target	**Species**	**Dilution**	**Company**
AKT (pan)	Rabbit	1:1000	Cell Signaling
DEPDC5	Rabbit	1:1000	abcam
GAPDH	Mouse	1:5000	Santa Cruz
Oct4	Rabbit	1:700	Genetex
Pax6	Rabbit	1:700	Genetex
Phospho-S6 (Ser240/244)	Rabbit	1:1000	Cell Signaling
Phospho-AKT (Ser473)	Rabbit	1:1000	Cell Signaling
S6 ribosomal protein	Rabbit	1:1000	Genetex
Sox2	Mouse	1:700	Genetex
Western Blot Secondary Antibodies			
Target	**Conjugate**	**Dilution**	**Company**
Anti-mouse	Horseradish peroxidase	1:5000	Cell Signaling
Anti-rabbit	Horseradish peroxidase	1:5000	Cell Signaling

3.4.2 mTOR hyperactivation experiments

2-3x10^6 cells were incubated for 90 min in Neurobasal A or Neurobasal A without amino acids (US Biological) supplemented with 3.7g/L Sodium Bicarbonate (Gibco). For rescue experiments, cells were incubated with additional 100nM rapamycin (Absource Diagnostics). Immediately following incubation, cells were collected in ice cold DPBS, centrifuged at 500xg and immediately lysed in RIPA buffer (see 3.4.2)

3.5 Ribonucleic Acid (RNA)

3.5.1 RNA extraction

RNA of cell lines was extracted using Nucelospin RNA kit (Macherey-Nagel) after immediate and direct lysis in cell culture plates. Steps were followed per the manufacturer's instructions and RNA was collected in RNase-free H_2O. Concentrations were measured using a nanophotometer (IMPLEN).

3.5.2 RT-qPCR

To verify gene expression in samples sent to RNA sequencing, real-time reverse transcription quantitative PCR (RT-qPCR) was performed. Complementary DNA (cDNA) was produced from RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher) by following the manufacturer's instructions. cDNA was then subjected to analysis using the Universal Probe Library set (human, probes #1-90, Roche) where 10ng cDNA, 260nM forward primer (Sigma), 260nM reverse primer (Sigma), 133nM UPL probe (Roche), 1.3X qPCR-probe-MasterMix-high-ROX (Steinbrenner Laborsysteme GmbH) was

mixed in a conical 96w PCR plate (BioCentrix) and analyzed with a StepOne Plus (Thermo Fisher) machine and the associated StepOne software (version 2.3; Thermo Fisher Scientific). PCR setup consisted of two enzyme activations steps (2 min, 50°C), a DNA denaturation step (10 min, 95°C) and 40 cycles of signal detection (15 s, 95°C and 1 minute 60°C). All experiments were performed with triplicates, normalized to the reference gene *PSMD7* and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Table 5; Livak and Schmittgen 2001).

Table 5 sgRNA oligos for lentiCRISPRv2 inserts

Primers for RT-qPCR			
Target	**Forward Primer**	**Reverse Primer**	**UPL probe**
DEPDC5	CTACAAGATAGTAACGGACAAAGAGC	CTGGAAGCTGGCGAAGTC	18
GFAP	AGAGGGACAATCTGGCACA	CAGCCTCAGGTTGGTTTCAT	29
PSMD7	CACGTGACCAGTGAAATTGG	CCACCGTCGTGTCTTTGATA	6
REDD1	CAGTGCCCTCCAAGACAGAG	TGGCTGCCTCAGTTTTCCAA	34

3.5.3 3'-mRNA sequencing

To observe the effects of *DEPDC5*-KO on the transcriptome, 3'-mRNA analysis was performed. RNA was extracted from three biological replicates of D62-NTC, DEPDC5-2.1, and DEPDC5-2.2 with or without rapamycin progenitors and after 1 month of neuronal differentiation. All samples passed quality check using a Tapestation 4200 (Agilent Technologies) with an RNA integrity number (RIN) >7. RNA libraries were generated with Quantseq 3'-mRNA Library Prep (Lexogen) and sequenced on a HiSeq 2500 V4 (High Output Mode) with a coverage of 10M Raw Reads per sample and a standard read length of 1x50bp (NGS Core Facility Bonn). Raw reads were quality controlled (FastQC; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), trimmed (trimmomatic; Bolger et al. 2014) and aligned to hg38 (Rsubread; Liao et al. 2019) using the standard settings as provided in the manual. Feature counts were extracted and used for subsequent statistical analysis (see 3.7.2.1). All samples passed quality control.

3.6 Immunocytochemistry

For visualization of markers of hNPCs and neurons, cells were seeded and differentiated as stated previously (see 3.1.3). Cells were fixed in 4% paraformaldehyde+4% sucrose (Electron Microscopy Sciences; Sigma) in 1X PBS with 0.9 mM calcium and 0.5 mM magnesium for 15 min at room temperature (RT). Fixed cells were then washed 3 times and submerged in 1X PBS until staining. Stains were performed by permeabilization with 0.1% Triton X-100 in PBS for 15 min at RT followed by

immediate blocking in 10% goat and donkey serum (Merck) in 1X PBS+0.02% Tween-20 for 1 hour at RT. Fixed cells were then submerged in 20µL diluted primary antibodies in antibody solution (5% goat and donkey serum in 1X PBS+0.02% Tween-20) overnight in a humidified chamber at 4°C followed by 3 washes with PBST and subsequent secondary antibody incubation in antibody solution for 1 hour at RT (Table 6). Stained cells were washed a final 3 times in 1X PBST, washed one time in double-distilled H_20 and mounted to ethanol washed microscope slides with Prolong Gold Antifade with DAPI (Thermo Fisher). Cells were visualized with a Nikon Eclipse Ti Confocal Microscope equipped with a spinning disc unit (CSUW1, Andor) and LED lasers at wavelengths 405, 488, 561 and 639. Visualization was done with Nikon Elements software (version 4.60.00 Build 1171).

Table 6 Immunocytochemistry antibodies

Immunocytochemistry Primary Antibodies			
Target	**Species**	**Dilution**	**Company**
Aquaporin 4	Rabbit	1:1000	Sigma
Beta Tubulin 3 (Tuj1)	Mouse	1:500	mouse
GFAP	Guinea pig	1:500	Synaptic Systems
MAP2	Mouse	1:500	Millipore
Nestin	Rabbit	1:500	abcam
Sox2	Mouse	1:500	Genetex
vGlut1/2	Rabbit	1:500	Genetex
Immunocytochemistry Secondary Antibodies			
Target	**Conjugate**	**Dilution**	**Company**
Anti-mouse	488	1:1000	Thermo Fisher
Anti-guinea pig	Cy3	1:1000	Millipore
Anti-rabbit	647	1:1000	Invitrogen

3.6.1 Morphology and cell counting

Analysis of cell sizes as well as neuronal, astrocyte and hNPC counts were done using FIJI ImageJ software (v. 1.53f51; Shindelin et al. 2012). Sholl analysis was performed with the neuroanatomy plugin Simple Neurite Tracer (version 4.0.3) with a radius step of 1, semi-log method and ‘Best fitting’ degree settings. Dendrite lengths were collected from sholl analysis measurements.

3.7 Statistical Analysis

3.7.1 Group Comparisons

Group differences between morphological analysis were tested using two-sample unequal variance T-test where significance was defined if $p < 0.05$. All samples were compared to their respective controls (NTC vs Dep2.1 or Dep2.2). Mean measurements and standard error of the mean were calculated for each morphological parameter.

3.7.2 RNA Sequencing

Data analysis can be visualized at https://kjpmolgenlab.github.io/CePTER_RNASeq/index.html.

3.7.2.1 Analysis Preprocessing

Count matrices were based on entrez gene ids and checked for non-unique annotations. Non-unique annotations were merged by sum. Visual inspection of count distributions per sample and per gene as well as clustering analysis of raw and normalized counts per million reads (cpm) were performed to check for technical outliers. We removed genes with no variance across samples or which were detected in less than 50% of the samples. After cleaning, the average read per sample was 7,195,819 (SD 345,374). In total 14,664 genes were removed due to low reads resulting in 13,731 genes passing quality criteria. Count normalization and the statistical analysis was performed on cleaned data using DESeq2 with the full model being:

$$cpm \sim intercept + factor(gRNA) + factor(DIFF) + factor(RAPA) + factor(Cell\ line)$$

To confirm replication of technical and biological replicates, hierarchical clustering (hclust) analysis was performed on the top 2000 genes by variance. The replicates were inspected based on the Euclidean distance, principal component analysis (GLM-PCA), and multidimensional scaling (MDS).

3.7.2.2 Weighted Gene Co-expression Network Analysis (WGCNA)

Normalized count data was variance stabilized (*getVarianceStabilizedData*) and log2 transformed to model normal distribution. Best softPower threshold for calculating the adjacency matrix was estimated based on the Scale free topological model fit ($R^2 > 0.8$) and the Mean connectivity (scree plot criterion). softPower was set to 6. We calculated the unsigned topological overlap matrix and calculated modules based on the fastclust (hierarchical clustering) dendrograms implementing the cutreeDynamic including the option that the PAM algorithm respects dendrogram structure in the identification of gene-sets. The minimum number of genes within a set was defined as 50. Module Eigengenes (first principal component) were calculated. Modules were merged if they clustered together with a distance < 0.2 (*mergeCloseModules*).

Differences between KO and NTC were tested for each condition with each cell line separately implementing linear modeling correcting p-values for the number of modules tested.

3.7.2.3 Group comparisons

For group comparison, raw counts were loaded into DESeq2 using the “DESeqDataSetFromMatrix” function. Differential expression was estimated using the function “DESeq” with standard options. P-values were false discovery rate (fdr) corrected for the number of genes tested. Genes were considered to be significant if the fdr was < 0.05 in both KO cell lines compared to the NTC control, respectively.

Resulting deregulated gene datasets were analyzed based upon the KEGG Mapper Tool (https://www.genome.jp/kegg/mapper/search.html), Entrez gene database summary https://www.ncbi.nlm.nih.gov/gene, or Genecard database summary (https://www.genecards.org/) and clustered to known pathway or functional mechanisms related to the mTOR pathway. Isolated genes were further investigated for ASD and epilepsy disease gene overlap.

3.7.2.4 Gene List enrichment testing

To test whether *DEPDC5*-KO is associated with the disease of interest, the differentially expressed genes were tested for enrichment using fisher exact test in published data sets of high-risk genes for epilepsy (Epi25 https://epi25.broadinstitute.org/; Ran et al. 2015), Autism Spectrum Disorders (https://gene.sfari.org/, de Rubeis et al. 2014, Iossifov et al.2014, Voineagu et al. 2011), Fragile X Syndrome (Darnell et al. 2011), Tuberous Sclerosis (Grabole et al. 2016, Martin et al. 2017), Intellectual Disability (Pinto et al. 2014, Parishak et al. 2013), or Schizophrenia (Fromer et al. 2016, Cocchi et al. 2015). Gene lists were considered as associated with *DEPDC5* if the odds ratio (OR) was greater 1 and the Bonferroni adjusted p-value < 0.05 (Fisher exact test).

GO term enrichment analysis was performed implementing the gprofiler2 (Raudvere et al. 2019) package. We used the correction_method=”g_SCS" to account for multiple testing as well as the diacyclic graph structure of the ontologies. All genes passing quality control in our analysis were set as a reference gene-universe.

3.7.3 Magnet Pipeline

To evaluate developmental age and brain region of the genes differentially expressed due to *DEPDC5*-KO, transcriptomic data was integrated into the MApping the Genetics of neuropsychiatric traits to the molecular NETworks of human brain (MAGNET) pipeline (https://molgenlab.shinyapps.io/MAGNET_lite_V2/; Yousaf et al. 2018). This in-house pipeline was

generated using the Allen Brain Atlas (Kang et al. 2011) using 1,340 tissue samples taken from one or both hemispheres of 57 postmortem brains varying over age. Transcripts were tested with Fisher's exact test for significant enrichment in the 29 lists of genes corresponding to co-regulated modules activated during brain development, as published in the original publication (adjusted p-value<0.05).

4. Results

4.1 Confirmation of the hNPC phenotype in WT

To confirm that the extracted cells were indeed hNPCs and did not express stem cell markers, WT D62 cells were evaluated for their proliferative capacity and ability to differentiate into neurons via immunoblotting and immunostaining (Figure 3A). As controls, the neural stem cell line CB153 and the human embryonic kidney cell line HEK293T were used to compare the expression of progenitor markers within D62 hNPCs. Western blotting of D62 hNPCs confirmed the expression of *DEPDC5* in all cell lines indicating its baseline presence among a variety of cell types.

The neural progenitor marker Sox2 was clearly expressed among all three cell lines. Sox2 is a transcription factor that is necessary for maintaining self-renewal and preventing differentiation, aspects common in all three cell lines. However, the pluripotency and stem cell marker Oct4 was not expressed within the D62 hNPCs indicative that the D62 cells have limited proliferative capacity and are not stem cells. The cells also showed expression of the neuroepithelial and progenitor markers Nestin and Pax6, indicative of a radial glial cell type. CB153 showed a lack of Pax6 expression but a strong Oct4 presence characteristic of an earlier neuroepithelial cell phenotype. HEK293T cells expressed Oct4 as this is also a marker for immortalized cells but as expected they do not express the neural subtype markers due to the differences in the tissues from which they were derived. Overall, the D62 cells have characteristics of neural progenitor cells and exhibit radial glial protein markers with limited self-renewal capacity and a lack of pluripotency.

In addition, D62 cells showed protein expression of the nuclear proliferation marker Ki67 which is absent in quiescent cells (Figure 3B). Only a few cells expressed Ki67 which goes in line with our observation of slow proliferation rates which had a doubling time of every 5 days. In proliferation state, D62 cells showed low expression of the immature neuronal marker Tuj1 which was mainly localized to the cell soma. After four weeks of differentiation, D62 cells expressed the radial glial/astrocyte marker GFAP and Tuj1 (Figure 3C). Thus, D62 cells express radial glia cell markers and proliferation markers during proliferation and can be differentiated into neurons after 4 weeks of differentiation.

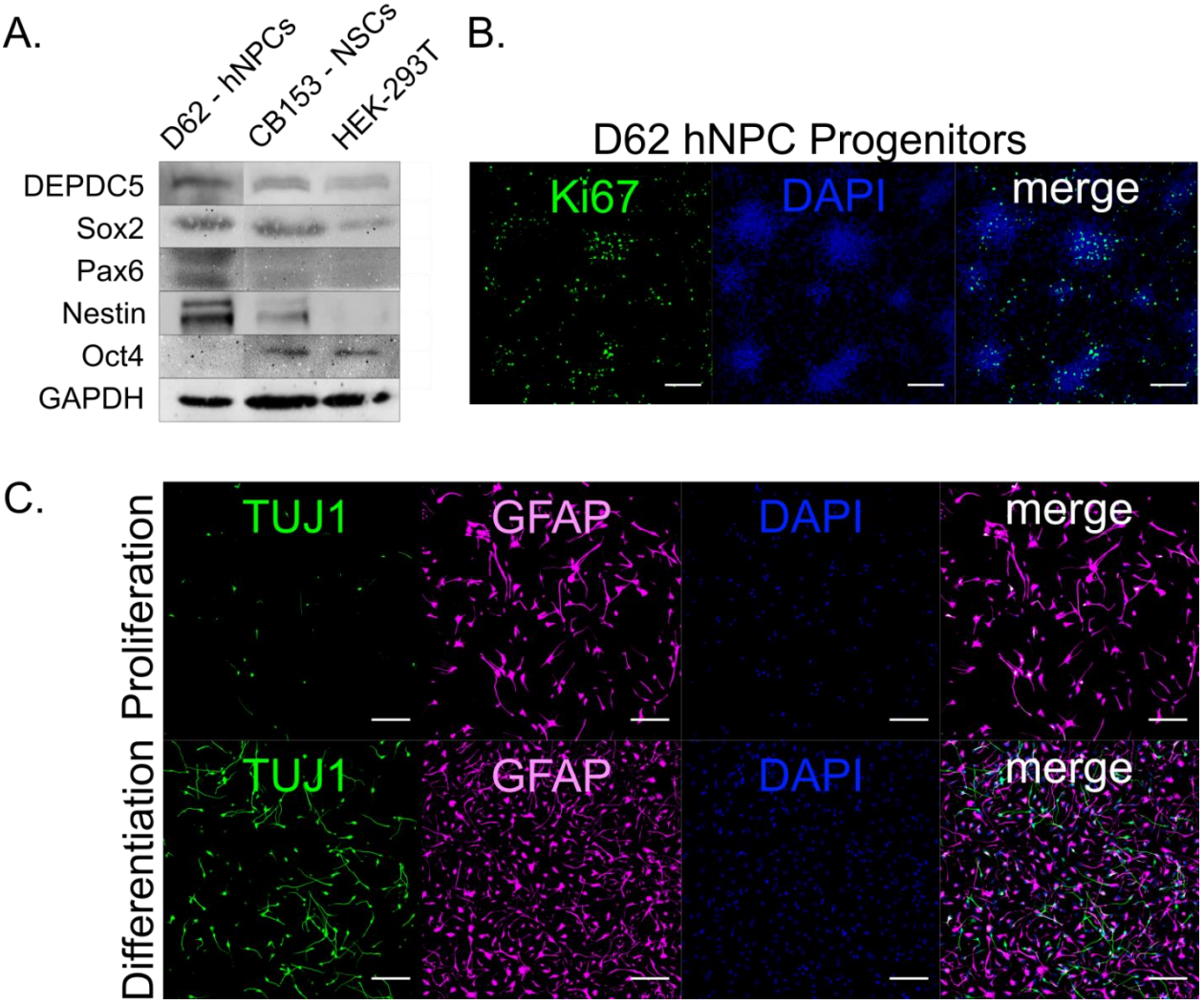


Figure 3 Confirmation of hNPC phenotypes
(A) Western Blot of D62 human neural progenitor cells, CB153 human neural stem cells and HEK293T human embryonic kidney cells stained for antibodies against DEPDC5, Sox2, Pax6, Nestin, Oct4 and GAPDH. (B) Immunocytochemistry of D62 progenitor cells stained against Ki67 and DAPI. (C) Comparison of immunocytochemical images of D62 progenitors during proliferation (top row) and four weeks of differentiation (bottom row) using markers for Tuj1, GFAP and DAPI. Scale bars: 100 µm. All stainings have been performed in biological replicates.

4.2 Inducing mutations in DEPDC5 utilizing CRISPR-Cas9

Because hNPCs have been known to have low transfection efficiencies similar to our own experience using nucleoporation, we generated D62 hNPCs with *DEPDC5* knockout mutations using lentiviral CRISPR-Cas9 constructs (see 3.2.1). Patient-specific *DEPDC5* mutations often result in gene disruption inducing premature stop codons (Baldassari et al. 2018). To address the comorbidity of autism and epilepsy, we targeted the region of a mutation identified in an Australian family exhibiting ASD, epilepsy or both. The mutation (c.21C>G) changes a tyrosine on exon 2 causing cessation of functionality of

DEPDC5. Two sgRNAs targeting *DEPDC5* exon 2, called Dep2.1 and Dep2.2 (see 3.2.1), were designed to initiate double-strand breaks as close as possible to the patient-specific mutation. The cut-site of Dep2.1 is 16 base pairs downstream from the Australian cohort mutation and Dep2.2 resides 12 base pairs upstream (Figure 4A). Successful insertions or deletions (indel) were confirmed in *DEPDC5* exon 2 with Sanger sequencing. Background peak formation and unspecific nucleotide changes observed indicate mutation induction from CRISPR-Cas9 in our bulk cultures (Figure 4B). Verification of *DEPDC5* knockout was checked with immunoblotting. *DEPDC5* was expressed in wild-type and the NTC but was absent in both Dep2.1 and Dep2.2 protein extracts (Figure 4C). Taken together, we were successful in inducing indel formation in *DEPDC5* exon 2 and confirmed the knockout of DEPDC5 in D62 hNPCs via western blot analysis.

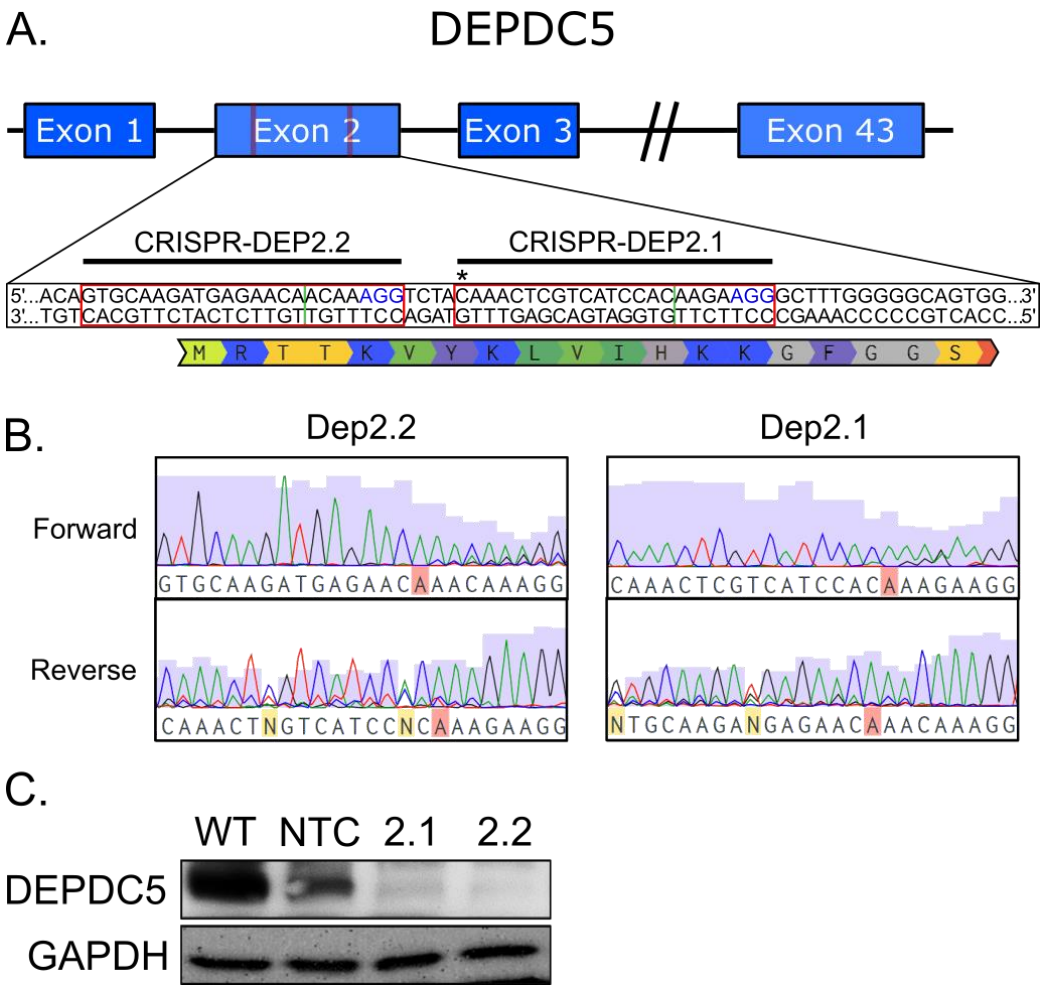


Figure 4 CRISPR-Cas9 induced DEPDC5 mutations in hNPCs
(A) Design of *DEPDC5* sgRNAs targeting exon 2. Expected cut sites are labeled in green. PAM sequences are in blue. *Australian cohort mutation (B) Sanger sequencing of Dep2.1 and Dep2.2 knockout cell lines (bulk cultures). Trace qualities are labeled as bar graphs (purple). (C) Immunoblotting of DEPDC5 protein expression levels compared with GAPDH (control).

4.3 Hyperactivation of mTOR due to *DEPDC5* knockout

To test if the loss of DEPDC5 causes mTOR hyperactivity, levels of phosphorylated S6 ribosomal protein (pS6) (Ser 240/244) were checked via Western blot (Figure 5). Knockout hNPCs incubated in standard proliferation or amino acid free media for 90 min displayed higher amounts of phosphorylated S6 when compared with NTC. This expression was attenuated with the addition of rapamycin reducing the phosphorylation to baseline levels in both media. The phosphorylation of AKT (pAKT; Ser473) was also checked as mTOR hyperactivity has a reverse feedback mechanism signaling downregulation of AKT. Expression of pAKT was decreased with pS6 upregulation confirming that this reverse feedback mechanism is intact. Rapamycin reverses this mechanism causing pAKT levels to increase due to inhibition of mTOR. This mechanism was also present in the NTC hNPCs where additional rapamycin would cause an increase in AKT phosphorylation. Together, this suggests that mTOR is hyperactive due to loss of DEPDC5 and can be attenuated by rapamycin.

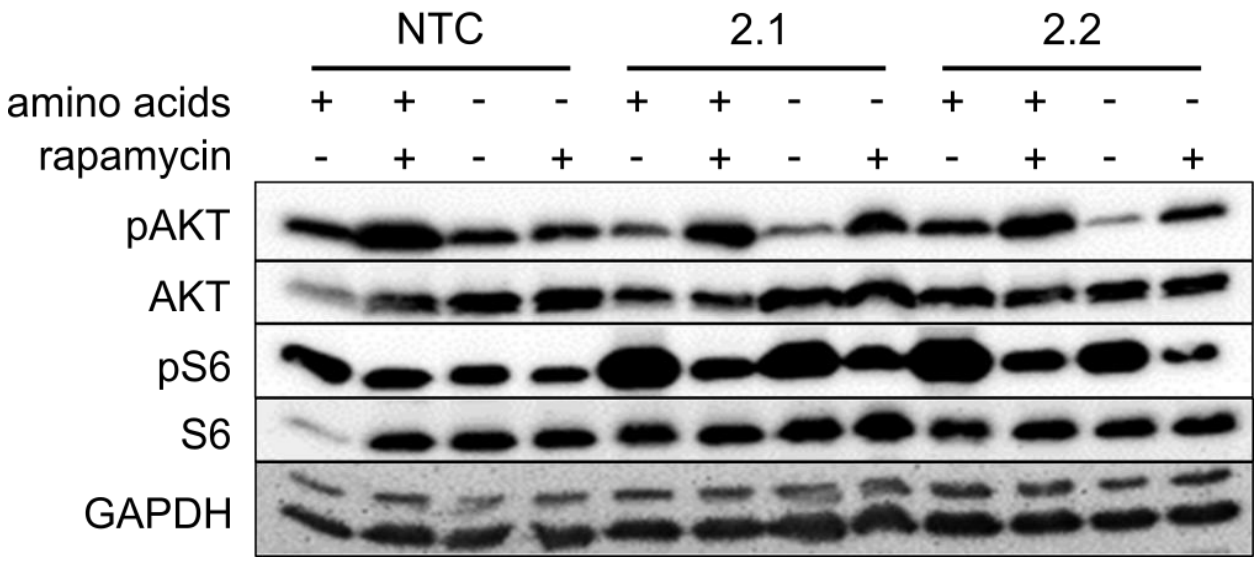


Figure 5 *DEPDC5* knockout causes phosphorylation of S6 in amino acid free media
Western Blot of Dep2.1, Dep2.2 and NTC incubated in proliferation or amino acid free media with or without the addition of 100nM of rapamycin. Blots were cropped to show relevant bands. n=3. (AKT: protein kinase B, S6: ribosomal protein S6, p: phosphorylated)

4.4 DEPDC5 knockout alters gene expression in progenitor and differentiated hNPCs

To investigate the effects of *DEPDC5*-KO on gene expression, RNA extracted from proliferating and differentiating hNPCs with or without rapamycin was sent for RNA sequencing. After preprocessing, Principal Component Analysis (PCA) was performed to visualize data clustering of our cell lines. Visual inspection showed separate clustering of the cell lines into proliferation and differentiation subtypes

(Figure S2). Heatmap clustering of distances normalized log2 counts indicated the same (Figure 6). Distances showed a separation between NTC and KO cell lines predominantly during differentiation. Clustering of NTC samples was also apparent during proliferation although there was an overlap with the KO samples advocating a more similar population of cells during NPC stages. Addition of rapamycin did show a partial reversal during proliferation stages as most of the KO cells with rapamycin clustered with the NTC; however, this was not apparent during differentiation. Lack of clustering of rapamycin treated KOs with NTC suggests incomplete recovery of loss of DEPDC5. Altogether, the largest difference occurred between the two developmental stages (proliferation vs. differentiation), NTC and KOs cluster separately during differentiation and rapamycin shows partial recovery during proliferation but not differentiation.

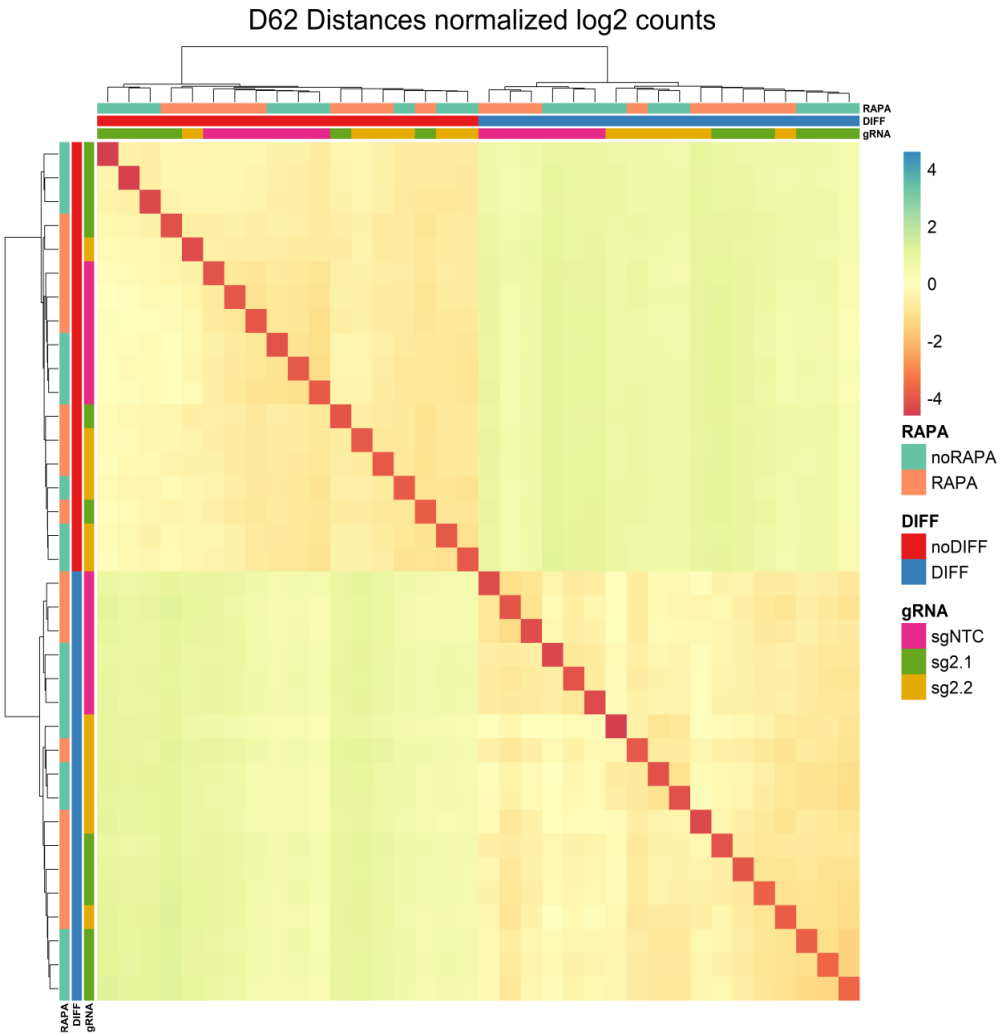


Figure 6 Preprocessing of D62 Transcriptomic Data of *DEPDC5*-KOs
Heatmap of unsupervised hierarchical clustering of distances normalized log2 counts between cell lines and conditions with main differences between proliferation and differentiation developmental stages.

4.5 Cell type specific gene expression of *DEPDC5*-KOs

Since heterogeneous hNPC cultures were used, an analysis of different cell type markers was performed. Known cell type specific markers for neuroepithelial/neural stem cells (*Oct4, Nanog*), neural progenitor cells (*Sox2*, *Nestin*, *Pax6*, *GFAP*), astrocytes (*GFAP*, *AQP4*, *ASCL1*), oligodendrocytes (*Olig2*), microglia (*TMEM119*), immature (*TUBB3*) and mature neurons (*MAP2*, *SYP*), excitatory neurons (*SLC17A7*, *GRIA2*), inhibitory neurons (*GAD1*, *GABBR2*), serotonergic neurons (*SERT1, HTR2B*), cholinergic neurons (*CHAT*, *CHRNA7*) and dopaminergic neurons (*TH*, *DRD2)* were compared with KOs vs. NTC during proliferation and differentiation (For gene names see List of genes/proteins). From observation, hNPCs did not show expression in markers for early neuroepithelial/neural stem cells nor for the different mature neuronal subtypes. However, cells were positive for radial glial markers *Sox2*, *Nestin, Pax6* and *GFAP* which were higher expressed in the differentiated population (Figure 7). Descriptive differences were also noted for oligodendrocyte markers, with higher levels amongst the progenitor population. Unexpectedly, markers for mature and immature neurons remained static regardless of proliferation and differentiation, suggesting that the cells have a baseline expression of neuronal genes indicative of an intermediate progenitor population spontaneously differentiating. Inhibitory markers showed higher baseline expression amongst the undifferentiated population. Lastly, despite confirming *DEPDC5* knockout at DNA and protein level, *DEPDC5* RNA was detected within all cell lines, suggesting that nonsense mediated decay leads to the reduced protein levels. There were no clear differences with the addition of rapamycin to any of the triplicates amongst gene expression markers. Thus, differentiated hNPCs are still a heterogeneous culture of neurons, astrocytes and hNPCs.

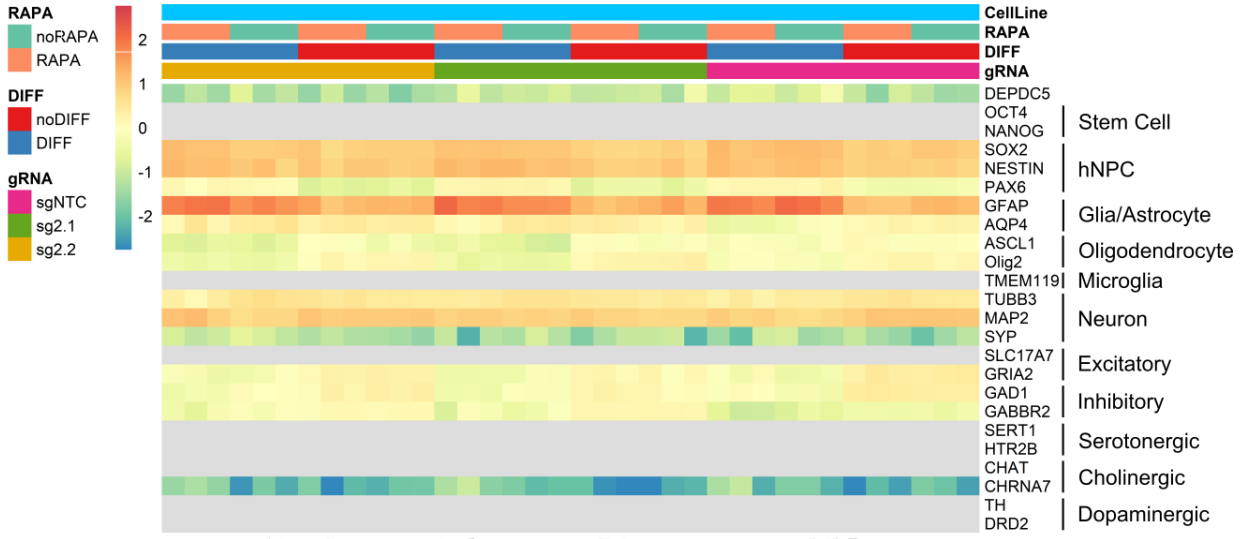


Figure 7 Gene expression data of cell type specific markers
RNA expression of different markers specific to neural stem cells, radial glia, astrocytes, immature neurons, oligodendrocytes, microglia, mature neurons and mature neuronal subtypes in *DEPDC5*-KO and NTC lines.

4.6 *DEPDC5*-KOs have large deregulation in gene expression due to mTOR hyperactivity

We next analyzed the amount of genes up- or downregulated in both *DEPDC5* mutations in comparison with NTC in both proliferating and differentiating conditions. We also analyzed the effect of rapamycin on deregulated gene expression (Figure 8). In total, 738 genes were deregulated due to *DEPDC5*-KO during both stages and all but 89 genes were recovered after rapamycin treatment (p-value<0.05). Deregulated genes in the proliferation dataset totaled at 237. After rapamycin treatment, 201 of these genes were rescued while 36 still remained significantly deregulated and 19 being specific to rapamycin induction. For differentiated *DEPDC5*-KOs, a total of 552 genes were found to be deregulated when compared to NTC controls. Among these, only 68 were still deregulated after rapamycin treatment while 484 genes were recovered. A total of 72 additional genes were identified to be rapamycin induced.

Overall, there was an overlap of 51 genes which were differentially expressed in both proliferation and differentiation stages, with 36 of these not being rescued by rapamycin. Interestingly, the differentially regulated genes induced by rapamycin administration were specific to the cell stage and did not overlap between proliferation and differentiation. Interestingly, the deregulation of the genes *NPY*, *SERPINE2*, and *STMN2* was observed through all conditions. Taken together, rapamycin significantly reduces the

overall effects of *DEPDC5*-KO and mTOR hyperactivity, but does not counteract all of the network deregulation.

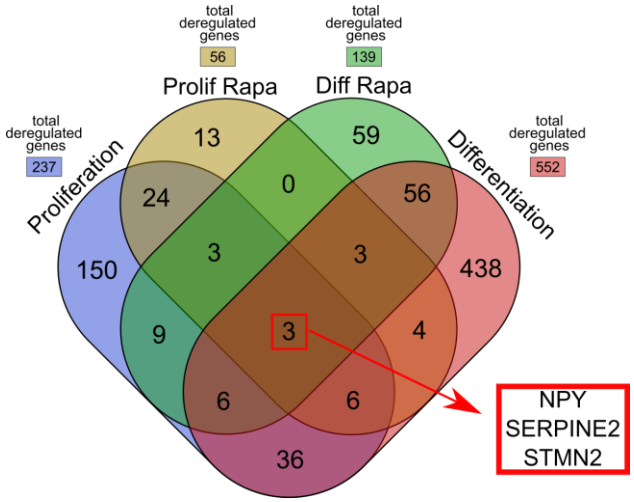


Figure 8 Venn diagram showing the amount of deregulation associated with *DEPDC5*-KO
DEPDC5-KO showed a significant reduction of deregulated genes when administered with rapamycin. Colored ovals contain genes which were identified in proliferation (blue), prolif. with rapamycin (yellow), differentiation (red) and differentiation with rapamycin (green) conditions. Consistent genes observed during all conditions were *NPY*, *SERPINE2* and *STMN2*. Overlapping ovals show the total amount of genes which were recovered by rapamycin administration while genes which were not attenuated are shown in the outer ovals.

4.7 KEGG pathway/GO enrichment analysis of deregulated gene pathways

To understand possible cellular pathways altered upon the knockout of *DEPDC5*, deregulated gene sets were analyzed manually by their gene descriptions or with the KEGG Mapper pathway tool and identified gene networks known to be related to mTOR were clustered together. Gene associations identified pathways in *p53*/cell cycle, AKT/mTOR, nucleotide and lipid metabolic processes and genes known for protein degradation and lysosomal processes. For proliferation and differentiation, a total of 13 and 27 mTOR/AKT genes were identified in upstream or downstream processes with the majority showing upregulation (Table 7, Table 8). Upstream mTOR processes identified a host of extracellular matrix proteins including collagen proteins (*COL4A1, COL4A2, COL6A2*), glycoproteins (*LAMA5, TNC, THBS1, THBS4*), receptor tyrosine kinases (*ERBB4, EFNA5, EFNB2*) along with growth factor receptors and interactors (*FGF1, NGFR SORCS2, PTN, PDGFRA*; For gene names see List of genes/proteins).

We grouped *p53* or cell cycle related genes and found 26 deregulated genes during proliferation and 36 during differentiation (Table 9). Interestingly, genes for lysosome and protein degradation were significantly downregulated and identified only in the differentiation dataset (

Table **10**). There were also 17 deregulated genes related to nucleotide and lipid metabolism during proliferation and 47 for differentiated *DEPDC5*-KOs (Table 11). Hence, loss of DEPDC5 causes disruption in multiple upstream and downstream pathways causing an overall disruption in normal cellular processes in neural cells which may be related to disease formation during development.

To verify the expression of RNA sequencing transcripts, we checked the expression of GFAP and *REDD1* using RT-qPCR. We identified positive correlations between *GFAP* (0.66) and *REDD1* (0.83) expression with p-values less than 0.05. Thus, RNA sequencing expression correlated with expression from RT-qPCR and confirms that the data is reproducible.

Offtarget effects were checked against deregulated genes for up to 4 mismatched basepairs. sgDEP2.1 identified 159 possible offtargets while 83 possible offtargets were found for sgDEP2.2. The proliferation dataset identified one gene (*DCX;* downregulated) as being an offtarget while 7 (*PDE4D, DLC1, DCX, C6orf118, SLC16A14, TCF4, EPB41L3;* all upregulated) were identified for differentiation. None were identified in proliferation with rapamycin and two (*DLC1, DCX;* upregulated) were found for differentiation with rapamycin. All identified overlapping genes were intronic or intergenic. As *DCX* was downregulated during proliferation and upregulated during differentiation, any offtarget effects appeared inept at gene disruption. Similarly, all other offtargets were upregulated suggestive of a lack of gene disruption.

Table 7 Proliferation Kegg Pathway genes related to PI3K/AKT/mTOR pathway

Proliferation mTOR/AKT Genes								
	Gene name	**Mean log2FC**	**Mean pvalue**	**Function**	**Relation to MTORC1**	**Function**	**Deregulation**	**Rapamycin recovery**
DDIT4 (REDD1)	DNA damage inducible transcript 4	9.51E-01	1.19E-02	Inhibition of MTORC1 is mediated by a pathway that involves DDIT4/REDD1, AKT1, the TSC1-TSC2 complex and the GTPase RHEB. Plays an important role in responses to cellular energy levels and cellular stress, including responses to hypoxia and DNA damage.	Upstream	Hypoxia / stress / amino acids / inhibits mTOR	Up	yes
SERPINE1	serpin family E member 1	1.23E+00	2.62E-15	Acts as a regulator of cell migration, independently of its role as protease inhibitor. It is involved in cellular and replicative senescence	Downstream	Hypoxia induced pathway of mTOR activation	Up	no
TNFRSF10B	TNF receptor superfamily member 10b	7.93E-01	1.46E-02	This receptor can be activated by tumor necrosis factor-related apoptosis inducing ligand (TNFSF10/TRAIL/APO-2L), and transduces an apoptosis signal. Promotes the activation of NF-kappa-B. Essential for ER stress-induced apoptosis.	Downstream	Apoptosis / p53 signaling	Up	yes
FGF1	fibroblast growth factor 1	7.03E-01	6.55E-05	Its binding to integrin, subsequent ternary complex formation with integrin and FGFR1, and the recruitment of PTPN11 to the complex are essential for FGF1 signaling. Induces the phosphorylation and activation of FGFR1, FRS2, MAPK3/ERK1, MAPK1/ERK2 and AKT1	Upstream	Growth factor activation	Up	yes
LAMA4	laminin subunit alpha 4	4.23E-01	2.88E-02	Laminins, a family of extracellular matrix glycoproteins, are the major noncollagenous constituent of basement membranes. They have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis.	Upstream	Cytoskeleton	Up	yes
MDM2	MDM2 proto-oncogene	5.17E-01	5.95E-03	E3 ubiquitin-protein ligase that mediates ubiquitination of p53/TP53, leading to its degradation by the proteasome. Inhibits p53/TP53- and p73/TP73-mediated cell cycle arrest and apoptosis by binding its transcriptional activation domain.	Downstream of AKT	AKT activation / p53 signaling	Up	yes
PTN	pleiotrophin	3.36E-01	7.08E-04	In adult hippocampus promotes dendritic arborization, spine development, and functional integration and connectivity of newborn granule neurons through ALK by activating AKT signaling pathway (By similarity)	Upstream	AKT activation / receptor binding	Up	yes
CDKN1A	cyclin dependent kinase inhibitor 1A	1.32E+00	3.26E-32	The encoded protein binds to and inhibits the activity of cyclin-cyclin-dependent kinase2 or -cyclin-dependent kinase4 complexes, and thus functions as a regulator of cell cycle progression at G1. Also known as p21.	Downstream	P21 / p53 activation	Up	no
COL9A3	collagen type IX alpha 3 chain	4.30E-01	3.59E-03	Type IX collagen, a heterotrimeric molecule, is usually found in tissues containing type II collagen, a fibrillar collagen	Upstream	Extracellular Matrix	Up	yes
RPS6KA2	ribosomal protein S6 kinase A2	6.61E-01	2.17E-03	This kinase contains two non-identical kinase catalytic domains and phosphorylates various substrates, including members of the mitogen-activated kinase (MAPK) signaling pathway.	Upstream	TSC2 inactivation	Up	yes
RPS6	ribosomal protein S6	-3.34E-01	1.62E-02	This gene encodes a cytoplasmic ribosomal protein that is a component of the 40S subunit.	Downstream	Ribosome biogenesis	Down	yes
PDGFRA	platelet derived growth factor receptor alpha	-6.96E-01	2.88E-04	Phosphorylates PIK3R1, PLCG1, and PTPN11. Activation of PLCG1 leads to the production of the cellular signaling molecules diacylglycerol and inositol 1,4,5-trisphosphate, mobilization of cytosolic Ca(2+) and the activation of protein kinase C.	Upstream	AKT activation	Down	yes
HK2	hexokinase 2	-1.45E+00	3.71E-03	Hexokinases phosphorylate glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways.	Downstream of AKT	Glucose metabolism / AKT activation	Down	yes

Table 8 Differentiation PI3K/AKT/mTOR genes deregulated due to *DEPDC5*-KO

Differentiation mTOR/AKT Genes								
Gene	**Gene name**	**Mean log2FC**	**Mean pvalue**	**Selected Summary**	**Relation to MTORC1**	**Function**	**Deregulation**	**Rapamycin recovery**
THBS4	thrombospondin 4	1.80E+00	2.57E-01	Thrombospondin family members are adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions. This protein forms a pentamer and can bind to heparin and calcium.	Upstream	Cell-cell interactions	Up-	yes
MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	6.06E-01	1.68E-01	MAGUK proteins participate in the assembly of multiprotein complexes on the inner surface of the plasma membrane at regions of cell-cell contact. The product of this gene may play a role as scaffolding protein at cell-cell junctions.	Upstream	PTEN binding	Up-	yes
ERBB4	erb-b2 receptor tyrosine kinase 4	2.58E+00	1.92E-01	This gene is a member of the Tyr protein kinase family and the epidermal growth factor receptor subfamily. The protein binds to and is activated by neuregulins and other factors and induces a variety of cellular responses including mitogenesis and differentiation.	Upstream	Tyrosine Kinase Receptor	Up-	yes
FGF1	fibroblast growth factor 1	9.76E-01	2.08E-01	FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion.	Upstream	Growth factor	Up-	yes
NR4A1	nuclear receptor subfamily 4 group A member 1	2.43E+00	3.86E-01	This gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. The encoded protein acts as a nuclear transcription factor.	Downstream	Phosphorylated by AKT (Pekarsky 2001)	Up-	yes
NR4A3	nuclear receptor subfamily 4 group A member 3	1.73E+00	3.42E-01	This gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. The encoded protein may act as a transcriptional activator.	Downstream	AKT phosphorylzation (Fu et al. 2007)	Up-	yes
TNC	tenascin C	1.96E+00	2.31E-01	This protein is homohexameric with disulfide-linked subunits, and contains multiple EGF-like and fibronectin type-III domains. It is implicated in guidance of migrating neurons as well as axons during development, synaptic plasticity, and neuronal regeneration.	Upstream	Extracellular Matrix	Up-	yes
MCL1	MCL1 apoptosis regulator, BCL2 family member	5.69E-01	5.97E-01	This gene encodes an anti-apoptotic protein, which is a member of the Bcl-2 family.	Downstream	Anti-apoptotic protein	Up-	yes
SERPINE1	serpin family E member 1	2.74E+00	9.95E-15	Acts as a regulator of cell migration, independently of its role as protease inhibitor. It is involved in cellular and replicative senescence	Downstream	Hypoxia induced pathway of mTOR activation	Up	no
ATF4	activating transcription factor 4	6.85E-01	1.74E-01	Core effector of the ISR, which is required for adaptation to various stress such as endoplasmic reticulum (ER) stress, amino acid starvation, mitochondrial stress or oxidative stress Promotes the transcription of genes linked to amino acid sufficiency and resistance to oxidative stress to protect cells against metabolic consequences of ER oxidation	Downstream	Stress	Up-	yes
GNG2	G protein subunit gamma 2	2.07E+00	7.19E-02	This gene encodes one of the gamma subunits of a guanine nucleotide-binding protein. Such proteins are involved in signaling mechanisms across membranes.	Upstream	G-protein activation of AKT	Up-	yes

Gene	Gene name	Mean log2FC	Mean pvalue	Selected Summary	Relation to MTORC1	Function	Deregulation	Rapamycin recovery
THBS1	thrombospondin 1	1.74E+00	2.25E-01	This protein is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix This protein can bind to fibrinogen, fibronectin, laminin, type V collagen and integrins alpha-V/beta-1.	Upstream	Cell-cell interactions	Up-	no
EFNA5	ephrin A5	2.09E+00	2.28E-01	Ephrin-A5, a member of the ephrin gene family, prevents axon bundling in cocultures of cortical neurons with astrocytes. The EPH and EPH-related receptors comprise the largest subfamily of receptor protein-tyrosine kinases and have been implicated in mediating developmental events, particularly in the nervous system.	Upstream	Tyrosine Kinase	Up-	no
EFNB2	ephrin B2	8.27E-01	5.61E-01	The ephrins and EPH-related receptors comprise the largest subfamily of receptor protein-tyrosine kinases and have been implicated in mediating developmental events, especially in the nervous system and in erythropoiesis.	Upstream	Tyrosine Kinase	Up-	no
FZD2	frizzled class receptor 2	-8.10E-01	4.65E-01	This gene encodes a protein that is coupled to the beta-catenin canonical signaling pathway. Increased phosphorylation of AKT via PI3K (Zins et al. 2016)	Upstream	AKT phosphorylation	Down-	yes
SLC3A2	solute carrier family 3 member 2	-9.87E-01	1.86E-09	The encoded transporter plays a role in regulation of intracellular calcium levels and transports L-type amino acids.	Indirect	Amino acid transport	Down-	yes
CASTOR1	cytosolic arginine sensor for MTORC1 subunit 1	-1.21E+00	4.30E-01	Functions as an intracellular arginine sensor within the amino acid-sensing branch of the TORC1 signaling pathway.	Upstream	Arginine sensor	Down-	yes
WNT2B	Wnt family member 2B	-2.30E+00	3.54E-01	This gene encodes a member of the wingless-type MMTV integration site (WNT) family of highly conserved, secreted signaling factors.	Upstream	Wnt pathway	Down-	yes
SLC7A5	solute carrier family 7 member 5	-1.98E+00	1.69E-21	The heterodimer with SLC3A2 functions as sodium-independent, high-affinity transporter that mediates uptake of large neutral amino acids such as phenylalanine, tyrosine, L-DOPA, leucine, histidine, methionine and tryptophan	Indirect	Amino acid transporter	Down-	yes
CDKN1A	cyclin dependent kinase inhibitor 1A	-9.93E-01	2.84E-09	This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-cyclin-dependent kinase2 or -cyclin-dependent kinase4 complexes, and thus functions as a regulator of cell cycle progression at G1.	Downstream	Phosphorylated by AKT (Ying et al. 2002)	Down-	yes
COL4A2	collagen type IV alpha 2 chain	-8.42E-01	1.75E-08	This gene encodes one of the six subunits of type IV collagen, the major structural component of basement membranes.	Upstream	AKT Activation	Down-	yes
COL6A1	collagen type VI alpha 1 chain	-6.95E-01	2.17E-01	The collagens are a superfamily of proteins that play a role in maintaining the integrity of various tissues. Collagens are extracellular matrix proteins and have a triple-helical domain as their common structural element.	Upstream	AKT Activation	Down-	yes

Gene	Gene name	Mean log2FC	Mean pvalue	Selected Summary	Relation to MTORC1	Function	Deregulation	Rapamycin recovery
COL6A2	collagen type VI alpha 2 chain	-1.38E+00	5.34E-01	The product of this gene contains several domains similar to von Willebrand Factor type A domains. These domains have been shown to bind extracellular matrix proteins, an interaction that explains the importance of this collagen in organizing matrix components.	Upstream	AKT Activation	Down-	yes
LAMA5	laminin subunit alpha 5	-9.51E-01	4.99E-01	This gene encodes one of the vertebrate laminin alpha chains. Laminins, a family of extracellular matrix glycoproteins, are the major noncollagenous constituent of basement membranes.	Upstream	Extracellular Matrix	Down-	yes
MDM2	MDM2 proto-oncogene	-8.17E-01	7.11E-02	This gene encodes a nuclear-localized E3 ubiquitin ligase. The encoded protein can promote tumor formation by targeting tumor suppressor proteins, such as p53, for proteasomal degradation. This gene is itself transcriptionally-regulated by p53.	Downstream	Activated by AKT	Down-	yes
SORCS2	sortilin related VPS10 domain containing receptor 2	-1.23E+00	2.89E-01	The heterodimer formed by NGFR and SORCS2 functions as receptor for the precursor forms of NGF (proNGF) and BDNF (proBDNF)	Upstream	Activation of AKT	Down-	yes
ATP6V1F	ATPase H+ transporting V1 subunit F	-5.60E-01	3.69E-01	This gene encodes a component of vacuolar ATPase (V-ATPase), a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles.	Indirect	v-ATPase	Down-	no
NGFR	nerve growth factor receptor	-2.27E+00	4.67E-01	Low affinity receptor which can bind to NGF, BDNF, NTF3, and NTF4. Forms a heterodimeric receptor with SORCS2 that binds the precursor forms of NGF, BDNF and NTF3 with high affinity, and has much lower affinity for mature NGF and BDNF (PubMed:24908487).	Upstream	Growth Factor Receptor	Down-	no

Table 9 Proliferation and Differentiation Kegg Pathway related genes to cell cycle/p53.

Deregulated cell cycle/p53 genes							
Proliferation				**Differentiation**			
Genes	**GO terms (BP)**	**Deregulation**	**Genes not recovered**	**Genes**	**GO terms (BP)**	**Deregulation**	**Genes not recovered**
CDKN1A **BBC3** **MEG3** **PTK2B** **SERPINE1** **MDM2** **NUPR1** **TNFRSF10B**	DNA damage response Apoptotic signaling by p53 Response to hypoxia	Up	**CDKN1A** **SERPINE1**	**RPRM** **BRINP1** **NR4A1** **CEP135** **CENPU** **CENPN** **CLSPN** **PCLAF** **MAGEH1** **CDKN3** **NEK11** **TMPO** **ECRG4** **BTG1** **USP16** **HMGCS1** **EPB41L3** **PRC1** **CENPF** **GADD45B** **SRF** **MCL1** **H3-3B** **KHDRBS1** **BEX1**	Centromere complex assembly Negative regulation of mitotic cell cycle Mitotic cell cycle process	Up	**BRINP1**
PSRC1 **CCDC8** **MCM6** **AURKB** **CENPE** **CENPF** **ESCO2** **HAUS1** **KNSTRN** **MKI67** **NUCKS1** **PRC1** **PTTG1** **RRM1** **SMC4** **TACC3** **TMEM260** **TPX2**	Mitotic spindle assembly Negative regulation of sister chromatid separation Regulation of mitotic nuclear division	Down	**PTTG1**	**PSRC1** **C6orf89** **HMG20B** **IFI27L2** **PHLDA3** **IFI6** **CDKN1A** **BAX** **BBC3** **RBMS3** **G0S2**	Positive regulation of IRE1-mediated unfold protein response Positive regulation of apoptotic signaling pathway	Down	**IFI6**

GO – gene ontology; BP – biological process

Table 10 Differentiation Kegg Pathway related genes to lysosome/protein degradation.

Deregulated lysosome/protein degradation genes							
Proliferation				**Differentiation**			
Lysosome/ protein degradation Genes	**GO terms (BP)**	**Deregulation**	**Genes not recovered**	**Lysosome/ protein degradation Genes**	**GO terms (BP)**	**Deregulation**	**Genes not recovered**
N/A	**N/A**	Up	**N/A**	**N/A**	**N/A**	Up	**N/A**
N/A	**N/A**	Down	**N/A**	**ATP6VE2** **ATP6VE1** **ATP6V1F** **GLMP** **CHMP3** **CLCN7** **UBXN6** **GRN** **TPP1** **GAA** **CTSH** **DTNBP1** **SLC7A5** **SLC3A2**	Phenylalanine transport Leucine import across plasma membrane Lysosome organization	Down	**DTNBP1**

GO – gene ontology; BP – biological process

Table 11 Differentiation Kegg Pathway deregulated genes related to nucleotide and lipid metabolism.

Deregulated nucleotide and lipid metabolism genes							
Proliferation				**Differentiation**			
Genes	**GO terms (BP)**	**De-regulation**	**Genes not recovered**	**Genes**	**GO terms (BP)**	**De-regulation**	**Genes not recovered**
MGLL **COMT** **COX6C** **DHCR7** **ACSBG1** **GATM** **PYGB** **SCD** **SCD5** **PTGDS**	Fatty acid biosynthetic process	Up	**SCD5**	**FUT9** **ADK** **NMNAT2** **GATM** **HMGCR** **HMGCS1** **LBR** **MAOB** **MGAT5** **PDE4D** **PFKFB3** **ELOVL2** **ST6GAL1** **GGCT** **B3GNT5**	Fatty acid elongation Fatty acyl-CoA biosynthetic process Cellular lipid metabolic process Nucleotide metabolic process	Up	**MAOB**
CPS1 **GUCY1B1** **HK2** **RRM1** **GALNT17** **ST6GAL1** **ST6GALNAC5**	Protein glycosylation	Down	**N/A**	**MGAT4B** **MDM2** **PLCD3** **ATP6V0E2** **DGKA** **GAPDH** **GAA** **B4GALT1** **GPX4** **GSS** **HEXA** **INPP5D** **NAGLU** **PIGT** **GATB** **PFKL** **PFKP** **PKM** **CHPF2** **NADSYN** **PTGIS** **GALNT17** **BLVRB** **TKT** **UPP1** **PLA2G4** **ATP6V0E1** **DHRS3** **ATP6V1F** **CHST10** **COLGALT2** **G6PD**	Glycolytic process Glucose metabolic process Glycoprotein biosynthetic process	Down	**PLCD3**

GO – gene ontology; BP – biological process

4.8 ASD and Epilepsy risk genes are enriched in genesets deregulated in *DEPDC5*-KO hNPCs

As individuals harboring mutations in *DEPDC5* were recurrently shown to be comorbid for multiple disease phenotypes, we analyzed disease gene datasets with our KO cells (Epi25 https://epi25.broadinstitute.org/, EpilepsyGene http://www.wzgenomics.cn/EpilepsyGene/, SFARI https://gene.sfari.org/, de Rubeis et al. 2014, Iossifov et al. 2014). *DEPDC5*-KO affects 22 risk genes associated with ASD and epilepsy in the proliferating cells and 54 risk genes in the differentiation of KOs without rapamycin (Figure 8). After attenuation with rapamycin most of these risk genes showed recovery to normal expression (Table S1). Interestingly, one gene associated with epilepsy, *GNAO1*, only appears to be differentially regulated in KO cells after treatment with rapamycin. Two risk genes, *CD151* and *PDLIM1*, are deregulated in both proliferation and differentiation datasets after treatment of KO cells with rapamycin, suggesting a possible mTOR related mechanism that is carried over through development, and triggered by the overexpression of mTOR. Interestingly, the immature neuronal marker *DCX* appears attenuated by rapamycin in proliferating cells but not the differentiated dataset, indicating mTOR having effects on early hNPC differentiation. However, the cell type specific deregulated expression of *GRIN2A* (neurons) and *AQP4* (astrocytes) also suggests mTOR independent mechanisms caused by loss of DEPDC5 that affect glutamatergic and glia development.

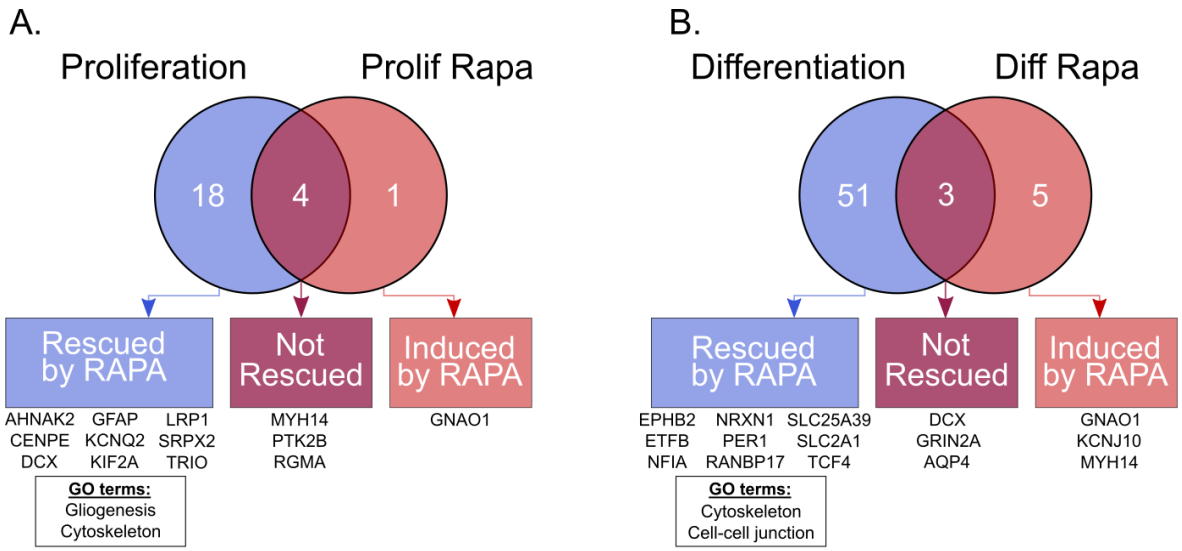


Figure 9 ASD and epilepsy risk genes associated with *DEPDC5*-KO.
Venn diagrams indicate total ASD/Epilepsy disease genes which were identified with *DEPDC5*-KO cells and their corresponding GO terms. (A) Disease genes identified in the proliferation data set with or without rapamycin (B) Deregulated *DEPDC5*-KO genes corresponding to Epilepsy/ASD data sets after 4 weeks of differentiation with or without rapamycin. *Entire lists (including mentioned genes) of differentially genes can be seen in* Table S1.

4.9 Comparison of disease gene datasets with *DEPDC5*-KO mutants

To analyze the overall effects of *DEPDC5*-KO in relation to specific disease types, associated gene lists were analyzed for enrichment within some of the largest disease studies addressing epilepsy, TSC, ASD, ID, and SCZ (see Material and Methods: 3.7.2.4). The data sets were cross referenced with significant deregulated genes associated with *DEPDC5*-KOs. An overlapping ASD dataset was observed for “Module 16” of Voineagu et al. which is a deregulated glial gene-network identified in ASD post-mortem tissue. This was recovered through rapamycin use for both proliferation and differentiation (Figure 10). We also observed significance in a Schizophrenia pruning dataset during differentiation dataset which showed resistance to rapamycin (Cocchi et al. 2015). Thus, the deregulation identified from DEPDC5-KOs is possibly comorbid with multiple diseases during development corresponding to neuronal and glial developmental processes.

Adjusted p-values also showed significant enrichment across most TSC datasets in all cell stages (Figure 10). *DEPDC5*-KO deregulated genes showed recovery in the Grabole et al. 2016 *TSC2* dataset through rapamycin use during proliferation. The Martin et al. 2017 dataset composed of TSC-induced subependymal giant cell astrocytomas (SEGAs) was significant during proliferation and differentiation and still showed significance after rapamycin treatment. Although no significance was observed from Martin et al. 2017 cortical tuber dataset during proliferation, differentiation showed significance with recovery through rapamycin use suggesting neuronal gene abnormalities during development (Figure 10B,D). Therefore, *DEPDC5*-KOs show the highest overlap in TSC datasets and have partial recovery through rapamycin use.

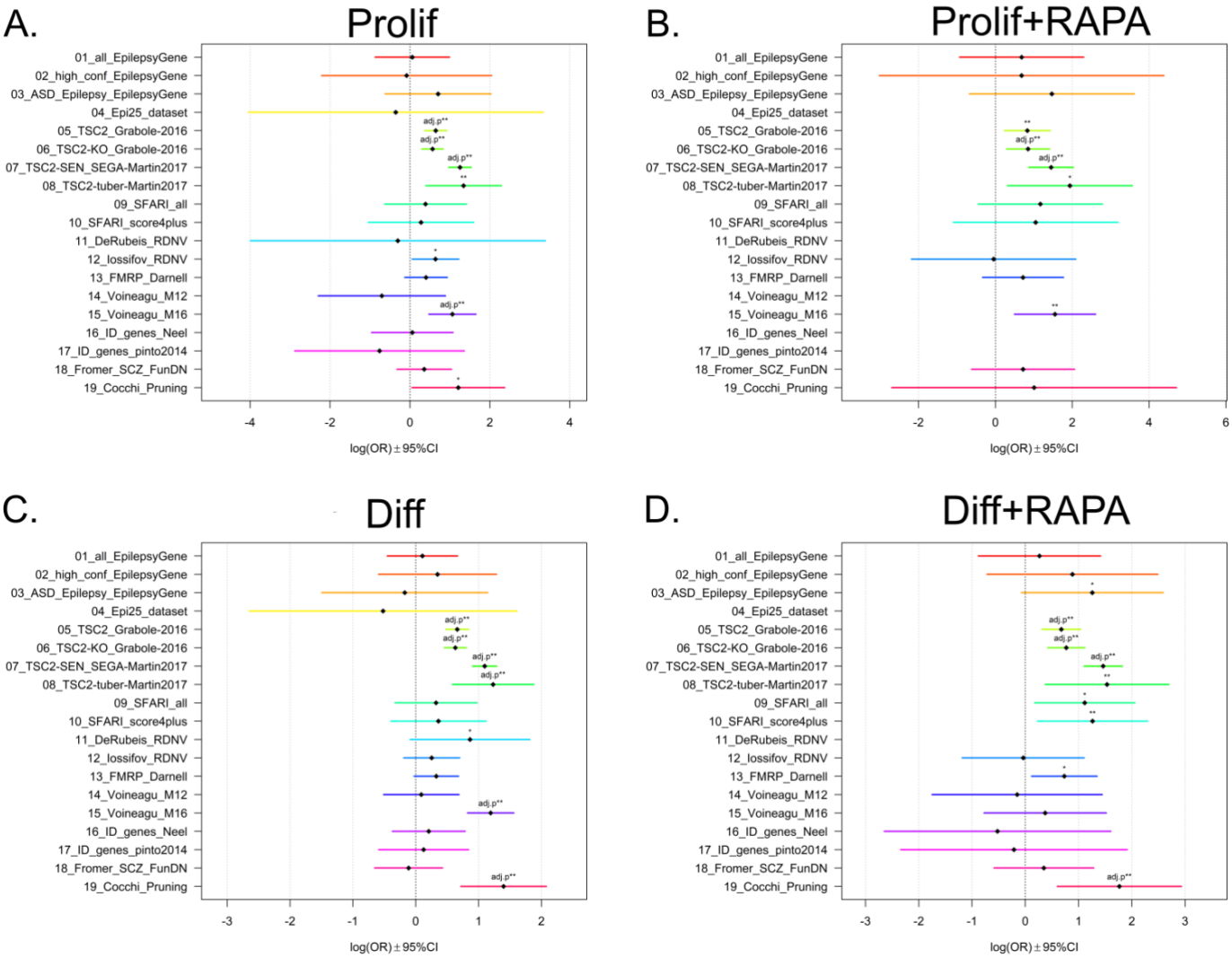


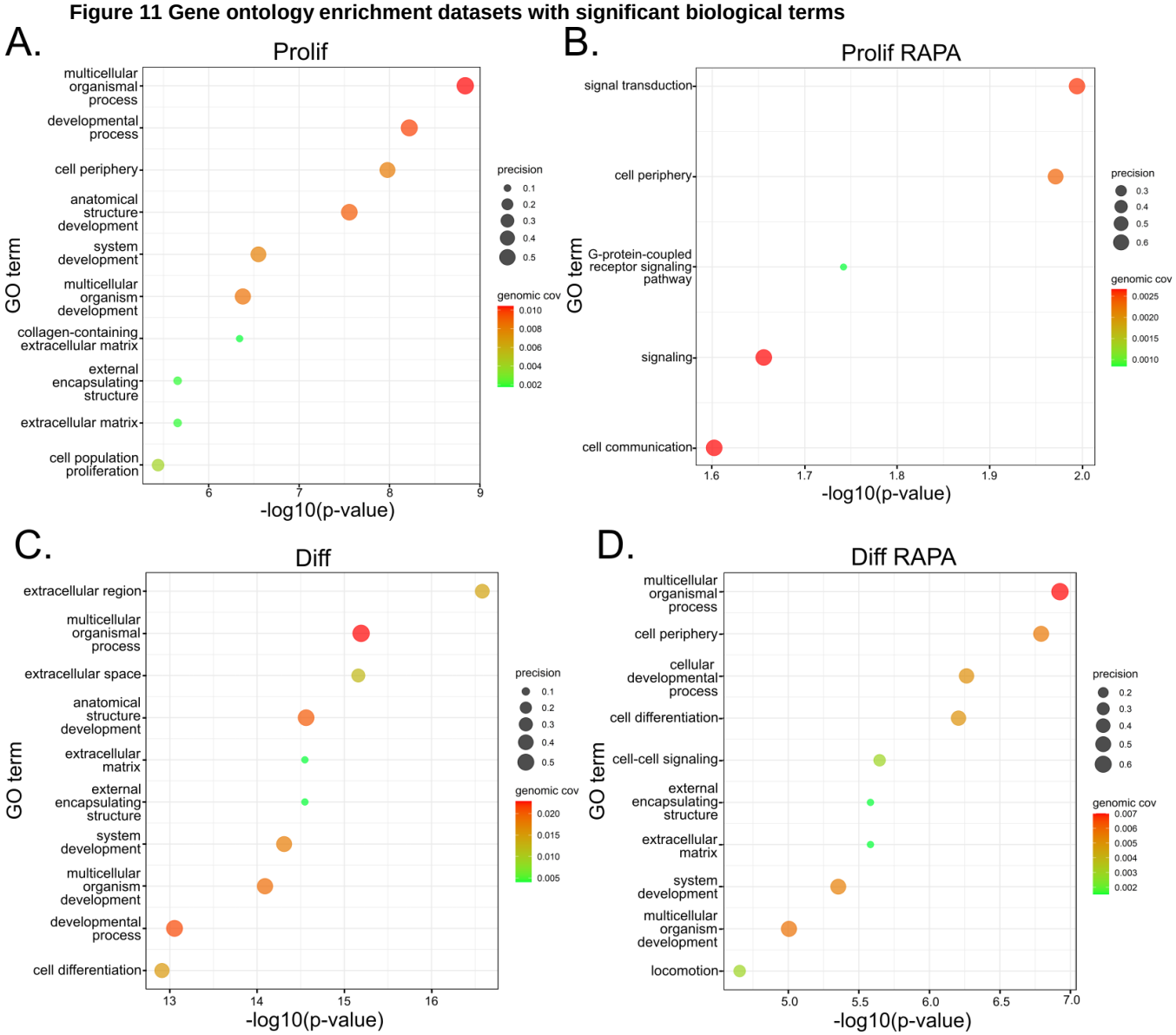
Figure 10 *DEPDC5*-KO shows significance for TSC2 disease in genome wide datasets
Deregulated genes from *DEPDC5*-KOs were compared to genomic studies for certain diseases in (A) proliferation (B) with rapamycin and (C) differentiation (D) with rapamycin. (*Epi25 https://epi25.broadinstitute.org/, EpilepsyGene: Ran et al. 2015, https://gene.sfari.org/, de Rubeis et al. 2014, Iossifov et al.2014, Voineagu et al. 2011, Darnell et al. 2011, Grabole et al. 2016, Martin et al. 2017, Pinto et al. 2014, Parishak et al. 2013, Fromer et al. 2016, Cocchi et al. 2015)*

4.10 Gene ontology term enrichment for differentially expressed genes

To investigate the deregulated networks imposed by *DEPDC5*-KO and to highlight associated biological functions of the differentially expressed genes, Gene Ontology (GO) enrichment was performed. Among the top ten significant associations (Bonferroni corr p-value < 0.05) GO terms for system development, developmental processes, anatomical structure development, extracellular matrix, external encapsulating structure and multicellular organismal process were identified in both proliferation and differentiation datasets (Figure 11A, B). Specific GO terms for *DEPDC5*-KO during proliferation were collagen-extracellular containing matrix, cell periphery and cell population proliferation (Figure 11A). Specific GO

terms identified during differentiation corresponded to extracellular region, extracellular space and cell differentiation (Figure 11C). Most of the significant GO terms during proliferation were attenuated through rapamycin use (Figure 11B). This is in contrast to differentiated *DEPDC5*-KOs where the addition of rapamycin to differentiating D62 cells could not rescue many of the GO terms (Figure 11D). However, the rapamycin added datasets had higher p-values in comparison to their non-rapamycin treated counterparts in both stages. In fact, in proliferating cells rapamycin administration resulted in fewer significant GO-terms with the lowest p-values suggesting that rapamycin does in fact reverse many of the biological disease phenotypes induced by *DEPDC5*-KO during proliferation but not during differentiation.

Figure 11 Gene ontology enrichment datasets with significant biological terms



Significant GO terms were listed in their relation to the significance from left to right in *DEPDC5*-KO cells undergoing (A) proliferation, (B) proliferation with rapamycin, (C) differentiation or (D) differentiation with rapamycin. Genomic coverage was reflected as high (red) or low (green) while precision measures were measured based upon circle size.

4.11 Weighted Gene Co-expression Network analysis of *DEPDC5*-KOs

Since GO analysis only produced results indicative of major changes in differentiation and proliferation and the overall developmental processes associated with them, we wanted to identify co-regulated genes in an unbiased fashion and analyze if other GO terms could give insight to the deregulation of *DEPDC5*-KOs. We performed WCGN analysis of RNA expression data (Figure 12A) and identified 8 clusters (Figure 12B). Unassigned genes were clustered into the grey module.

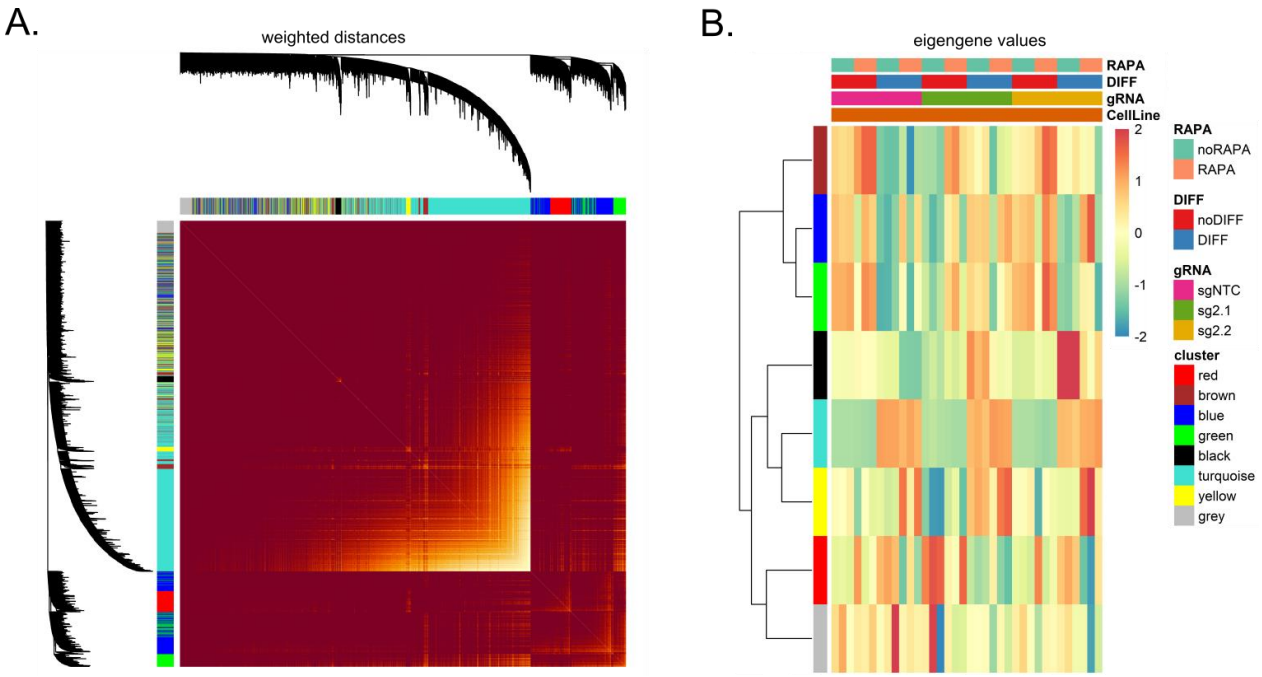


Figure 12 WCGNA of *DEPDC5*-KOs compared to NTC
(A) Hierarchical clustering of the weighted distances of DEPDC5 deregulated genes prior to merging. (B) Heatmap of the resulting 8 clusters after merging and their eigengene values.

Resulting modules were then tested for changes in expression between *DEPDC5*-KOs vs. NTC amongst all the variable conditions. This identified significance amongst the black and brown modules. The black module contained similar GO terms as seen before, namely “cell differentiation” and “cell development”. During proliferation stage, NTC expression remained static while *DEPDC5*-KOs showed significant downregulation even with rapamycin attenuation. During differentiation, the black module *DEPDC5*-KOs showed a change towards overexpression in comparison to controls which was reversed to NTC levels using rapamycin. Analysis of the brown module identified “protein production”, “protein transport” and “ribosome” specific modules (Figure 13). *DEPDC5*-KOs during proliferation showed decreased expression in the brown module which was reversed but dramatically overshoots NTC expression. Rapamycin

addition during differentiation in the brown module reverses the difference in expression closer to NTC levels. Therefore, rapamycin addition normalizes the expression in the proliferation black module and recovers most of the differences in the brown module. Without the rapamycin addition, the black and brown modules for *DEPDC5*-KOs show significant deregulation in comparison to NTC.

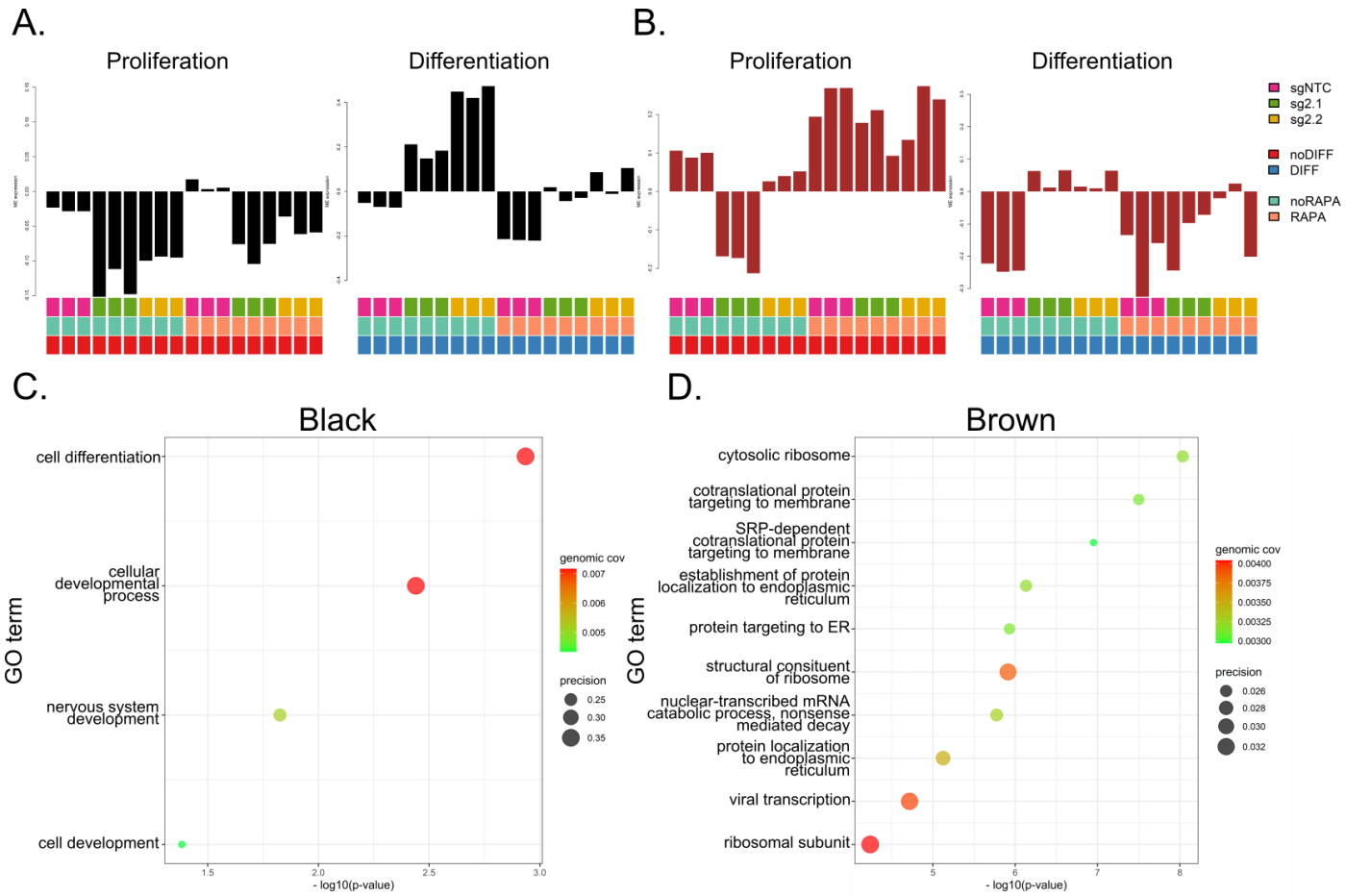


Figure 13 WCGNA identifies two modules with significance between *DEPDC5*-KOs and NTC.
Expression values of proliferation and differentiation with and without rapamycin for the (A) black and (B) brown modules. Associated GO terms for the (C) black and (D) brown modules. Insignificant modules can be seen in Figure S3.

4.12 *DEPDC5*-KOs exhibit increased differentiation and number of neurons

To investigate the hypothesis resulting from Gene Ontology and cell marker analysis indicating a disturbance of developmental processes and cellular differentiation, we investigated gene expression of known cell type specific markers. Patterns between the KOs and NTC during proliferation and differentiation stages exhibited a switch in expression between hNPC, neuron, oligodendrocyte and astrocyte markers. Although we couldn't observe consistent presence of some hNPC markers for both stages, we did observe the two hNPC markers *NES* and *SOX2* during proliferation and differentiation respectively (Table 12, Table 13). *NES* was shown to be upregulated during proliferation while *SOX2* was

shown to be downregulated during differentiation when comparing KO vs. NTC expression. Interestingly, the glial marker *GFAP* has an opposing pattern where it is upregulated during proliferation and downregulated during differentiation in KOs (also see Figure 7). This indicates that KOs have higher expression of progenitor markers during proliferation compared to NTC.

Immature neuron expression of *DCX* was strongly downregulated during proliferation and became upregulated during differentiation in comparison with controls (Table 13). We also observed significant upregulation during differentiation in other neuronal markers (*DCX, TUBB3, GRIA4, GRIN2A;* For gene names see List of genes/proteins). This pattern was also apparent from the increase of the astrocyte marker *AQP4* during differentiation while showing downregulation of the astrocyte dominant excitatory amino acid transporter 1 (*SLC1A3)* during proliferation suggesting rapid differentiation of neurons and astrocytes due to *DEPDC5*-KO.

Upon analysis using immunofluorescence, *DEPDC5*-KOs showed more MAP2 positivity when compared with controls (Figure 14A). Simultaneously, we observed significantly ($p<0.01$) increased ratios of MAP2 positive neurons in *DEPDC5*-KOs which were recovered through the use of rapamycin (Figure 14B). Upon observation, pS6 stains of differentiated cells confirmed increased hyperactivity amongst KO MAP2 positive somas which was not apparent in the NTC (Figure 14B). This indicates that insufficient *DEPDC5* results in an increase in neuronal differentiation, possibly due to an increase in downstream processes related to mTOR.

Table 12 Progenitor Cell Type Specific Markers from Transcriptomics

Gene	Gene name	Mean log2FC	Mean p-value	Selected Summary	Marker	Deregulation	Rapamycin recovery
DCX	doublecortin	-8.01E-01	6.71E-06	The encoded protein appears to direct neuronal migration by regulating the organization and stability of microtubules.	Immature neurons	Down-	yes
PAX6	paired box 6	-8.66E-01	6.44E-03	Activity of this protein is key in the development of neural tissues, particularly the eye.	Progenitor cells	Down-	yes
GFAP	glial fibrillary acidic protein	6.65E-01	2.49E-03	It is used as a marker to distinguish astrocytes from other glial cells during development.	Glia	Up-	yes
NES	nestin	5.93E-01	4.00E-07	This gene encodes a member of the intermediate filament protein family and is expressed primarily in nerve cells.	Progenitor cells	Up-	yes
OLIG1	oligodendrocyte transcription factor 1	7.61E-01	7.46E-03	Promotes formation and maturation of oligodendrocytes, especially within the brain.	Oligodendrocytes	Up-	yes
SLC1A3	solute carrier family 1 member 3	-4.51E-01	5.43E-07	Also known as EAAT2. This gene encodes a member of a high affinity glutamate transporter family.	Astrocyte	Down-	yes

Table 13 Differentiation Cell Type Specific Markers from Transcriptomic

Gene	Gene name	Mean log2FC	Mean pvalue	Selected Summary	Marker	Deregulation	Rapamycin recovery
DCX	doublecortin	2.83E+00	5.75E-20	The encoded protein appears to direct neuronal migration by regulating the organization and stability of microtubules.	Immature neurons	Up-	no
TUBB3	tubulin beta 3 class III	1.13E+00	9.56E-02	This protein is primarily expressed in neurons and may be involved in neurogenesis and axon guidance and maintenance.	Immature neurons	Up-	yes
SOX2	SRY-box transcription factor 2	-4.81E-01	5.87E-01	This intronless gene encodes a member of the SRY-related HMG-box (SOX) family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate.	Progenitor cells	Down-	yes
GFAP	glial fibrillary acidic protein	-7.46E-01	3.77E-12	It is used as a marker to distinguish astrocytes from other glial cells during development.	Glia	Down-	yes
ZNF488	oligodendrocyte transcription factor 1	-1.46E+00	2.51E-01	Transcriptional repressor. Plays a role in oligodendrocyte differentiation, together with OLIG2.	Oligodendrocytes	Down-	yes
GRIA4	glutamate ionotropic receptor AMPA type subunit 4	1.28E+00	2.16E-01	Glutamate receptors are the predominant excitatory neurotransmitter receptors in the mammalian brain and are activated in a variety of normal neurophysiologic processes.	Neurons / Astrocytes	Up-	yes
GRIN2A	glutamate ionotropic receptor NMDA type subunit 2A	2.27E+00	1.97E-01	NMDA receptors are both ligand-gated and voltage-dependent, and are involved in long-term potentiation, an activity-dependent increase in the efficiency of synaptic transmission.	Neurons / Astrocytes	Up-	no
AQP4	Aquaporin 4	2.23E+00	2.26E-02	This gene encodes a member of the aquaporin family of intrinsic membrane proteins that function as water-selective channels in the plasma membranes of many cells.	Astrocyte	Up-	no

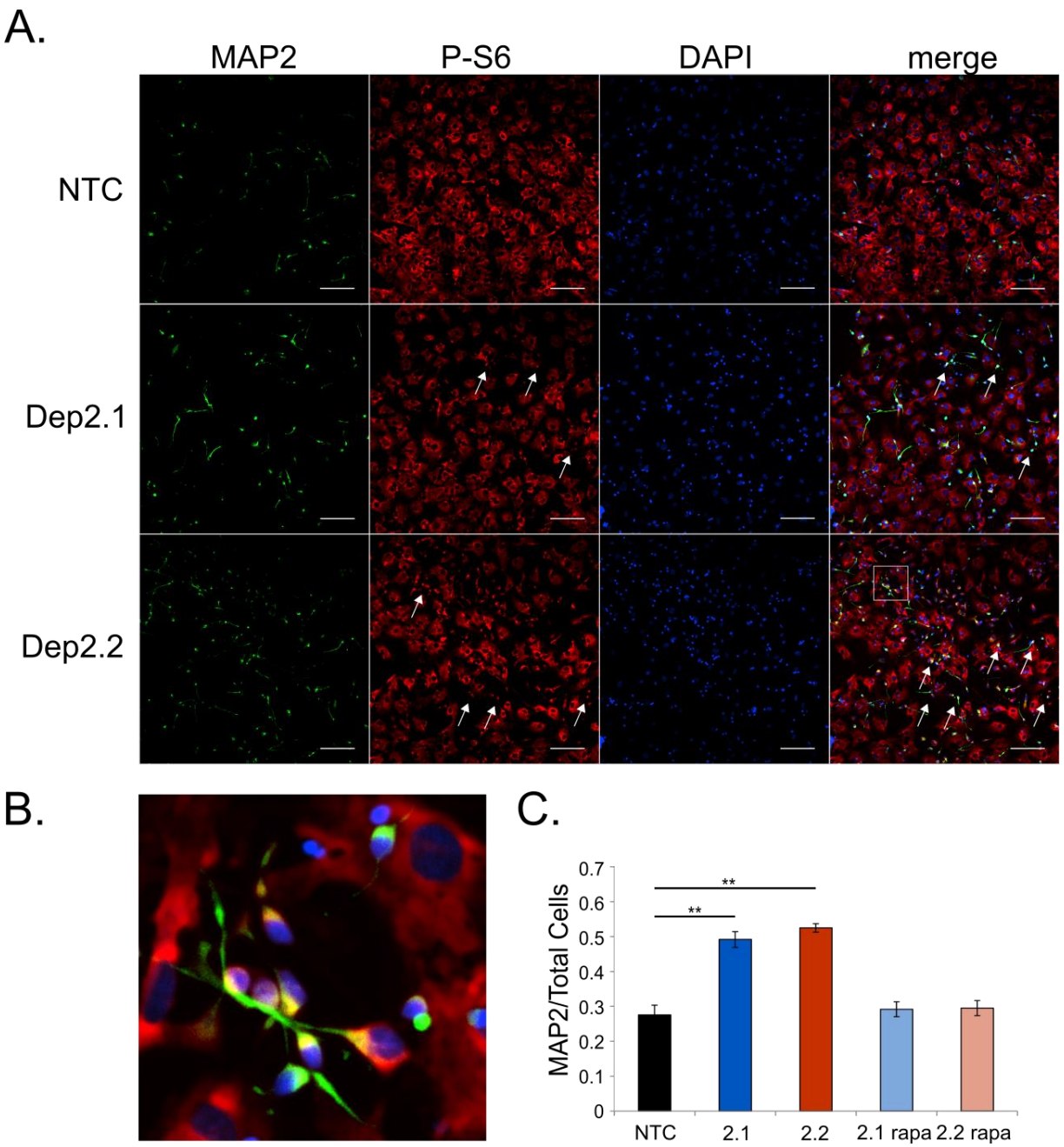


Figure 14 *DEPDC5*–KO cells produce higher amounts of neurons
DEPDC5-KO hNPCs were differentiated for one month and stained with MAP2 and p-S6. (A) KO cultures show higher amounts of MAP2 positive neurons and observed co-localization with pS6 (white arrows) in the cell soma. (B) An enlarged view of Dep2.1 neurons showing possible colocalization of pS6 and MAP2. (C) Ratios of MAP2 positive neurons vs. total cells reveal an increase of neurons in KO cell lines which was reversed by the chronic administration of rapamycin. Groups were compared with Students t-test compared to control **p<0.01. Error bars represent ± SEM.

4.13 *DEPDC5*-KOs show increased dendritic arborization with decreased soma and axon length

Previous studies have reported increased dendritic arborizations resulting from *DEPDC5* mutations (Fusco et al. 2020). To investigate these findings, we analyzed the neurite projections from MAP2 positive somas in differentiated *DEPDC5*-KOs. The neurite length was significantly (p<0.01) decreased in

DEPDC5-KOs. When the cells were treated with rapamycin chronically during differentiation, the neurites produced even shorter projections and did not recover the decreased length (Figure 15A). Sholl analysis of the mean maximum intersections did show an increase in neurite arbors of *DEPDC5*-KO neurons. Yet this also was not attenuated from the treatment of rapamycin (Figure 15B). Together this shows that differentiation of *DEPDC5*-KOs from hNPCs produced shorter neurite lengths and higher neurite arborization but cannot be recovered through the chronic administration of rapamycin suggesting the loss of DEPDC5 causes morphological changes independent of mTOR.

As mTOR hyperactivation has been reported to significantly increase soma size in *TSC2* and *DEPDC5*-KOs (Iffland et al. 2020) we measured the soma sizes of nestin positive cells and MAP2 positive neurons. Interestingly, we found reductions in the soma sizes in both NPCs and neurons ($p<0.01$; Figure 15C, D). When rapamycin was administered, overall soma sizes failed to return to normal, also suggesting the cell size differences observed in our system were not related to mTOR.

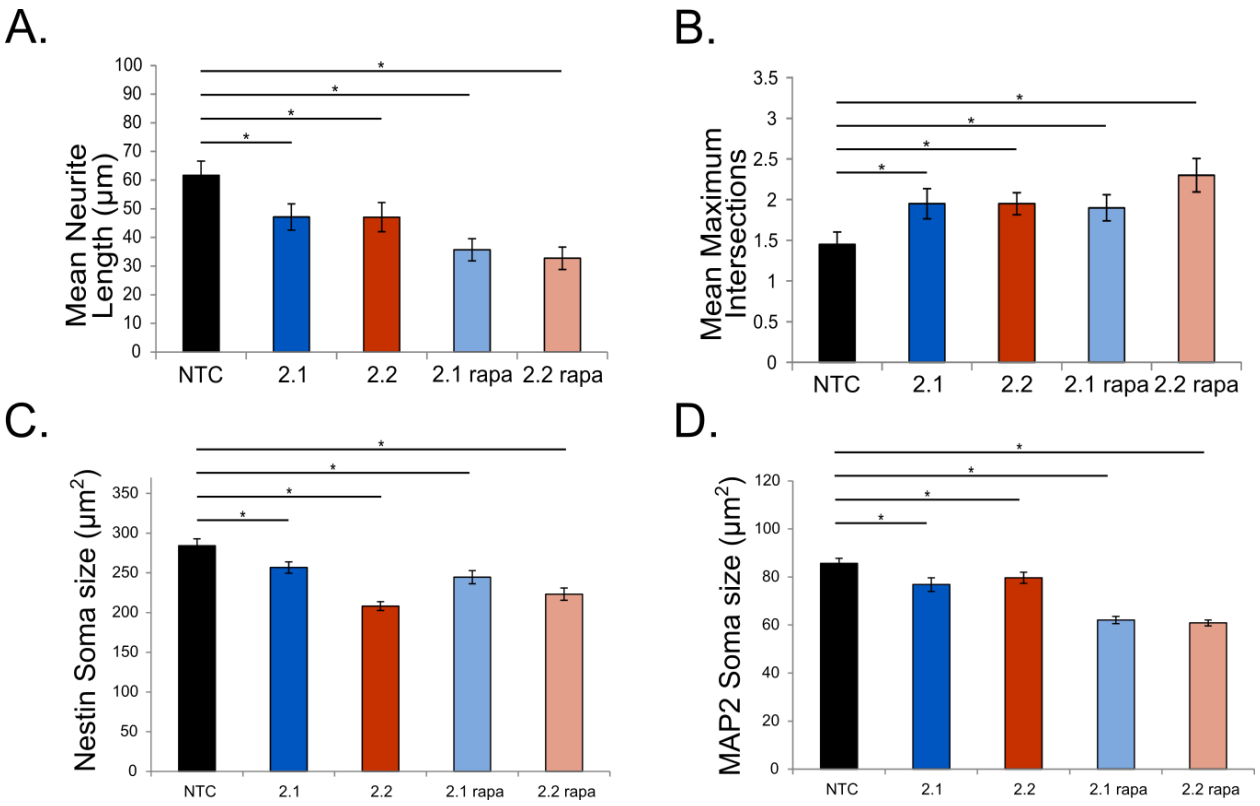


Figure 15 *DEPDC5*-KO show dendritic arborization and smaller soma sizes
(A) Neuron neurite length show decreases in length compared to controls which do not recover with the addition of rapamycin. (B) Mean maximum intersections are increased in *DEPDC5*-KOs and do not return to normal with chronic administration of rapamycin. (C) KO NPC soma sizes display smaller areas in controls which rapamycin fails to reverse. (D) MAP2 soma sizes were measured showing the same effect as NPCs. Students t-test, $p<0.05$. Error bars represent ± SEM. (20 cells were counted for A, B; more than 150 cells were counted for C, D).

4.14 *DEPDC5*-KO deregulated gene expression during development

In order to understand the developmental expression of the significant genes identified in *DEPDC5*-deficient hNPCs, we used spatial and temporal gene brain expression data incorporating the previously published 29 co-regulated gene modules using parts of the in house pipeline MAGNET-lite (Kang et al. 2011, Yousaf et al. 2018). Modules were generated from post-mortem tissue taken from multiple stages of development and their corresponding gene expression. Deregulated *DEPDC5*-KO genes in proliferation stages are enriched for genes in modules 1 (321 total genes) and 2 (2545 total genes) of the Kang et al. dataset while genes affected in differentiated KOs associated with modules 1, 2 and 18 (78 total genes) (Figure 16A). Eigenvalues show the highest activation of module 1 in the hippocampus (HIP) and amygdala (AMY) while having lower expression in the orbitofrontal cortex (OFC), dorsal frontal cortex (DFC), medial frontal cortex (MFC) and cerebellar cortex (CBC). Module 2 showed expression in later developmental stages with minor expression in the mediodorsal nucleus of the thalamus (MD), HIP and CBC. The module that appears affected by *DEPDC5*-KO only during differentiation stages, module 18, shows similar expression to module 1 in early stages with the inclusion of the MD and striatum (STR) with consistent expression in the HIP, AMY and CBC.

We then utilized the MAGNET-lite pipeline to select the top connected genes amongst each module and mapped the deregulated genes into this hub network. Among the top 36 genes ranked by network connectivity the hub gene *TOP2A* was affected in both proliferating and differentiating KO cells. The gene *NUSAP1* was also present as a network hub gene for proliferating KO cells. In module 2, we identified the differentially expressed hub genes *ACSBG1* and *TF* for the proliferation and differentiation datasets respectively. The differentiation dataset identified the six deregulated hub genes: *SLC12A4, FKBP10, RFX4, BMP7, NOTCH1*, and *LRP10* for module 18. Together, these hub genes are likely to be the main drivers during *DEPDC5*-KO and may be essential to causing cellular disruption and disease.

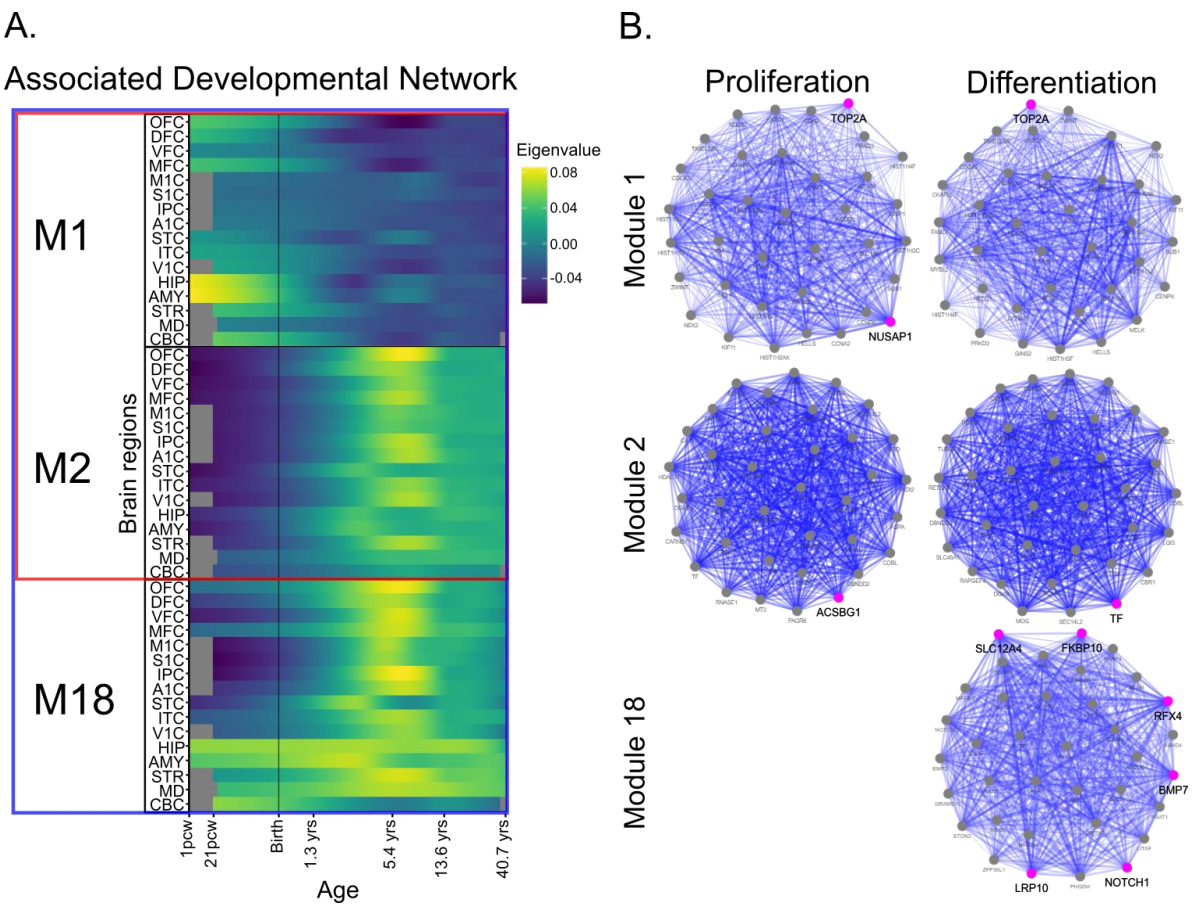


Figure 16 Spatially and temporally expressed genes corresponding to *DEPDC5*-KO
(A) The associated developmental networks correspond to module 1 and 2 for both proliferation and differentiation *DEPDC5*-KO expressed genes while module 18 is differentiation specific (B) Hub gene networks developed for the three identified modules in proliferation and differentiation datasets. Only adjacency >0.4 are shown to depict network density. *OFC: orbital prefrontal cortex; DFC: dorsolateral prefrontal cortex; VFC: ventrolateral prefrontal cortex; MFC: medial prefrontal cortex; M1C: primary motor (M1) cortex; S1C: primary somatosensory (S1) cortex; IPC: posterior inferior parietal cortex; A1C: primary auditory (A1) cortex; STC: superior temporal cortex; ITC: inferior temporal cortex; V1C: primary visual (V1) cortex; HIP: hippocampus; AMY: amygdala; STR: striatum.*

5. Discussion

5.1 Primary hNPCs are suitable to study early development

In this study, primary human cells extracted from a developing human fetal cortex exhibited a radial glial phenotype and developed into neurons after 1 month of differentiation. As expected, undifferentiated D62 cells expressed the proliferation marker Ki67 and showed expression of the neural progenitor markers Nestin, SOX2, GFAP as well as PAX6 at protein level. This indicates that undifferentiated cells are in fact exhibiting an early neurodevelopmental stage. After 1 month of differentiation, immature and mature neurons expressed their respective markers, TUJ1 and MAP2, showing that the cells can be induced to become more mature subtypes and can model the developmental processes of cell maturation in the fetal brain.

Differentiation of D62 cells led to RNA expression of the immature neuronal markers *DCX*, *TUJ1* (*TUBB3*) and the expression of NMDA and AMPA receptors related to neuronal excitability. However, the observed increase in cell density from immunocytochemical stainings and overall continuous expression of the progenitor/astrocyte marker GFAP also suggests that the differentiation of the cells not only results in neurons, but continuously expands upon the previous progenitor population potentially through asynchronous division. Conversely, GFAP was downregulated during differentiation, suggesting a decrease in the underlying proliferation of neural progenitor cells. Transcriptomic analysis suggests the formation of astrocytes after 1 month of differentiation as identified by the mature astrocyte marker *AQP4* and previous downregulation of the astrocyte dominant excitatory amino acid transporter, *SLC1A3,* during proliferation. We thus assume that our differentiated cultures contain astrocytes creating a cell-cell network necessary for developmental processes. However, this heterogeneous batch culture made unbiased cell counting from immunological stainings difficult as *AQP4* resides on the cell membrane forming tight junctions and creates a challenge with identifying the boundary between different cell types. Therefore, follow-up experiments on separated cell types (e.g. single cell RNA-Seq) would allow further characterization of this cell model.

Differentiated primary human neural progenitor cells have been shown to be functionally similar in gene expression and development over time with post mortem brain tissue during GW 15-21 (Stein et al. 2014). This accurate representation of human developmental processes provides a unique model which more closely relates to *in vivo* development. Though the continuous expansion of these cells often results in

eventual senescence, this was not observed in our population where growth was maintained past passage 32. Thus, D62 cells develop characteristics similar to *in vivo* development and are suitable in modeling early human brain development *in vitro*. Therefore, the rest of the discussion will focus on *DEPDC5*-KOs and their relation to NTC.

5.2 hNPC *DEPDC5*-KOs compared to NTC

5.2.1 Successful generation of DEPDC5-KO hNPCs via CRISPR-Cas9 lentiviral transduction

We have shown the successful indel formation at two loci of exon 2 in the *DEPDC5* gene using CRISPR-Cas9. Subsequent protein analysis confirmed successful KO of DEPDC5 in that expression of the target protein was not visible while being present in control cell lines. We also identified few offtargets overlapping with deregulated genes. However, all offtargets resided in intronic or intergenic regions and were upregulated indicating that the overlap was coincidental and not due to loss of function.

Interestingly, transcriptome analysis showed expression of *DEPDC5* with little variation across knockout models and controls. Thus, it can be hypothesized that the mutated mRNA undergoes nonsense mediated decay (NMD) resulting in degradation of mRNA transcripts prior or during protein synthesis. Based on the absence of *DEPDC5* protein we can exclude that repair mechanisms restore mutated mRNA and allow protein synthesis (Karamyshev and Karamysheva et al. 2018). Reinforcing this, WGCNA of the KO cell lines also indicates rapid alterations caused by *DEPDC5*-KO and GO terms related to ribosomes, protein production and nonsense mediated decay. This correlation fits our findings and we thus conclude that *DEPDC5* was successfully knocked out in the hNPC lines.

5.2.2 KO of DEPDC5 increases activity of mTOR

Western Blot experiments show hyperactivation of the mTOR pathway induced by insufficient DEPDC5 protein when incubated in media with and without amino acids. Increased phosphorylation of S6 during amino acid depletion corroborates previous studies of KO models of *DEPDC5* and *TSC2* and thus confirms the suitability of our model (Hughes et al. 2017, Costa et al. 2016). KO mouse models of *DEPDC5* and *TSC1/PTEN* in hippocampal neurons also show increased pS6 activity similar to our observations of S6 phosphorylation in MAP2 positive cells (Yuskaitis et al. 2019, Westen et al. 2014). Administering rapamycin reverses the hyperactivation of mTOR and recovers the increased phosphorylation of S6 in progenitors, reinforcing that the hyperactivity in *DEPDC5*-KOs is indeed

mTORC1 related. Therefore, our investigation at the functional level confirms that loss of DEPDC5 modulates mTOR activity.

5.2.3 *DEPDC5*-KO impairs epilepsy and ASD associated processes

Analysis of transcriptome data generated from *DEPDC5*-KOs showed deregulation in a variety of different cellular mechanisms including protein and ribosome biogenesis, ER membrane transport, PI3K/AKT/mTOR pathway, p53/cell cycle, nucleotide and lipid metabolism or lysosome/protein degradation. This finding was confirmed at single gene, GO term, KEGG, and WGCN analysis. All processes have been identified as having relation to mTOR (Saxton and Sabatini et al. 2017). An analysis of possible consequences related to neurological diseases is elaborated for the most prominent pathways identified.

5.2.3.1 Protein and ribosome biogenesis

Interestingly, WGCNA identified the brown module related to protein synthesis with GO terms pointing to ribosomes, protein synthesis, ER targeting and nonsense mediated decay processes as being downregulated during proliferation and upregulated during differentiation in *DEPDC5*-KOs. *DEPDC5* is an inhibitor of mTOR which regulates protein production and ribosome biosynthesis through its activation of the S6 and 4E-BP1 pathways (Holz et al. 2005, Biever et al. 2015). We identified S6 as being significantly ($p<0.002$) downregulated in proliferating *DEPDC5*-KOs which has been implicated in alterations in dendritic spine density causing ASD (Lu et al. 2021, Lo et al. 2020). In a recent study comparing iPSCs and post mortem tissue of ASD patients, alterations in protein targeting to ER" and "ribosome biogenesis" were also identified as mechanisms related to ASD (Griesi-Oliveira et al. 2020). Concurrently, associations of ribosome biogenesis with neurodevelopmental diseases are becoming more prominent and provide potential targets for treatment (Hetman and Slomnicki 2019). We also found that rapamycin reverses most of these alterations during proliferation and differentiation indicating that the alterations are due to mTOR overactivation. Thus, loss of DEPDC5 alters protein and ribosome biogenesis and is a consequence of mTOR deregulation. This is likely is a driving factor of abnormal cellular processes and could be linked to developing ASD during early development.

5.2.3.2 PI3K/AKT/mTOR

We confirmed that loss of DEPDC5 leads to deregulated expression of PI3K/AKT/mTOR pathway genes (Bar-Peled et al. 2013). Despite the lack of specific mTOR subunit differential expression, we observed

consistent changes in genes regulating upstream and downstream processes of mTOR (see Table 7, Table 8). Downstream processes will be addressed in the following subsections (see 5.2.3.3, 5.2.3.4, 5.2.3.5). Our identification of upstream deregulated genes during proliferation and differentiation related to extracellular matrix and signaling mechanisms of mTOR is indicative of reactive mechanisms caused by *DEPDC5* loss. This explains how mTOR can cause alterations at the plasma membrane and disrupt large cellular networks. We identified gene expression differences in growth factor receptors, collagen proteins, and glycoproteins all related to the extracellular matrix.

The extracellular matrix is essential for proper cortical development and folding processes in the brain and is the essential scaffold allowing neuron migration and differentiation. The repressed neuron migration observed in *DEPDC5*-KO rat models and decreased neuronal density in deeper cortical layers in mice models are thought to be due to alterations in the extracellular matrix (Marsan et al. 2016; Yuskaitis et al. 2018). Altered FGF, NGF or BDNF signaling has been implicated in a wide range of neurodevelopmental diseases including ASD and is often associated with abnormal cortical development (Turner et al. 2015). Altered expression of collagen genes has been shown to occur in a valproic acid rat model of autism similar to the deregulated collagen gene expression in our study (Olde Loohuis et al. 2017). Along with this, extracellular glycoproteins have also been linked to ASD (Dwyer and Esko 2017). Thus, *DEPDC5*-KOs result in altered extracellular matrix and growth factor signaling gene expression which could cause developmental abnormalities in scaffolding and neuronal migration thus generating the developmental abnormalities observed in ASD, TSC and epilepsy.

5.2.3.3 P53/cell cycle genes

KEGG pathway analysis revealed the interaction between *DEPDC5-KO*s and *p53* or cell cycle related mechanisms. The interplay between mTOR and *p53* is complex and alterations in feedback mechanisms between the two have been shown to lead to epileptic seizures (Cui et al. 2021). *p53* acts as a modulator of stress conditions such as DNA damage, hypoxia, oxidative stress or oncogene activation. We identified *MDM2*, a main inhibitor of *p53* expression, in our transcriptomic analysis. Upregulation of *p53* and downregulation of *MDM2* has been shown to be a common mechanism in human hippocampal samples of temporal lobe epilepsy which was also observed during *DEPDC5*-KO differentiation (Engel et al. 2007). This mirrors other findings regarding protein deregulation mechanisms where ER stress has been shown to cause upregulation of *MDM2* and jeopardizes protein translation leading to decreased neural activity

during seizures (Liu et al.2019). Likely, the disbalanced DEPDC5 availability in patients induces ER stress which causes the activation of *p53* genes something we also observed from WCGNA analysis (Figure 13).

In addition, changes in *p53* expression were further corroborated in GO-term analysis emphasizing “cell population proliferation” and “system development”. Loss of *p53* is associated with variations in cell population and differentiation in multiple studies; *DEPDC5*-KO cells clearly showed enhanced differentiation of MAP2 positive cells potentially resulting from these *p53* abnormalities (Henriksson et al. 2020, Navarro et al. 2020). Interestingly, brain organoid experiments in *p53* knock-down iPSCs also corresponded with abnormal structure development and changes in neuronal migration indicating clear relation to developmental mechanisms (Navarro et al. 2020). Taken together, the rapid proliferation and differentiation of *DEPDC5*-KOs is likely to result from abnormal *p53* expression caused by mTOR hyperactivity and in turn can be a developmental cause of epilepsy and ASD.

5.2.3.4 Nucleotide and lipid metabolism

The significance of lipid-metabolic expression alterations caused by loss of DEPDC5 in both proliferation and differentiation stages relates to the complex mechanisms tied to the mTOR pathway. Particularly GO term analysis identified processes related to lipid metabolism (fatty acid elongation, cholesterol biosynthetic process, glycolipid metabolic process, membrane lipid biosynthetic process) and nucleotide synthesis (NAD biosynthetic process) in both cell developmental stages. Both pathways are influenced by mTOR downstream activation and deregulation and have been shown to cause neurological disease (Lamming and Sabatini 2013, Valvezan et al. 2017). As mTOR is a known regulator of cell growth, changes in activity would correspond with alterations in subsequent lipid and nucleotide production to fulfill cellular demands.

Lipid synthesis occurs in the smooth ER, after which lipids are often exported to the other organelles of the cell (Pichler and Emmerstorfer-Augustin et al. 2018). Such interplay also correlates with possible mechanisms discussed previously for ribosome and protein biogenesis at the ER. Studies analyzing lipid metabolism have shown association with epilepsy and ASD phenotypes. Reduced gene expression of lipids has been reported in human hippocampal sections taken from patients with temporal lobe epilepsy and altered fatty acid metabolic pathways have also been reported in individuals with autism suggesting common deregulation between both diseases (Ajith et al. 2021, Tamiji and Crawford 2010). Cholesterol and fatty acid acylation both play roles in establishing lipid-raft platforms allowing modifications for

neurotransmitter receptors (Borroni and Barrantes et al. 2016). Due to the significant downregulation of these mechanisms in *DEPDC5*-KOs it is likely that lipid-raft destabilization could generate receptor imbalances and would result in the classical excitatory/inhibitory imbalances seen in zebrafish *DEPDC5*-KOs (Swaminathan et al. 2018). A lack of sufficient lipids would also correspond to the decreases in cellular size. Thus, lipid imbalances are likely to be a mechanism underlying abnormal neuronal function leading to ASD or epilepsy phenotypes.

GO terms also found the upregulation of nucleotide synthesis due to loss of DEPDC5. Nucleotides are essential for the formation of nucleic acids along with being essential for cell-cell communication mechanisms. Thus, purine metabolism enzymes have gained more focus and mutations have been implicated in epileptic seizures (Garcia-Gil et al. 2018). Adenosine signaling provides cross talk between neurons and astrocytes using adenosine kinase (*ADK*), a gene overexpressed upon *DEPDC5*-KO differentiation. *ADK* overexpression is related to spontaneous seizures and temporal lobe epilepsy due to loss of neuronal excitability and neuroprotection by astrocytes (Boison et al. 2012). Increased mTOR activity drives increased ribosomal RNA synthesis which depletes nucleotide pools and can also trigger DNA repair mechanisms and *p53* activity linking the previously mentioned pathways (Hastak et al. 2008, Valvezan et al. 2017). Nucleotide depletion would correspond with our observation of an upregulation of nucleotide synthesis as the KO cell lines are attempting to restore homeostasis. DEPDC5-KOs also identified upregulation of the stress response factor *ATF4* which is directly phosphorylated by mTORC1 driving downstream purine synthesis (Lui and Sabatini et al. 2020). Therefore, nucleotide imbalances play a potential role in *DEPDC5* related epileptic seizures.

5.2.3.5 Lysosome/protein degradation

Lastly, we identified consistent downregulation of a host of lysosome transporters and protein degradation mechanisms. Again, mTOR activity has been shown to be a negative regulator of lysosomal function and of protein degradation comparable to our transcriptomic observations (Zhou et al. 2013, Zhao et al. 2015). Either increased or decreased acidification has resulted in decreased neurite elongation and loss of excitatory synapses leading to epileptogenesis in *TSC1* mouse models (McMahon et al. 2012, Fassio et al. 2018). Other studies have identified disrupted neural tube development, ubiquitinated protein accumulation and an overall hyperproliferation due to misregulated transcription factor recycling (Fassio et al. 2020, Cecconi et al. 2007, Fimia et al. 2007). This also corresponds with the identified GO terms

“cellular proliferation” and “system development” alterations caused by *DEPDC5*-KOs. Therefore, inhibition of lysosomal activity and of the subsequent protein degradation as induced by the loss of DEPDC5 can alter formation of proper neuronal morphology and cellular distribution during development.

5.2.3.6 Summary

Loss of DEPDC5 causes downstream deregulation of a variety of cellular processes ranging from mTOR upstream and downstream mechanisms, DNA and protein synthesis, alterations in lipid and nucleotide synthesis and protein degradation mechanisms within the cell. Disruption in any of these pathways has been associated with epilepsy and ASD phenotypes. Therefore, it can be assumed that mitigating such mutations with drugs might be challenging as interference with one pathway could also deregulate other pathways. Rapamycin and other mTOR inhibitors have been promising in mitigating the diseases related to mTOR deregulation and showed significant promise in our study. However, careful considerations need to be deliberated in treatment as it doesn’t attenuate mTOR independent mechanisms.

5.2.4 Protective mechanisms and reverse feedback activations due to DEPDC5-*KO*

An aspect of our transcriptomic data is the lack of specific mTOR or AKT genes/subunits identified. Despite discovering many of the same downstream pathways related to mTOR hyperactivity, the direction of expression of many of the genes conflicts with previous studies. Our analysis mainly identified downstream or upstream regulators which can affect or be affected by mTOR function and many genes which are known to be direct regulators showed similar expression to controls. For example, the known mTOR-4EBP-1 pathway and the many genes related were not observed. Transcriptomic and proteomic analysis of loss of DEPDC5 in zebrafish identified similar lack of mTOR or AKT genes (Swamanathan et al. 2018).

Studies analyzing protein and RNA expression found positive correlations between both in two separate analyses of the same data set resulting in 40% or 56%-84% overlap in expression (Liu et al. 2016). To explain the differences in expression direction, we suggest a mix of reactive mechanisms caused by mTOR deregulation and reverse pathway mechanisms as possible explanations (Figure 17).

5.2.4.1 mTOR hyperactivity causes activation of protective mechanisms

First, it is possible that our transcriptomic data does not fully reflect the cellular mechanisms occurring and the cells are exhibiting a reactionary mechanism to *DEPDC5* loss. Many of the known pathways related to

mTOR hyperactivity are identified in our study (ribosome biogenesis, nucleotide synthesis, lipid synthesis, lysosome biogenesis, glycolysis, autophagy). Western blot analysis of mTOR hyperactivity and the clear mitigation through rapamycin use emphasizes this. Therefore, downregulation of protein production such as RPS6 and downregulation of ribosomal proteins during differentiation could correspond with cell survival mechanisms to mitigate mTOR hyperactivity. The dramatic downregulation of amino acid transporters *SLC7A5, SLC3A2, SLC25A39 and SLC38A10* could also be the result of counteracting the loss of DEPDC5 as decreases in amino acids would normally cause mTOR activity to cease. *SLC7A5* and *SLC3A2* were also deregulated in a study analyzing mTORC1 function through *ATF4* loss indicating that these transporter imbalances are likely related towards nucleotide imbalances caused by mTOR deregulation (Torrence et al. 2021). *SLC7A5* and *SLC3A2* form a heterodimer which functions as a sodium-independent neutral amino acid transporter. It is responsible for the transport of phenylalanine, tyrosine, threonine, cysteine, L-DOPA, tryptophan, histidine, methionine and leucine while also exchanging glutamine (Scalise et al. 2018). This allows sufficient leucine concentration in the lysosome which recruits v-ATPase and allows mTOR to bind to the Rag GTPases. Similarly, we also found other v-ATPases such as *ATP6V0E1*, *ATP6V0E2* and *ATP6V1F* to be downregulated in *DEPDC5*-KO cells.

Identification of *RPS6* downregulation in progenitor cells is opposite to what was observed through western blot analysis. This suggests mechanisms where loss of DEPDC5 and subsequent increased mTOR activity results in transcription changes attempting to circumvent functional changes within the cell. This hypothesis is further reinforced by the upregulation in *REDD1* during differentiating cells as *REDD1* reacts to cellular stress and negatively regulates mTOR activity through TSC1/2. *REDD1* was also deregulated in a transcriptome study of mouse ESCs using *ATF4* KOs causing an mTORC1 feedback loop (Torrence et al. 2021). Therefore, cellular survival mechanisms are attempting to counteract mTOR activity on the transcriptional level and through rapamycin the effects are fully restored. Similar to other studies, amino acid sensors which inhibit *DEPDC5* were identified (Torrence et al. 2021; Swaminithan et al. 2018). We also saw deregulation in the *DEPDC5* inhibitor *CASTOR1* which binds to arginine causing DEPDC5 inhibition. This could either be a mechanism which tries to inhibit mTOR activity through the amino acid sensing pathway, or could be related to the heavy deregulation in nucleotide synthesis mechanisms caused by mTOR activity.

5.2.4.2 Negative feedback loop through loss of DEPDC5

The other hypothesis is that mTOR hyperactivity is mitigated through reverse feedback mechanisms during differentiation. Other cellular pathways which work parallel to mTOR are interconnected and recent evidence also suggests that many contain reverse feedback mechanisms to modulate the cellular environment and signals (Ghomlaghi et al. 2021, Rozengurt et al. 2014). For example, though AKT can repress TSC1/2 expression, it can also activate transcription factors in the cell nucleus.

mTOR activity has been known to induce cellular stress by activating the DNA damage response along with ER stress caused by increased protein translation. These mechanisms were observed from our analysis. Feedback loops attempting to circumvent mTOR hyperactivity are able to suppress these mechanisms and in fact change the expression of all associated genes.

In mouse skeletal muscle cells, *REDD1* has been shown to be induced through energy stress responses where it acts to inhibit ATP-demanding processes such as glycogen storage and protein synthesis, similar to GO terms observed in hNPCs (Britto et al. 2018). This mitigated repression prevents cellular growth and allows activation of p53 mechanisms to repress mTOR activity through *TSC1/2*. Though whether DNA damage responses or ER stresses cause such suppression, the overall effects would result in mTOR hypoactivity and thus causes the same downstream mechanisms to become downregulated. This would correlate with transcriptomic data during the differentiation dataset and explain the increased downregulation in mTOR downstream processes. Changes related to decreased cell size would also fit to this explanation as decreased mTOR activity has been shown to result in smaller cell sizes. Thus, a switch to mTOR hypoactivity during differentiation is a new and novel finding not yet reported in previous studies.

Further analyses at proteomic level should help clarify which of these hypotheses are accurate and to help understand the functional mechanisms and how they relate to gene expression, ASD and epilepsy.

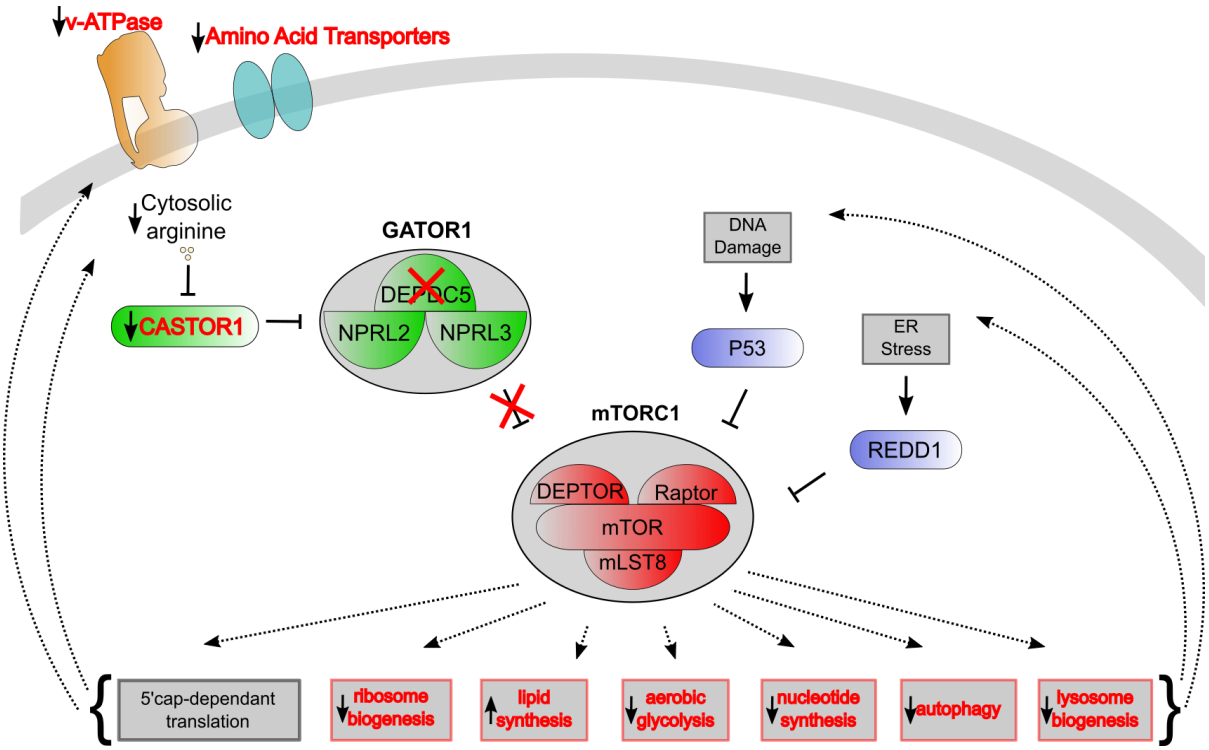


Figure 17 Schematic overview of protective and feedback mechanisms due to DEPDC5-KOs
Loss of DEPDC5 causes an increase in mTOR hyperactivity. This deregulation affects multiple downstream pathways which cause feedback and protective mechanisms to activate attempting to mitigate or prevent the effects caused by DEPDC5 loss. Activation of *p53* and *REDD1* attempt to inhibit mTOR activity. Concurrently, decrease in amino acid transporters causes amino acid sensors (*CASTOR1*) to decrease expression levels.

5.3 Rapamycin largely rescues effects caused by loss of DEPDC5

One of the most significant findings is the amount of deregulated gene expression recovered through the use of rapamycin. This supports that rapamycin reverses the effect caused by *DEPDC5* mutations and partially stabilizes the mTOR deregulation. However, the lack of a complete recovery does show the limitations of using rapamycin as a drug and offers a rationale as to why it is ineffective for some patients with *DEPDC5* mutations.

KO differentiation data sets show similar developmental GO terms with and without rapamycin treatment however significance was reduced and effect sizes were smaller with rapamycin attenuation. GO terms specific for cellular developmental process, multicellular organismal process, extracellular matrix and system development processes remain present. This suggests that even early recovery of mTOR does not fully alleviate the system development changes induced through loss of DEPDC5. Studies involving children affected by TSC with epilepsy showed a reduced seizure frequency over the period of 2 years with rapamycin treatment; however only 25% were seizure free (Zou et al. 2014). Although we are

modeling a much earlier stage of development, this could be because rapamycin mitigation does not fully prevent the abnormal system development mechanisms associated with mTOR hyperactivity and can only induce partial recovery during differentiation. This is contrary to what was observed with our morphological analysis where neuron proliferation was alleviated through rapamycin use during differentiation suggesting contrary effects for gene expression and observed morphology. Therefore, the underlying system development alterations appear to be unrelated to neuron development and could be affecting other cell types.

Deregulated genes not rescued by rapamycin administration resulted in GO terms related to “cell-cell signaling”. Interestingly, three genes which were differentially expressed in our cell lines regardless of developmental stage or treatment with rapamycin were neuropeptide Y (*NPY*), Serpin Family E Member 1 (*SERPINE1*) and the stathmin proteins (*STMN1/STMN2)*.

5.3.1 NPY

NPY is a neuromodulator which is prominently found in GABAergic neurons and functions as a chemical messenger which mediates stress resilience (Sajdyk et al. 2004). Low expression of *NPY* has been observed in both ASD and epilepsy patients while *NPY* restoration can recover anti-social behaviors exhibited in ASD patients while also reversing suppressing epileptic seizures (Cattaneo et al. 2020, Ramanathan et al. 2004). Interestingly, *NPY* consistently remained downregulated through all experimental conditions in *DEPDC5*-KOs and could thus underline the imbalances observed from ASD and epilepsy patients exclusive of mTOR. This significant finding could be underlying the developmental disease phenotypes observed from *DEPDC5* mutations separate from MTOR.

5.3.2 SERPINE1

SERPINE1 functions as a protease inhibitor involved in inhibition of plasminogen and necessary for proper cell adhesion. Cohorts of high functioning ASD patients identified increased *SERPINE1* levels in blood samples compared with controls (Okazaki et al. 2022). We also found *SERPINE1* to be upregulated in all conditions except differentiation with rapamycin which overshot its recovery. Studies analyzing genomic variants of ASD individuals identified mutations in the *SERPINE1* gene as risk susceptible to ASD (Campbell et al. 2009). *SERPINE1* was also associated with seizure frequency in dissections of tissue from human temporal lobe epilepsy patients (Chen et al. 2020). hiPSC-derived interneurons treated with endogenous *SERPINE1* showed increased migration when compared to controls suggesting its role

in developmental mechanisms which could lead to neurodevelopmental disease (Genestine et al. 2021). Thus, *SERPINE1* is a prime candidate for ASD and epilepsy which we hypothesize is related to *DEPDC5* but independent of mTOR.

5.3.3 STMN1/STMN2

Stathmin 2 (*STMN2)* acts as a cytoskeletal regulator, is implicated in neurite outgrowth and is often use as a marker for neurons. Proliferating *DEPDC5*-KOs show a significant downregulation of *STMN2* compared to controls and a significant upregulation during differentiation compared to controls. This could be reflecting its role in microtubule assembly during neuron differentiation. Interestingly STMN2 was found to be deregulated in a comprehensive analysis of 12 genome-wide gene expression profiling studies of multiple epilepsy patient tissue (Mirza et al. 2011). However, because of its use as a neuronal marker, *STMN2* has not been investigated as frequently as its family member Stathmin 1 (*STMN1*).

Interestingly, *STMN1* was also identified in our analyses but was recovered with rapamycin during differentiation. However, the presence of both stathmin members in all but one condition suggests that deregulation in microtubule assembly may be common with *DEPDC5* loss. In addition, mosaic mutations in the *STMN1* gene have been identified with large whole-exome-sequencing studies from 2300 families with at least one child with ASD (Krupp et al. 2017). Thus, alterations in expression of stathmin proteins which could not be rescued by rapamycin could explain why rapamycin failed to recover morphological abnormalities within nestin and MAP2 positive cells. They also could be mechanisms for why *DEPDC5* cohorts fail to respond to rapamycin treatment.

5.4 *DEPDC5*-KOs have increased neurites, smaller dendrite lengths and decreased cell soma sizes

Morphological analyses show that loss of DEPDC5 produced aberrations. We observed an increase in neurite arborization similar to other studies of mTOR hyperactive models (Jaworski et al. 2016; Brewster et al. 2013). Increases in quantities of neurites are thought to be linked to excitatory/inhibitory imbalances brought about by mTOR hyperactivity (Jaworski et al. 2005). Mitigation using rapamycin has been shown to attenuate the increased branching in *TSC2*-KO hiPSCs and mouse *DEPDC5*-KD N2aC cells (Iffland et al. 2018; Nadahur et al. 2019). However, this was not observed in hNPC *DEPDC5*-KOs as we saw little change in rapamycin application. This suggests that changes in neurite morphology underlying loss of

DEPDC5 in our model might be related to functions that are not fully mTOR dependent. Further experiments are needed.

For neurite lengths, we observed a decrease during hNPC *DEPDC5*-KO differentiation. This was also observed from mTOR hyperactivity in differentiated *TSC2*-KO iPSCs but was not confirmed in a similar study (Li et al. 2017; Winden et al. 2019). Interestingly, in our hNPC *DEPDC5*-KO neurons, rapamycin did not recover the effects of neurite length but reduced the overall length further. As no other DEPDC5 or TSC study observed the effect of rapamycin on neurite length, it is unfeasible to draw conclusions. However, when attempting to recover decreased dendrite lengths in *Raptor*-KD hippocampal mouse neurons using endogenous Raptor protein, Urbanska and colleagues also did not see a recovery to WT (Urbanska et al. 2012). This suggests that neurite lengths are often altered through mTOR deregulation but this phenotype cannot be recovered through restoring mTOR activity.

Cell size is often dictated by mTOR activity and loss of DEPDC5 in animal and in human cell models has resulted in increased cell sizes (Iffland et al. 2018; Klofas et al. 2020; Yuskaitis et al. 2018). However, our hNPC DEPDC5-KO model showed reduced cell sizes in both nestin and MAP2 positive cells. When we attenuated these cells with rapamycin, nestin positive cells saw no change while MAP2 positive cells decreased further in size, similar to our observed neurite lengths. Analysis of *DEPDC5*-KO transcriptome data shows very little genes which could induce this morphological change across all conditions used in this study. However, the mechanism may be related to *STMN1/2* as these relate to microtubule related dynamics and are deregulated regardless of developmental stage or rapamycin treatment in our data. Microtubules are necessary to establish the cytoskeleton of cells and alterations would change the developmental structure of the cells. This would explain how *DEPDC5*-KOs continuously exhibit smaller sizes when compared with controls due to *STMN1/2* deregulation occurring even with the addition of rapamycin. Another possible explanation is that the overall changes in length and size in our model could be due to mTOR independent processes elaborated on previously (see 5.3).

5.5 *DEPDC5*-KOs have higher differentiation rates

The finding that *DEPDC5-KO* mutants resulted in higher differentiation and expression of neuronal and glial subtype genes was consistent with the known functions of mTOR (Table 12, Table 13, Figure 14). Studies from mouse radial glia *TSC1/2* knockouts show increased SVZ neural progeny, impaired maturation and premature differentiation of neurons and astrocytes (Magri et al. 2011, Carson et al.

2012). mTOR has been known to be an essential regulator of the cell cycle and neural progenitor populations and activation of its downstream partner p70S6K has been implicated in early differentiation of ESCs (Easley et al. 2010). Recent experiments also show that mTOR is significantly upregulated in outer radial glial cells and loss changed cell populations in human iPSC-derived organoid cultures, although variably (Andrews et al. 2020).

In our dataset, differentially expressed genes during proliferation in *DEPDC5*-KOs vs. NTC showed downregulation of genes associated with neurons and astrocytes (*DCX, SLC1A3*) while showing increased progenitor markers *(NES, GFAP)*. This expression pattern was reversed during differentiation and *DEPDC5*-KOs showed higher expression for immature neurons (*DCX, TUBB3*) and mature astrocytes (*AQP4*) with subsequent downregulation of progenitor cell markers (*SOX2, GFAP*). Therefore, progenitor expression is higher in *DEPDC5*-KOs during proliferation and they also have higher expression of neurons and astrocytes during differentiation. We confirmed the higher differentiation rates through cell counting of MAP2-positive neurons and found that this effect was reversed with addition of rapamycin. Thus, the loss of DEPDC5 results in increased neuronal differentiation which can be causally linked to the proposed upregulation in mTOR activity and ASD.

An ongoing hypothesis is that neurodevelopmental disorders are the product of alterations of genes regulating proliferation and differentiation, which cause developmental abnormalities and lead to disease (Ernst et al. 2016). This is reinforced by our data indicating significant changes in cell type specific markers through loss of DEPDC5. Similarly, the rapid differentiation of our KO cell lines corresponds with other phenotypes seen with ASD patients having more neurons in the prefrontal cortex (Courchesne et al. 2011). Enhanced differentiation was also seen in an iPSC model of *TSC2* and in drosophila supporting that rapid proliferation and differentiation is a product of mTOR hyperactivity (Potter and Xu 2001, Li et al. 2017). Lastly, the compounding evidence supporting that ASD-related genes are involved in the transition of stem/progenitor cells to fate-restricted cell lines emphasize the timing and transition of differentiation heavily influences ASD development (Mutch et al. 2010, Saffary and Xie 2011, Xie et al. 2016, Bian et al. 2013, Lv et al. 2013). Therefore, the quantity of resulting cells or the timing of differentiation could be the pathological mechanisms associated with disease phenotypes from *DEPDC5* loss.

5.6 Significance of disease related gene datasets and their overlap

5.6.1 ASD and epilepsy gene datasets

This study identified that DEPDC5-*KO* alters 69 disease genes related to ASD and epilepsy with only 7 remaining significant after rapamycin treatment during proliferation and/or differentiation connecting the mutational loss of DEPDC5 with ASD and epilepsy (Figure 9). This study identified multiple ASD and epilepsy genes resulting from loss of DEPDC5. Two genes identified through the research done by Iossifov and colleagues, *CD151* and *PDLIM1*, were present in both proliferation and differentiation stages and were both recovered through rapamycin attenuation. *CD151* is a known molecule related to signal transduction and cellular development while *PDLIM1* is involved in neurite outgrowth. Little has been studied in their roles for ASD or epilepsy and it would be interesting to probe how DEPDC5 loss is related.

In addition, we identified significant overlap of genes associated with *DEPDC5*-KO in the developmental stages with Voineagu module 16. Module 16 is composed of markers for astrocytes and activated microglia and has been shown to be deregulated in ASD. We did observe uncorrected significant overlap ($p<0.05$) with the risk genes identified in the large ASD cohorts (Iossifov and DeRubeis datasets). These datasets contained many genes for mature cell types which may explain the lack of significance. Current hypotheses suggest that ASD related neurodevelopmental abnormalities can take place around 22 post-conception weeks judging from abnormal head growth and at a stage where glial and cortical lamination occur (Ploeger et al. 2009, Regev et al. 2020). As this hNPC model characterizes early development and most genetic studies lack comparable early development genetic data, this would explain lack of significant overlap in the larger ASD and epilepsy datasets. Longer differentiations into more mature neurons may reveal some of the channels and receptors known to be associated with epilepsy and ASD phenotypes.

An interesting finding is that our dataset identified orthologs of genes similar to known ASD risk genes. Mutations in chromatin-remodeling and transcription genes (*ADNP*, *FOXP2*, *CHD2*, *CHD7*, *CHD8)* have been reported to be associated with ASD (Basilico et al. 2020). This study found that loss of DEPDC5 resulted in the deregulation of similar orthologs as ASD disease genes (*ADNP2, FOXJ1, CHD5)* and could have disease significance in future analyses. Likewise, ubiquitin proteasome system genes such as *UBE3A*, *TRIP12* and *USP7* have also been associated with ASD (Basilico et al. 2020). Here we found similar orthologs in differentiated KOs such as *UBE2T*, *UBE2C*, *TRIP6* and *USP16* which might have

similar functions to identified disease genes. Hence, this exploratory transcriptome analysis has also highlighted numerous orthologs which could be related to disease mechanisms undiscovered in ASD and epilepsy.

5.6.2 TSC datasets

There were in total 649 genes which were deregulated due to mTOR hyperactivity and 89 genes were not attenuated with the use of rapamycin suggesting some of the disease mechanisms caused by loss of DEPDC5 result in processes independent of mTOR. Unsurprisingly, most deregulated genes are expressed during development as identified from GO terms (system development, neurogenesis, ECM). Therefore, most of the mTOR hyperactive related mechanisms in *DEPDC5*-KO and TSC point towards abnormal development caused by mTOR processes. Further, the findings in our model also overlapped with differentially expressed genes identified in TSC related subependymal giant cell astrocytoma (SEGA) and cortical tuber development. These abnormal cells are characterized by enlarged cell sizes and form dense masses which are often expressive of neuronal and glial markers leading to the hypothesis that they are derived from radial glial cells (Zimmer et al. 2020). They also do not fully develop into mature neurons. Though we halted our differentiation after one month, it would be interesting if this phenotype would also be a product of longer *DEPDC5*-KO differentiation due to the similarity in mechanisms and clear overlap of cellular markers in our sample.

5.6.3 Schizophrenia

The dataset relating to schizophrenia generated by Cocchi and colleagues also significantly overlapped with our differentiated *DEPDC5*-KO genes. Rapamycin attenuation had no effect on reducing significance. The original dataset was a collection of pruning genes related to schizophrenia patients. Interestingly, of the top 5 genes identified to be associated with schizophrenia in the original report, an overlapping gene, *THBS4,* was also identified in our analyses. This gene relates to the extracellular matrix and is highly related to cell-cell interactions. The significant overlap with the Cocchi gene list suggests possible mechanisms related to synaptic pruning and its relationship to mTORopathies. Interestingly, individuals with familial focal epilepsy caused by *DEPDC5* mutations have been reported to have comorbidities with schizophrenia as well.

5.6.4 Summary

We found several ASD and epilepsy genes when comparing the large disease gene datasets with deregulated genes from *DEPDC5*-KOs during proliferation and differentiation. Many of these genes were attenuated through rapamycin administration during both stages. Comparisons with large genetic datasets show the largest correlation with the *TSC* patient datasets. As epilepsy and ASD are quite heterogeneous in their disease-causing mechanisms and fairly widely sub-categorized in their phenotypes, the lack overlap between *DEPDC5*-KO gene expression is not surprising. *DEPDC5* itself is a disease-causing gene that resides in the mTOR pathway related mechanisms, as is *TSC*. Not all epilepsies and ASDs are related to the mTOR pathway. Therefore, the significance in gene expression abnormalities exhibited from *TSC* and *DEPDC5* mutations could be similar in terms of treatment.

5.7 Spatial and temporal expression of DEPDC5 disease genes

Our enrichment analysis on Kang pathway modules identified gene-modules and indicated the expression of the deregulated DEPDC5 associated genes over a spatial and temporal signature during human brain development. Genes identified upon loss of DEPDC5 overlapped with those in modules 1 and 2 for both the proliferation and differentiation datasets. Module 1 and module 2 correspond to cell cycle regulation and synaptic transmission where the first module is expressed specifically during early prenatal development and the latter postnatally in all regions. This corresponds with the development of neural progenitors, as structures such as the hippocampus, amygdala and the developing cortex are derived from radial glial cells. Cell cycle related genes in the cortex would correspond to the developing embryo's increased proliferation and extension of intermediate cells from the SVZ necessitating cell cycle transitions and differentiation. The enrichment of *DEPDC5*-KO associated genes in Module 2 (early developmental synaptic mechanisms) mirrors the rapid development and differentiation in our KO cell lines. Relating to autism and epilepsy, the deregulation due to *DEPDC5* mutations could thus lead to early cell cycle abnormalities during development and affect synaptic transmission which may present already after birth. This resembles the effects seen through electrophysiology studies in *DEPDC5*-KO mice showing excitatory and inhibitory imbalances in early stages (De Fusco et al. 2020).

Corresponding hub genes to our dataset are *TOP2A* and *NUSAP1* which both have roles in the nucleus. *TOP2A* is a topoisomerase which functions in breaking and rejoining DNA strands, preventing knotting or tangles and helping to maintain the structure of chromatids during mitosis (Nielsen et al. 2020). *NUSAP1*

has roles in spindle microtubule stabilization and chromosome segregation during metaphase and anaphase of mitosis (Mills et al. 2017). Interestingly, *TOP2A* and *NUSAP1* both show downregulation in *DEPDC5*-KO cells suggesting interference during mitosis which was also reported due to mTOR hyperactivity (Hsieh et al. 2018). However, currently no association with ASD or epilepsy has been reported.

Module 18, which is expressed early after birth in the development of hippocampus and amygdala, appeared to be specific for *DEPDC5*-KO associated genes altered during differentiation and is classified as “cell morphogenesis; glycine, serine and threonine metabolism” along with “biological adhesion molecules”. Glycine, serine and threonine metabolism correlate to the function of GATOR1 as an amino acid sensor process. One can thus suggest that its temporal expression is necessary in some of the early processes in the hippocampus and amygdala, mainly during early developmental stages after birth. This reinforces that timed expression of these “cell morphogenesis” genes are necessary in early development and disruptions could underlie disease phenotypes.

5.8 Limitations of the study

As any model, this study does not come without some limitations. Here, we utilized extracted tissue and cells undergoing development taken from a donated fetus. Ideally passage numbers are kept low in extracted progenitor populations as they can undergo spontaneous differentiation into intermediate progenitors or later stages. This study conducted transductions at around passage 11 and subsequent RNA sequencing and analysis at around passages 14-17. Although careful considerations were taken, some cells likely underwent spontaneous differentiation as exhibited from the mix of cell type specific markers observed from RNA sequencing analysis and immunocytochemistry. Cells exhibited mixed staining for glia and progenitor markers making it difficult to identify astrocytes. This was why we opted to study batch culture analyses as single cell sorting could not separate specific populations with the markers used in this study. Therefore, we could also not distinguish the specific indels induced through CRISPR-Cas9 due to batch culture. We also observed that phNPCs required higher confluencies than immortalized cell lines. Low confluencies resulted in apoptosis or senescence. This prevented the ability to expand single cell populations and therefore prevented analysis of *DEPDC5* heterozygotes.

phNPCs also continued to proliferate during differentiation further resulting in a mixed population of progenitors, astrocytes and neurons. AQP4 immunocytochemical stains revealed astrocyte populations.

However, reducing the cell density through enzymatic digestion failed to break apart the cell networks and thus prevented re-seeding the cells for microscopic analysis. Therefore, we could not do analysis on AQP4 positive populations.

Another issue comes with the integration of the CRISPR-Cas9 vector and continuous expression of the DEPDC5 sgRNA and Cas9 within the cells. Continuous Cas9 expression could cause issues with the overall machinery of the cell which is why proliferation experiments were carried out as soon as selection occurred and RNA could be extracted for sequencing. Differentiation experiments were also performed immediately but required 1 month before RNA extraction. We controlled for this by using our NTC vector which also would integrate and express Cas9. We also checked sgRNA off-targets to ensure that no other exonic genes were targeted and none of the off-targets were related to development or neuronal processes.

5.9 Outlook

As some of the main effects from mTOR hyperactivation in phNPCs were specific to the overall development of specific cell types, it would be interesting to also differentiate the cells to later stages to identify if the cells prefer development of specific neuronal cell types. *DEPDC5*-KO in zebrafish have elucidated GABAergic branching defects resulting in premature death and altered inhibition (Swaminathan et al. 2018). Investigating cell specific defects would also help confirm that these mechanisms are translated to human cells as well. Single-cell sequencing of mature neurons would provide insight into the altered cellular mechanisms within excitatory and inhibitory neurons elaborating on how these processes manifest intracellularly. Also, confirmation of these excitatory/inhibitory imbalances could be further studied through the use of electrophysiology, where changes in synaptic transmission would also help understand the mechanisms related to mTOR hyperactivity.

Importantly, the identified mechanisms in our study suggest a wide variety of pathways and mechanisms related to mTOR hyperactivity. We found that many *DEPDC5*-KO genes are expressed oppositely to what is expected and suggested that these mechanisms are related to feedback mechanisms or are reactive to loss of DEPDC5. We could confirm this using proteomic approaches or western blot analysis to observe the activity of the identified pathways and their genes as many are activated through phosphorylation. This would confirm the overall functionality caused by loss of DEPDC5. This would also allow the observation of mTOR pathway subunits and how their expression relates to their activity.

Extraction of phNPCs at an earlier time point may allow the extraction of earlier subtypes such as neuroepithelial cells which have been shown to have more stem-like properties and are easier to transfect and expand. It would be interesting to generate homogenous earlier phNPC cultures through cell-sorting techniques. Alternatively, the advancement of patient-specific iPSCs and cerebral organoid cell models have allowed *in vitro* studies to glimpse into the realm of patient-specific mutations and development using human cells. iPSCs could be taken from *DEPDC5* mutation carriers and could be compared if their disease phenotypes are consistent or vary between patients. This could help target the dosage and drugs needed to reverse DEPDC5 loss. It would also be interesting to analyze functional missense mutations as this has not been performed with *DEPDC5* carriers. Organoid generation could also help the understanding of the clear defects in DEPDC5 cellular migration which cannot be observed clearly in a 2D culture for ASD and other diseases (Urresti et al. 2021, Klaus et al. 2019, Yuskaitis et al. 2017). As animal models report migration abnormalities caused by loss of DEPDC5, confirming this in a 3D organoid culture could give insights into the later human developmental stages not answered in this study.

6. Conclusion

Here, we investigated the effects of loss of DEPDC5 using a human cell line extracted from fetal cortex. Characterizations of these cells showed that they exhibited radial glial markers and could further be differentiated into neurons. Utilizing CRISPR-Cas9 technology, knock-out of DEPDC5 targeting exon 2 showed overall hyperactivation of the mTOR pathway. When we used the mTOR inhibitor rapamycin, the cells reversed their hyperactivation of mTOR. We performed transcriptome analysis on proliferating and differentiating *DEPDC5*-KOs with and without attenuation of rapamycin to understand the function of the DEPDC5 loss using an early developmental model. We found 237 deregulated genes during proliferation and 552 during differentiation. Attenuation with rapamycin significantly reduced the amount of deregulated genes. We found an overall deregulation of mTOR mechanisms relating to increased translation mechanisms, ribosome biogenesis, lipid synthesis, aerobic glycolysis, nucleotide synthesis, autophagy and lysosome biogenesis, proliferation and differentiation mechanisms, system development and anatomical structure development for both developmental stages. Deregulated genes showed overlap with risk genes for epilepsy, TSC, SCZ and ASD confirming its association with the disorders. Loss of DEPDC5 also resulted in an mTOR dependent increase in neuronal differentiation which was attenuated through rapamycin induction. We also identified alterations of cell size, dendrite length and increased dendritic arborization but failed to recover the effect through rapamycin utilization indicating mTOR independent mechanisms related to neuronal development. Together our results suggest that loss of DEPDC5 in human progenitor cells underlies the phenotypic expression of epilepsy and ASD gene expression abnormalities and expands on the current knowledge of its role in neurodevelopmental pathology. While this is the first study in an isogenic human cell model, these findings need to be repeated in a more mature cell model to also capture the electrophysiological effects together with a more detailed characterization of the proteomic consequences of DEPD5-loss.

7. Appendix

7.1 Supplementary Figures: Results

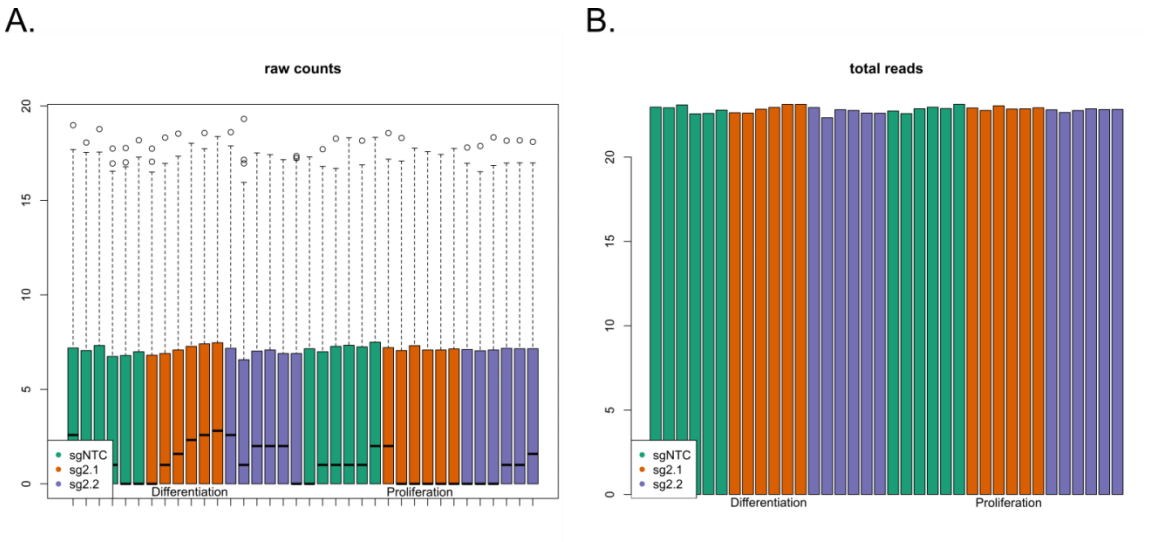


Figure S1 Total RNA raw counts and reads of the D62 NTC, Dep2.1 and Dep2.2
(A) Total raw count data of RNA transcripts (average reads per sample 7,195,819 SD± 345,374). (B) Total read data of D62 NTC, 2.1, 2.2 under differentiation and proliferation.

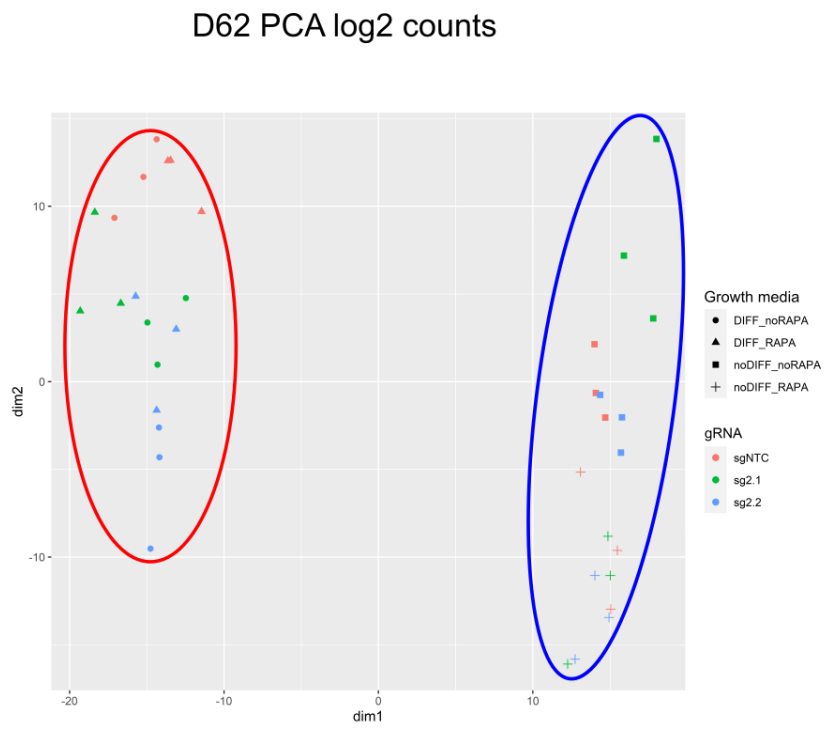


Figure S2 PCA analysis of the log2 counts of D62 *DEPDC5*-KOs and NTC.
Clustering of the cell lines separate based upon proliferation (blue circle) and differentiation (red circle).

Table S1 Risk genes for epilepsy or ASD in Proliferation and Differentiation *DEPDC5*-KO cells

Epilepsy and ASD genes in *DEPDC5*-KOs during Proliferation						
Rescued by Rapa					**Rapa Independent**	**Rapa Induced**
AHNAK2	CENPE	KCNQ2	MYOF	SYNE2	DCX	GNAO1
CD151	DDR2	KIF2A	PCDHB16	TRIO	MYH14	
CDCA7L	DNAH5	LRP1	PDLIM1		RGMA	
DCX	HTATIP2	MKI67	SRPX2		PTK2B	
Epilepsy and ASD genes in *DEPDC5*-KOs during Differentiation						
Rescued by Rapa					**Rapa Independent**	**Rapa Induced**
A2M	CPZ	JADE2	NSUN7	SH2D3C	DCX	GNAO1
AKAP6	DNAH11	KIF2A	PDLIM1	SLC25A39	GRIN2A	ITPR1
ATP1A2	EPHB2	MYH1	PER1	SLC2A1	AQP4	KCNJ10
ATP1B1	ETFB	MYH9	PIGT	SLCO1C1		MYH14
BMP4	FLNA	NFIA	PPP1R3B	TCF4		MFSD2A
CD151	GFAP	NCOR2	RANBP17	TOPORS		
CLCN7	GRN	NKAIN3	RBMS3			
COL4A2	GSE1	NR2F1	RNF213			
COL6A2	HNRNPF	NRXN1	SFPQ			

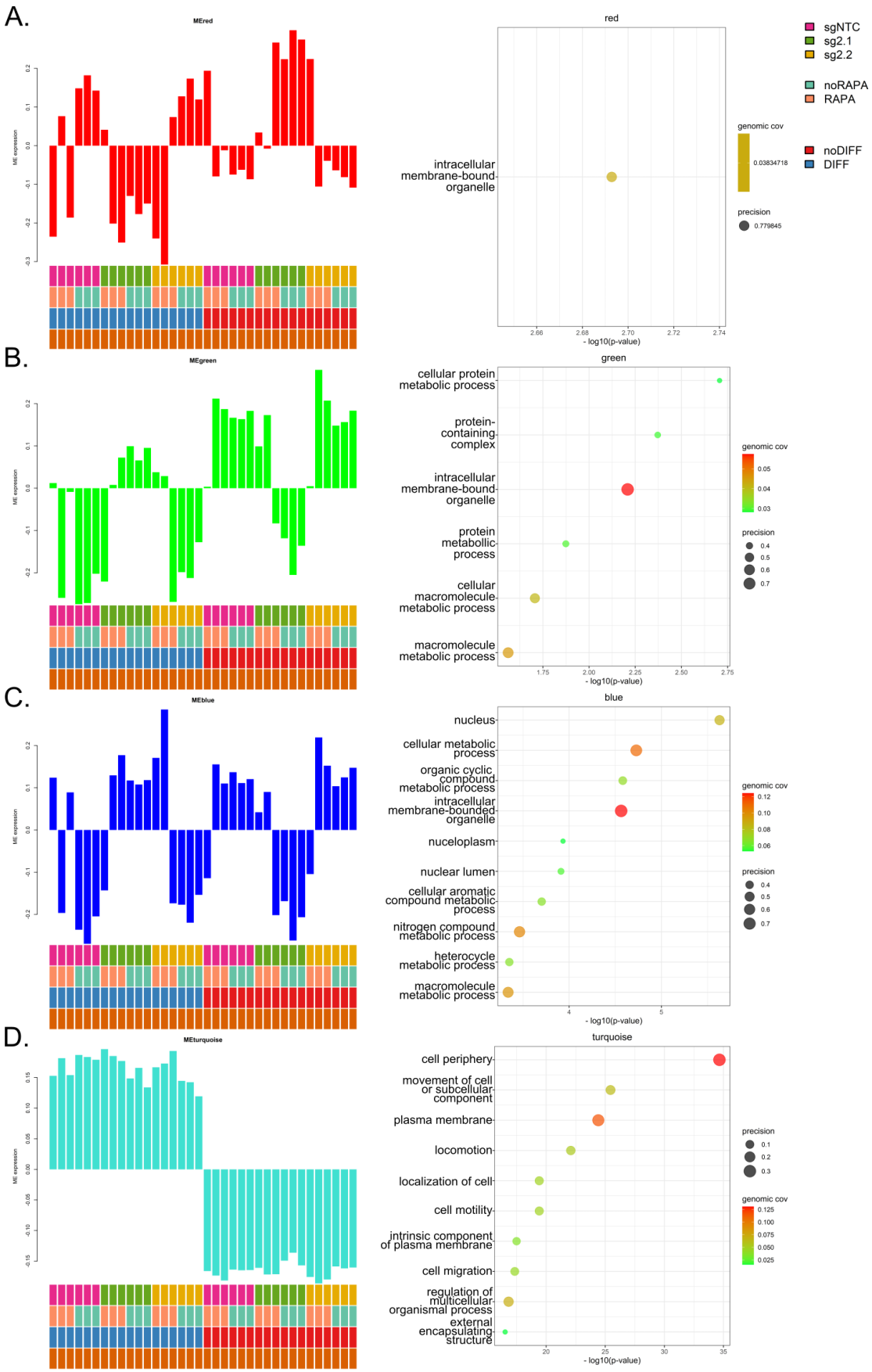


Figure S3 Eigengene of the other 4 identified modules from WCGNA and their respective GO terms. Eigengene values and GO terms for module (A) Red (B) Green (C) Blue (D) Turquoise (E) Yellow (F) Gray. Yellow and Grey modules did not yield and significant GO terms

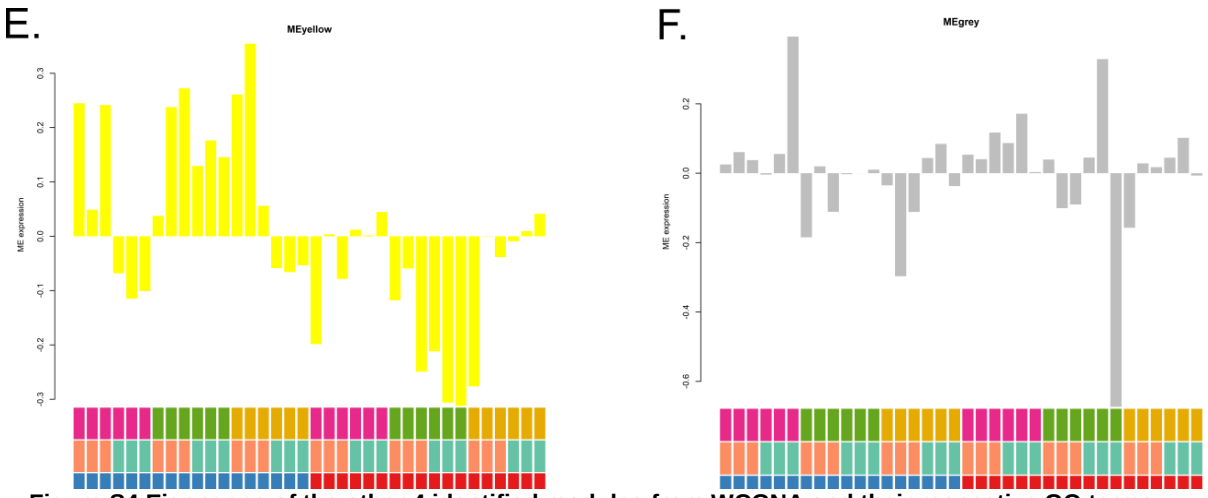


Figure S4 Eigengene of the other 4 identified modules from WCGNA and their respective GO terms. Eigengene values and GO terms for module (A) Red (B) Green (C) Blue (D) Turquoise (E) Yellow (F) Gray. Yellow and Grey modules did not yield and significant GO terms

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