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Analysis of Genes Responsible for Neuronal Morphological Changes in Down Syndrome hiPSC-derived Neurons

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Abstract

Down syndrome (DS) is a complex neurodevelopmental disorder caused by the trisomy of chromosome 21. DS is the largest genetic cause of intellectual disability, which occurs in varying severity among affected individuals. It is associated with a variety of developmental and cognitive defects, including reduced brain size, impaired synaptic function, and altered neuronal morphology. A number of genes have been identified to play critical roles in the development and maintenance of neuronal morphology, and alterations in the expression of some of these genes are implicated in the morphological changes observed in DS. Knocking out or altering their expression leads to significant changes in dendritic length, spine density, and branching complexity, providing further evidence for their role in DS pathophysiology. Specifically, Down syndrome cell adhesion molecule (DSCAM) is located on chromosome 21 and known to regulate neuronal development and synaptic connectivity. Recent advances in stem cell technology have enabled the generation of human induced pluripotent stem cell (hiPSC)-derived neurons, providing a powerful tool for studying the genetic mechanisms underlying DS pathophysiology. Herein, morphological changes in DS human induced pluripotent stem cell (hiPSC)-derived neurons are quantified, and the contribution of DSCAM to these changes is examined. In this study, we use hiPSCs from an individual with DS and isogenic control hiPSCs and differentiate them into glutamatergic cortical neurons. First, we find that there are morphological changes in developing Down syndrome hiPSC-derived glutamatergic neurons, as compared to isogenic control neurons. We also find that there is an increased expression of DSCAM in DS neurons. Next, a siRNA knockdown of the human DSCAM gene was performed, to reduce levels of DSCAM to those found in isogenic control neurons, but the knockdown was unsuccessful. Overall, this study highlights the importance of studying the genetic mechanisms underlying DS

pathophysiology using hiPSC-derived neurons. This paper also reviews recent studies investigating other genes responsible for such neuronal alterations. The identification and investigation of candidate genes involved in altered neuronal morphogenesis in DS may lead to the development of novel therapies for this disorder. Our findings suggest that targeting DSCAM may be a potential therapeutic approach for improving neuronal morphology in DS.

Introduction

Down syndrome

Down syndrome (DS), or trisomy 21, is a neurodevelopmental disorder caused by an extra copy of chromosome 21 and characterized by many phenotypes that impact various organ systems. Intellectual disability is a ubiquitous phenotype of DS. Moreover, DS is the most common genetic cause of intellectual disability (ID), although it varies in severity between individuals (Chiotto et al., 2019). Of interest to the experiments described herein are neuroanatomical abnormalities in the cerebral cortex that alter the cognitive profile of an affected individual. Understanding the ID associated with DS is dependent on gaining knowledge about the underlying mechanisms and morphologies that result in improper development and anomalous brain function.

The cerebral cortex of the brain is known to be responsible for higher-level processes of the human brain, such as learning, reasoning, and problem-solving. Therefore, the disruption of its development can contribute to ID (Juric-Sekhar et al., 2019). Alterations that have been identified in the DS cortex include reduced volume, lower neuron density, and altered dendritic spines (Contestabile et al., 2010). Moreover, the ID associated with DS has been linked to the developmental deficit of reduced cortical volume (Bletsch et al., 2018).

It is also well-established that brain connectivity is altered in DS. Brain connectivity is the pattern of synaptic links between neurons, associating various anatomically segregated brain regions. The development of neuronal processes, such as dendrites, axons, and axonal branches, directly determine how neuronal networks receive and process information. Some neuropathologies that occur in neurodevelopmental disorders include changes in the complex morphology of the dendritic tree (Martínez-Cerdeño, 2017) and axonal elongation (Lasser et al., 2018). Consistently altered subcellular dendritic patterning has been observed in murine and human models of DS (Benavides-Piccione et al., 2004; Takashima et al., 1989), which demonstrates a need to further investigate the molecular mechanisms that drive this aberrant growth.

Relevant to DS, disrupted connectivity within the cerebral cortex has been found to result in functional anomalies which may underlie ID (Pujol et al., 2015; Xu et al., 2020). Neural and synaptic plasticity is the ability of neurons to continually change the strength of connections in response to activity. The development of such connections between neurons is also altered in DS (Martínez Cué and Dierssen, 2020) which provides further insight regarding the intellectual disability phenotype of this disorder. Changes in plasticity are often caused by alterations present in the dendritic spines of neurons (Newpher and Ehlers, 2009) and is dependent on an adjustment of the balance of excitatory and inhibitory (E/I) synapses (Carcea & Froemke, 2013; Froemke, 2015). Increased inhibition (and consequential E/I imbalance) has been linked to DS-associated cognitive deficits in mouse models (Mitra et al., 2012). Glutamate is the most abundant excitatory neurotransmitter in the brain, playing a crucial role in learning and memory, which are also major functions of the cerebral cortex. The aforementioned developmental differences in DS neuronal morphology are prominent in excitatory cortical neurons, which are important to brain

cognition and known to be relevant in DS (Takashima et al., 1981; Falsafi et al., 2016). Thus, our studies herein focus on alterations at the cellular level in glutamatergic cortical neurons during brain development.

Growth cones and cellular cues

Developing neurons extend out multiple processes, the longest of which eventually develops into the axon. The axon uses electrochemical communication to send information to other neurons. However, before neurons can communicate, they must find their synaptic partners. This process occurs during development and requires that the axon be guided to its synaptic target. Growth cones contain both microfilaments and microtubules (Buck and Zheng, 2002) and are found at the end of extending axons. Growth cones have numerous finger-like protrusions, termed filopodia, which have receptors for extrinsic guidance cues. Guidance cue signaling through these receptors results in repulsion or attraction, causing the axon to extend toward or away from a given direction. Such steering is caused by intracellular signaling cascades and local translation of proteins that ultimately regulate microfilaments and/or other cytoskeletal proteins (Wang et al., 2016; Dent et al., 2011).

Netrin-1 is a laminin-like protein that has been studied for its role in axonal guidance (Guthrie 1997). Current findings show that as a chemotropic factor, increasing concentrations of netrin-1 may modulate the recruitment of receptors and intracellular pathways relevant to axon extension (Boyer and Gupton 2018). Multiple netrin receptors have been identified in vertebrates, including DCC (deleted in colorectal cancer), neogenin, the UNC5 protein family (Moore et al., 2007), and DSCAM (Ly et al., 2008). These receptors are known to interact with

various other receptors and intracellular signaling molecules to mediate the growth cone response to netrin-1.

Down syndrome cell adhesion molecule (DSCAM) is a transmembrane receptor from the immunoglobulin superfamily found in vertebrates (Yamakawa et al., 1998). Human DSCAM is located within the critical region of chromosome 21, which has been associated with the ID phenotype of DS (Pritchard and Kola, 1999). DSCAM overexpression has been confirmed in the brains of murine models of DS and humans with DS; furthermore, the dysregulation of DSCAM has been linked to ID (Jia et al., 2011; Saito et al., 2000). DSCAM is known to be expressed in the central nervous system and serves a crucial role in its development (Montesinos, 2014). It is also widely expressed in both adult mouse brains and adult human brains (Barlow et al., 2001; Yamakawa et al., 1998). Furthermore, DSCAM is expressed in commissural axons, acting as a receptor required for netrin-1-dependent axonal extension (Ly et al., 2008; Liu et. al, 2009). However, netrin-1 is a bifunctional guidance cue and thus can also mediate repulsion through a DSCAM/UNC5 complex (Purohit et al., 2012). DSCAM also mediates cell-cell interactions by promoting cell adhesion through an extracellular domain that forms either homophilic or heterophilic interactions with other proteins (Purohit et al., 2012; Figure 1).

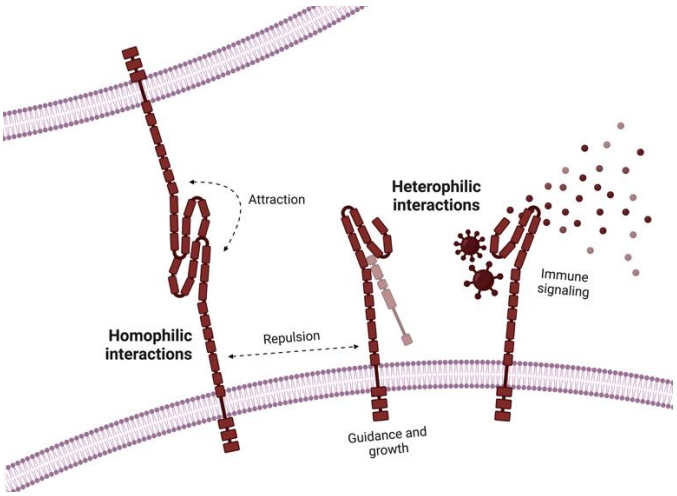

Figure 1. Homophilic and heterophilic interactions of DSCAM. Adapted from Schmucker & Chen, 2009.

As there is a growing body of knowledge surrounding DSCAM's role as a netrin-1 receptor and a cell adhesion molecule, it is of interest to increase our understanding of its functions in axon growth and guidance. For example, netrin-1, acting through DCC, induces the local translation of *Dscam* mRNA within mouse hippocampal growth cones. Furthermore, overexpression of DSCAM protein within mouse hippocampal growth cones results in reduced axon length (Jain and Welshhans, 2016). These findings provide the basis for our investigations of whether increased DSCAM contributes to the reduced connectivity that occurs in Down syndrome.

The human induced pluripotent stem cell model

Human induced pluripotent stem cells (hiPSCs) are a powerful model to study disease-related molecular mechanisms that can drive various outcomes at the genotypic and phenotypic levels. hiPSCs are created by obtaining somatic cells and forcing ectopic expression of transcription factors specific to pluripotency; this reprograms the somatic cells to a pluripotent stem cell state (Takahashi et al. 2007). By following specific protocols, hiPSCs can then be

induced to differentiate into nearly any cell type within the human body. Of relevance to this study, creating hiPSCs from individuals with Down syndrome can be used to study the altered neural development. They can also be used to examine how changes in gene expression lead to altered neural development by affecting extrinsic cues and intracellular signaling. Importantly, hiPSCs can also be used to study potential genetic rescue mechanisms (Wu et al., 2022). Apart from this, they are also valuable in designing and testing the efficacy of various drugs and treatments for DS (Lee et al., 2020).

hiPSC models are particularly useful in studies where functional tissues are not accessible, such as the human brain. Using an hiPSC model can also alleviate some of the discrepancies that manifest in using murine or other vertebrate models to study the features of DS, as there are physiological differences between animal models and their human counterparts. For example, rodents share large syntenic regions with human Hsa21, but do not share the complex cognitive phenotypes of humans with DS (Lee et al., 2020). As hiPSCs are developmentally immature, they can be followed through the process of maturation to replicate what may be observed *in vivo*. However, these are 2D cultures and as such, they do not replace the need for organoid or animal models. This is because 2D cultures cannot accurately replicate microenvironment interactions which are integral to holistically understanding the progression of neurodevelopmental disorders.

hiPSCs can also be used to generate organoid models of DS, which have been studied to try to improve our understanding of how embryonic brain development is altered in DS. For example, hiPSC-derived organoids have been found to support findings of reduced cortical volume in DS individuals (Li et al., 2022). They have also been used to study other molecular

pathways and gene expression, as well as generate models of pathogenesis (Tang et al., 2021; Xu et al., 2019; Bansal et al., 2022).

Treatment and rescue mechanisms in Down syndrome

Down syndrome mouse and hiPSC models are being used to study how abnormal cellular and molecular mechanisms of neurons can be rescued. Many potential mechanisms, including genetic corrections and molecular inhibitors, have been identified to try to reverse or minimize the effects of this chromosome triplication and related genes implicated in Down syndrome. Experiments targeting increased expression of the APP gene in mice have demonstrated rescue effects on endosomal phenotypes that may lead to inflammatory responses characteristic of DS and the associated pathogenesis of Alzheimer's disease (Chen et al., 2020). Furthermore, harnessing the power of XIST expression (and consequent wrapping and silencing of the extra chromosome 21) has been used to correct triplication dosages in neural stem cells (Czermiński and Lawrence, 2020).

One of the genes known to be overexpressed in Down syndrome is DYRK1A (Dowjat et al., 2006), which has been identified as a promising target for rescuing various brain defects (Feki and Hibaoui, 2018). Two studies have examined using epigallocatechin-3-gallate (EGCG), a naturally occurring polyphenol found in green tea, for its extensive effects on reversing many of the damaging effects of the DYRK1A gene (Guedj et al., 2009; De Toma et al., 2019). EGCG treatment was found to inhibit DYRK1A activity, increase tau phosphorylation, and rescue cognitive defects in mice. Additionally, behavioral deficits associated with DS were rescued in a mouse model using fluoxetine, which increased neurogenesis (Clark et al., 2006). Fluoxetine is a selective serotonin reuptake inhibitor that increases availability of the neurotransmitter serotonin

in the brain, which has been studied for its potential to improve cognitive function in mice with Down syndrome (Bianchi et al., 2010).

Other examples of modulation include the use of siRNAs (as studied in Ly et al., 2008) or CRISPR-Cas9 (as studied in Tang et al., 2021) to knockdown DSCAM expression. The effects of DSCAM overexpression on other genes, which may contribute to morphological changes, has also been studied. For example, DSCAM triplication in DS has been found to deregulate PAK1 and its phosphorylated form, p-PAK1 in mice (Pérez-Núñez et al., 2016). This was observed to lead to altered morphology, including a decrease in dendrite length and branching, as well as a decrease in the number and length of axons. PAK1 is a gene found downstream from DSCAM which is known to regulate the neuronal cytoskeleton and dendrite morphology (Pan et al., 2015). This finding was then applied to Down syndrome-iPSC derived cerebral organoid models in Tang et al., 2021. This study found that reduced neurogenesis in Down syndrome cerebral organoids was rescued by the use of a small molecule inhibitor, FRAX486, to inhibit PAK1.

This thesis focuses on defining how developing neuronal morphology is altered in human iPSC-derived glutamatergic neurons. Human iPSC-derived glutamatergic neurons were used because glutamatergic neurons are altered and contribute to the changes in cognitive function that occur in Down syndrome. Herein, it is also examined whether the morphological abnormalities of Down syndrome neurons can be rescued by reducing the overexpression of DSCAM. Using RNA interference, hiPSC-derived glutamatergic cortical neurons were treated with DSCAM siRNA and the effects on various neuronal morphologies were analyzed.

Materials and methods

Cell culture

Two lines of hiPSCs were maintained at the University of South Carolina for the following experiments. These were derived from cryopreserved samples of the UWWC1-DS1 (or DS1, Down syndrome) and UWWC1-DS2U (or DS2U, control) hiPSC lines (WiCell) which were preserved in mFrESR™ serum-free cryopreservation media. The cells were incubated at 37ºC with 5% CO_2 and cultured in mTeSR™ (Stem Cell Technologies). The cell culture protocol used was originally developed by Shi et al., 2012 and modified by Volpato et al., 2018. The modified protocol is used for neuronal differentiation.

Revival: Cryopreserved samples were partially thawed. Using a micropipette, the contents of one preserved tube (from one line) were added, dropwise, to a 15 mL centrifuge tube. 11 mL of chilled mTeSR™ media (StemCell Technologies) was slowly added to wash the cells. This mixture was centrifuged at 200 rpm for 5 minutes. Supernatant was removed, and the cell pellet was combined with 1.5 mL of fresh mTeSR™. The pellet was then plated into Matrigel-coated 6-well cell culture dishes (Corning). About 0.5 mL of the pellet and mTeSR mixture was added dropwise to a single well, resulting in three wells used per line. This was repeated for the second line of cells. Both cell lines were cultured in the same 6-well dish and then moved to separate plates as maintenance and splitting progressed. Media was changed after 2 days of growth.

Maintenance: The hiPSC lines required scraping and media change every day after the 2-day period following revival. To maintain, any developing differentiation and large colonies were scraped regularly, standing media was aspirated, and replaced with 2 mL of mTeSR™ daily.

Passaging and splitting: Cells were passaged about every 7 to 8 days. Before beginning, Matrigel-coated dishes were prepared. 1 mL of GCDR (Stem Cell Technologies) was added to the wells and aspirated after 6 minutes. 1 mL of mTeSR™ was added and cells were scraped using a lifter. For passaging, the contents of two wells were plated onto the new dishes using a micropipette at 50-70 µL/well, depending on original confluency of the wells. When splitting, the contents of one well were divided into two. Media was replaced 48 hours after passaging or splitting.

Differentiation: Cells are ready after passaging a few times, reaching about 80-90% confluency. To passage for differentiation, 1 mL of dPBS was added to each well after removing old media. This was immediately aspirated off and incubated with 1 mL of GCDR for 5 minutes at room temperature. After aspirating off the GCDR, 1 mL of mTeSR™ plus Y-27632 (ROCK inhibitor, Sigma-Aldrich) was added. 2 wells were scraped, combined, and added to one well on a Matrigel-coated dish.

Neural induction: 24 hours after passaging for differentiation, the cells were checked for 100% confluency. Once confirmed, the cells are washed with PBS and introduced to 2 mL of neuronal induction media (NIM) for 12 days. This NIM was made every five days by combining 25 mL of neuronal maintenance media (NMM), 25 µL of Dorsomorphin, and 25 µL of SB431542 (an inhibitor of the TGF-β/Activin/NODAL pathway).

Neuronal stage: After hiPSCs have been passaged through the intermediate stage (day 12 of NIM), the cells are added into neural maintenance media (NMM), which contains DMEM:F12 plus glutamax, among other nutrients and components for growth and maintenance. This is done by adding 200 µL of Dispase directly into the media and incubating at 37ºC for 3 minutes. Using a micropipette, the cells are removed while keeping the sheet as intact as possible. The sheet was

added to a 15 mL centrifuge tube containing 10 mL of fresh NIM. The mixture was allowed to settle for a few minutes, after which the supernatant was discarded. 4 mL of fresh NIM was added to the tube slowly, being careful not to break up the large clumps. Two pre-coated laminin wells were filled with 2 mL each. The cells were incubated overnight at 37ºC to reattach, and the media was changed to NMM plus FGF2 the next day (made by adding 4 µL of FGF2 aliquot in 20 mL of NMM). This FGF2 treatment was continued for 4 days, with media refreshed at 48 hour intervals. After 4 days of NMM plus FGF2 treatment, if neuronal rosettes start to meet or a neural crest begins to appear, the cells are split into two wells using the same Dispase protocol as above.

Passaging to single cells: At about day 25 after induction, neurons on the outer rim of the rosettes become visible and are ready to be passaged to single cells. Cells are first washed with PBS after removing media, and then dissociated with 0.5 mL of accutase per well. Cells were incubated at 37ºC for 5 minutes. To detach, the cells were pipetted up and down, and then added into a 50 mL centrifuge tube containing 10 mL of neural maintenance media. Cells were centrifuged at 400g for 5 minutes, washed with fresh NMM, and resuspended in 2 mL of fresh NMM. The cells were transferred to laminin-coated wells. The media was replaced the day after plating, and then in 48 hour intervals. At this point, the cells could be passaged and split at 90-100% confluency, approximately every few days, until ready for final plating.

Gene knockdown using DSCAM siRNA: For gene knockdown, pre-designed human DSCAM Accell siRNA and non-targeting control siRNA (NTC) were obtained from Dharmacon. These siRNA solutions were added to NMM. The cells were incubated with either 1 µL of the human DSCAM siRNA or the siRNA NTC for 96 hours in vitro.

Final plating: At about day 35 after neural induction, the cells were at a high confluency and ready for final plating. Cells were washed using 2 mL of PBS per well. 0.5 mL of accutase was added per well and allowed to incubate for 5 minutes at 37ºC. After gently pipetting the cells to detach, they were added into 10mL of NMM. This was centrifuged for 5 minutes at 400g and resuspended in NMM. Cells were then counted using a hemocytometer and Trypan blue. Cells were plated onto PLL and laminin-coated coverslips (Carolina Biologics) in a 12-well plate, at about 10,000-15,000 cells per coverslip. Media was replaced the day after plating, and then at 48 hour intervals.

Immunocytochemistry

Fixation: Cells were fixed onto the coverslips by adding fresh 4% paraformaldehyde with sucrose (in PBS) for 17 minutes. Coverslips were washed three times using PBS + 5mM MgCl2, incubating for 5 minutes at room temperature.

Immunostaining The coverslips were placed in TBS50 for 5 minutes, then permeabilized in 0.3% TBS50/Triton X-100 for 5 minutes and placed in IF buffer for 5 minutes. Coverslips were blocked in blocking buffer for 1 to 2 hours. Coverslips were then incubated cell side down on a drop of primary antibody (in IF buffer), which could be incubated overnight at 4ºC or 1 hour at room temperature. Four 5-minute washes with IF buffer were performed. Coverslips were then incubated cell side down upon a drop of secondary antibody (in IF buffer). Four more 5-minute washes of IF buffer were performed. The coverslips were rinsed briefly in 1X PBS two times. Then, after a brief rinse in DEPC water, the coverslips were carefully blotted dry and mounted on a drop of mounting media (stored at -20ºC) upon a microscope slide. The slide was dried overnight at room temperature in a cool, dry place. The following primary antibodies were used: mouse anti-TRA-1-60 (1:500; Abcam), rat anti-CTIP2 (1:500; Abcam), rabbit anti-DSCAM

(1:500; Sigma) and mouse anti-TUBB3 (1:1000; DSHB). The following secondary antibodies were used: donkey anti-mouse 488 (1:1000; Invitrogen), donkey anti-rat Cy3 (1:500; Jackson Immuno), DAPI (1:1000; Sigma), goat anti-rabbit Alexa 488 (1:1000; Thermo Fisher Scientific), and goat anti-mouse Alexa 568 (1:1000; Thermo Fisher Scientific).

Image acquisition

Slides were imaged using a Nikon Ti2E inverted microscope and a Hamamatsu Orca Fusion camera in stitched images at 20x with 15% overlay. Fluorescence intensity and other cellular attributes were measured using the DIC, TxRed (for TUBB3), and FITC (for DSCAM) channels. Morphological attributes and fluorescence were analyzed using ImageJ. Data was recorded, averaged, and normalized to the control group in Microsoft Excel. Graphs and statistical analysis were performed in GraphPad Prism.

Results

All experiments herein were performed using two hiPSC lines: one created from an individual with Down syndrome and an isogenic control line. This experiment is an n of 1 differentiation. Therefore, no statistical analyses were performed on the data, other than normalization to the control sham group. The results should be considered in the context of no replications and suggests future replicative experiments need to be completed.

Determination of cortical glutamatergic neuronal identity via CTIP2

Coup-TF interacting protein 2, or CTIP2 (also known as Bcl11b), is known to be expressed in a subset of layer V neurons in the cerebral cortex along with other markers of glutamatergic markers, such as VGLUT1 (Alcamo et al., 2008; Lai et al., 2008; Franco et al.,

2011). Therefore, it is used as a marker of cortical glutamatergic neuronal identity. TRA-1-60 is a cell surface antigen expressed on induced pluripotent stem cells and is commonly used as a marker of pluripotency, as it is lost as differentiation proceeeds (Schopperle & DeWolf, 2007). We performed immunocytochemistry on these hiPSC lines for CTIP2 and TRA-1-60 and found that these cells expressed the appropriate markers at each developmental stage (**Figure 2**). Before differentiation, hiPSCs expressed TRA-1-60; whereas after neuronal differentiation, hiPSC-derived neurons expressed CTIP2.

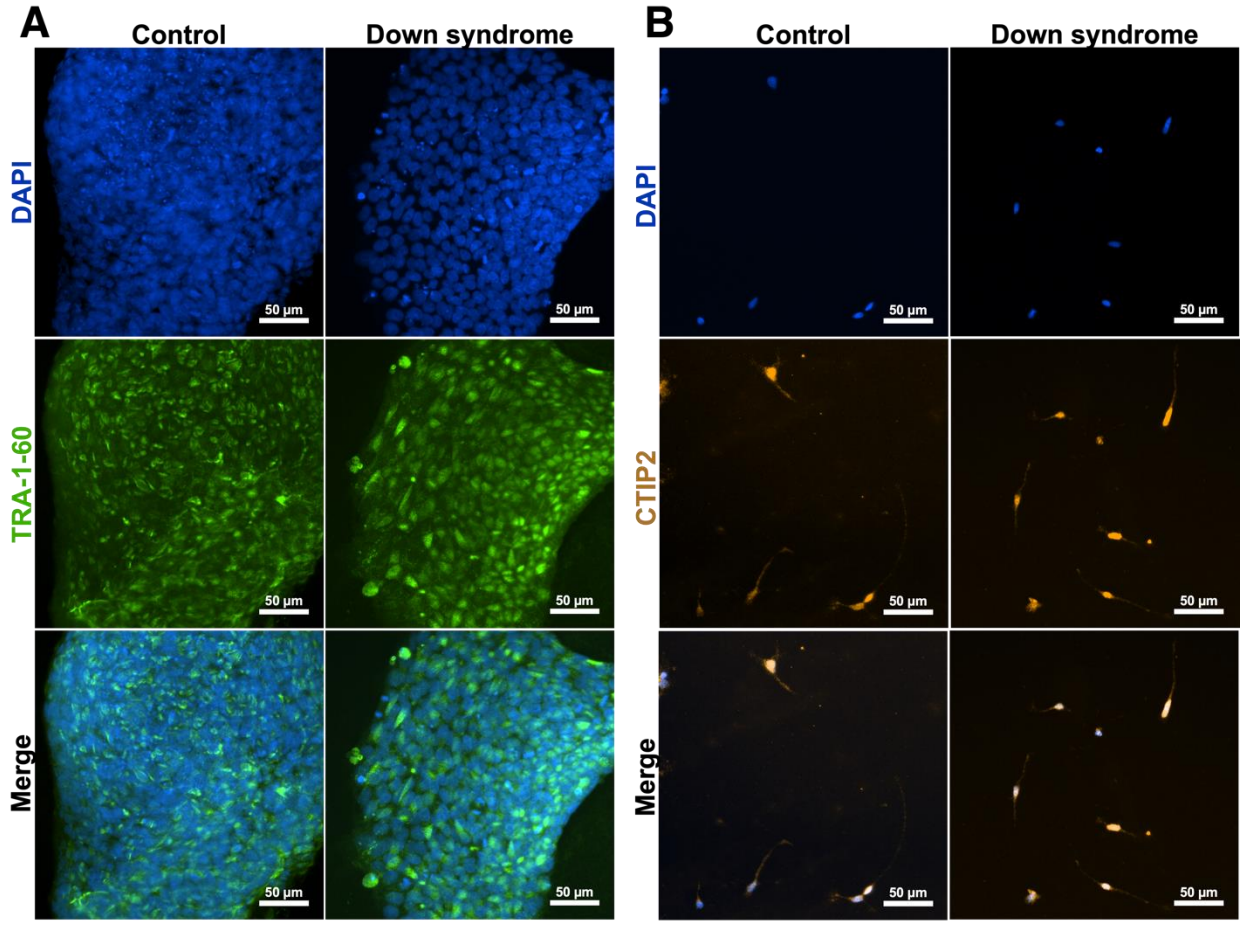

Figure 2*. The hiPSC lines used express pluripotency and neuronal markers at appropriate stages.* **A)** hiPSC colonies were stained for TRA-1-60. Scale bars, 50µm. Brightness and contrast were adjusted to optimize visibility.

B) hiPSC-derived neurons were stained for CTIP2 . Scale bars, 50μm. Brightness and contrast were adjusted to optimize visibility.

DSCAM is overexpressed in Down syndrome hiPSC-derived neurons

DSCAM has been found to be overexpressed in various DS samples, including post-mortem human tissue samples, murine models, and some neuronal cell lines (Saito et al., 2000; Jia et al., 2011; Pérez-Núñez et al., 2016). Therefore, we sought to examine the same in herein. In this experiment, Control and DS neurons were used in the following groups: no treatment (Sham), scrambled siRNA, and DSCAM siRNA. We then quantified DSCAM expression by performing immunocytochemistry, which stained cell samples for the DSCAM protein (see Methods and Materials). DSCAM was significantly increased in the soma of DS hiPSC-derived neurons, as compared to the isogenic control neurons (**Figure 3; Sham Control vs. Sham Down syndrome**). DSCAM expression was also increased in DS hiPSC-derived neurons treated with scrambled siRNA as compared to control hiPSC-derived neurons treated with scrambled siRNA (**Figure 3)**. Thus, this experiment further validates that DSCAM is overexpressed in DS and establishes the hiPSC model as suitable to study for the development of therapeutics and target mechanisms related to DSCAM.

However, we were unable to knockdown DSCAM using siRNAs in the DS neurons. There was not a significant reduction in DSCAM expression in the DSCAM siRNA-treated DS hiPSC-derived neurons as compared to their Sham and scrambled counterparts (**Figure 3**). This suggests that the knockdown was not successful.

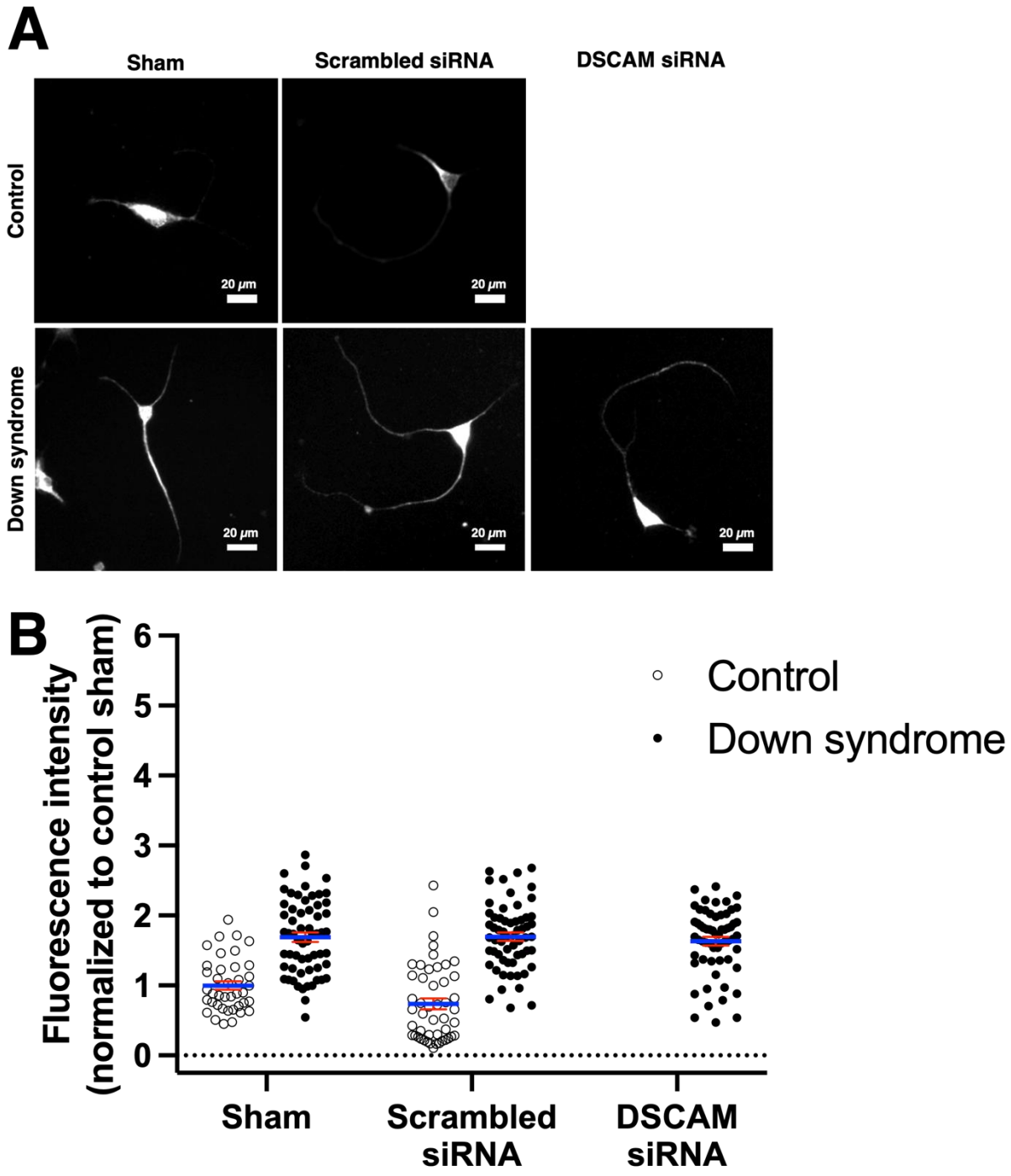

Figure 3. *DSCAM protein expression is increased in DS hiPSC-derived neurons.* **A)** Neurons were stained for DSCAM, and fluorescence in the soma was quantified. Scale bars, 20μm. Brightness and contrast were adjusted to optimize visibility. **B)** Quantification of DSCAM normalized to control. DSCAM expression is increased in DS neurons. For DS neurons treated with DSCAM siRNA, DSCAM expression is not different when compared to

DSCAM siRNA group, suggesting knockdown was not successful. Control Sham, n = 39 neurons; Control Scrambled siRNA, n = 48 neurons; DS Sham n = 60 neurons; DS scrambled siRNA, n = 60 neurons; DS DSCAM siRNA, n = 60 neurons.

Morphological deficits observed in Down syndrome hiPSC-derived neurons

Because DSCAM overexpression has been linked to morphological alterations in other models, the effect of DSCAM on such attributes was observed between the two cell lines. There were observed differences in axon length and overall neurite length between DS and control neurons. The axon length and overall neurite length was increased in DS neurons (**Figure 4A, B, and C**), as compared to Control neurons. Some DS neurons also exhibited increased branching in their axons compared to control neurons (**Figure 3A**), but quantification did not show any difference (**Figure 4E)**. The development of a prominent axon and increased branching can be used as a general indicator of excitatory neuron development (Shi et al., 2012). These results suggest that Down syndrome, and potentially DSCAM overexpression, may be linked to morphological deficits.

The soma area and dendritic morphology of the neurons were also analyzed. The mean soma area is very slightly reduced in the DS lines (**Figure 4D**). Dendritic morphology (length and number) was not different between the lines (**Figure 4F and 4G**). We were unable to make any conclusions about the DS neurons treated with DSCAM siRNA in relation to these morphology parameters because the knockdown was unsuccessful (**Figure 4B**).

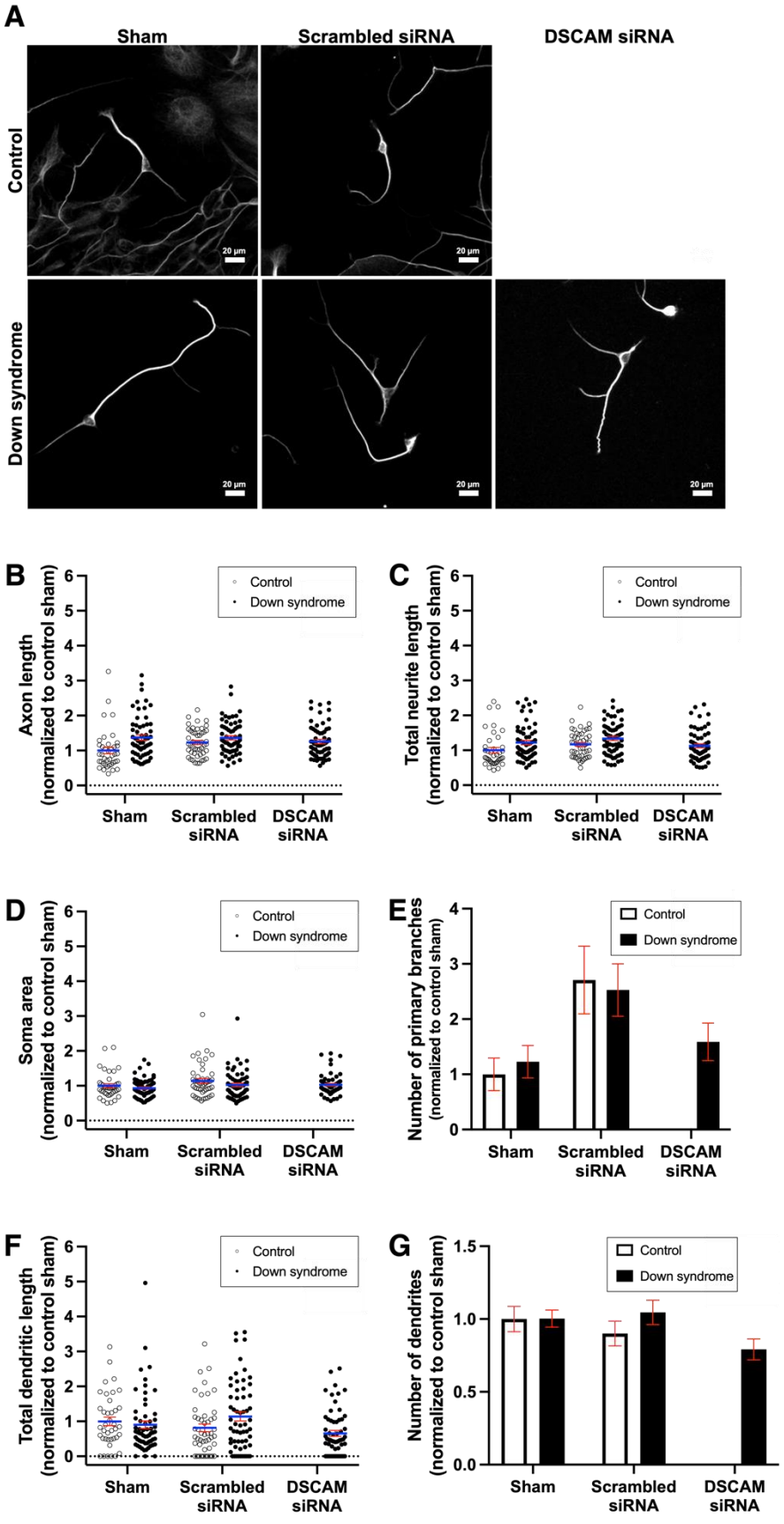
A
Sham
Scrambled siRNA
DSCAM siRNA
Control
Down syndrome
20 µm
B
Axon length
(normalized to control sham)
C
Total neurite length
(normalized to control sham)
D
Soma area
(normalized to control sham)
E
Number of primary branches
(normalized to control sham)
F
Total dendritic length
(normalized to control sham)
G
Number of dendrites
(normalized to control sham)
Control
Down syndrome
Sham
Scrambled siRNA
DSCAM siRNA

Figure 4. **A)** Neurons were stained for β-tubulin. Axon length, number of axon branches, number of dendrites, total dendrite length, total neurite length, and soma area were quantified. Scale bars, 20 μm. Brightness and contrast were adjusted to optimize visibility. **B-C)** Down syndrome hiPSC-derived neurons have longer axons and total neurite length. **D)** Mean soma area is slightly reduced in Down syndrome hiPSC-derived neurons. **E-G)** There was no difference in axon branching, dendrite length, and number of dendrites between the hiPSC-derived control and DS neurons. Control Sham, n = 39 neurons; Control Scrambled siRNA, n = 48 neurons; DS Sham n = 60 neurons; DS scrambled siRNA, n = 60 neurons; DS DSCAM siRNA, n = 60 neurons.

DSCAM may regulate morphology via TUBB3

We also examined the expression levels of β-tubulin III (TUBB3), which is a protein primarily expressed in neurons and plays an important role in the development and maintenance of neuronal structures and function. It is a major constituent of microtubules, which are cytoskeletal structures that provide stability and shape to the neuron and are essential for a variety of functions, including axon guidance (Tischfield et al., 2010). It is a common neuronal marker. After staining for TUBB3 using immunocytochemistry, it was found that TUBB3 expression is increased in DS neurons (DSCAM siRNA, scrambled siRNA and Sham groups; **Figure 5**). However, it was also upregulated in Control neurons treated with scrambled siRNA. This makes the results difficult to interpret because we would expect that the Control neuron/Sham group would be the same as the Control neuron/scrambled siRNA group. This suggests that the scrambled siRNA may be having an effect on TUBB3 expression; this means that it is not possible to make a conclusion about the Down syndrome neurons based on this data.

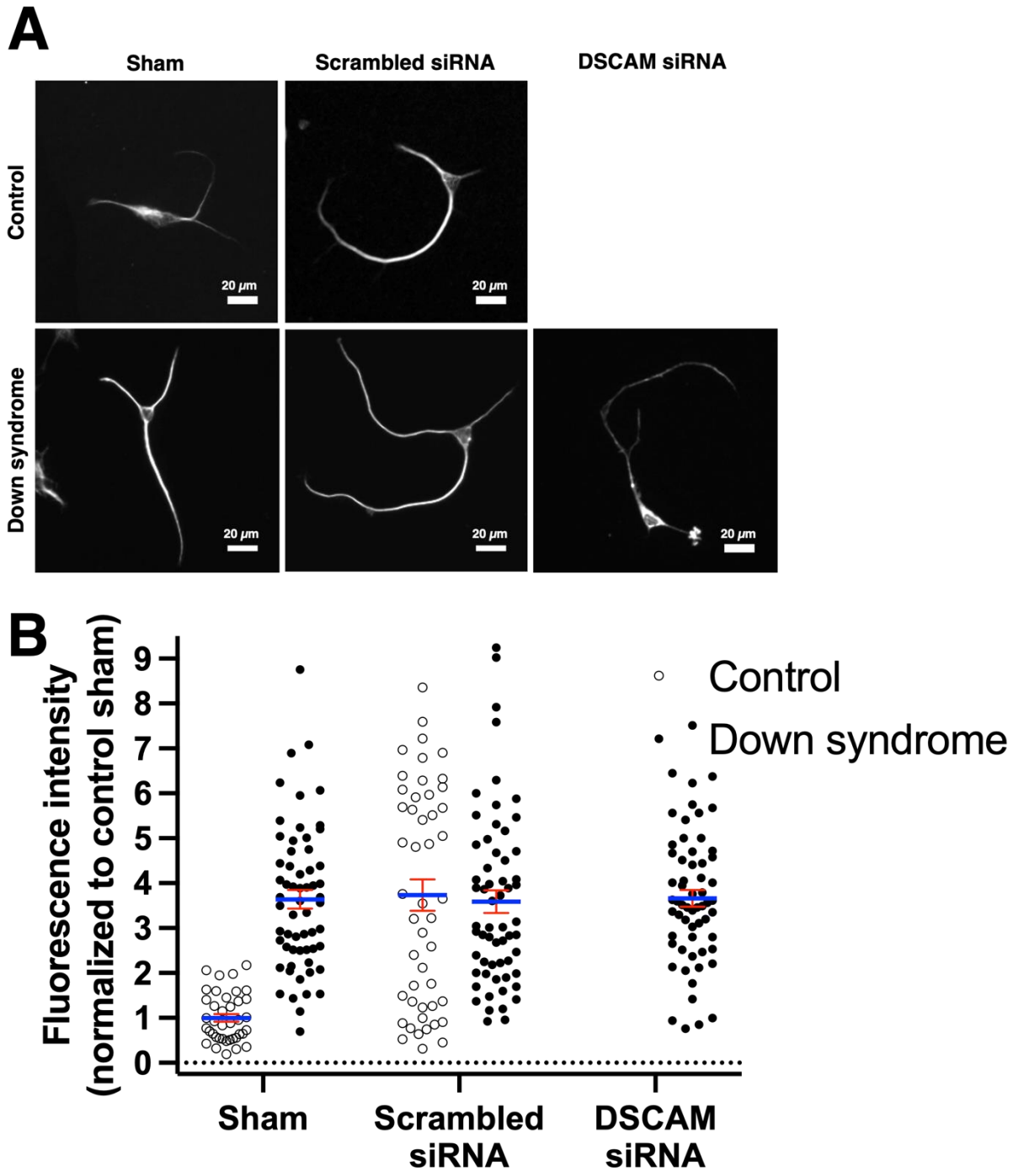

Figure 5. *TUBB3 protein is expressed in higher amounts in control neurons treated with scrambled siRNA and all DS hiPSC-derived neurons., as compared to sham control neurons.* **A)** Neurons were stained for β-tubulin, and fluorescence in the soma was quantified. Scale bars, 20μm. Brightness and contrast were adjusted to optimize visibility. **B)** Quantification of TUBB3 normalized to control. TUBB3 expression levels are higher in control

neurons treated with scrambled siRNA and all DS hiPSC-derived neurons, as compared to sham control neurons. Control Sham, n = 39 neurons; Control Scrambled siRNA, n = 48 neurons; DS Sham n = 60 neurons; DS scrambled siRNA, n = 60 neurons; DS DSCAM siRNA, n = 60 neurons.

Discussion

DSCAM in Down syndrome

Down syndrome (DS), or trisomy 21, is a genetic neurodevelopmental disorder characterized by the presence of an additional copy of all or part of human chromosome 21 (HSA21). One of the genes present on this chromosome is DSCAM (Down syndrome cell adhesion molecule), which is crucial to various developmental processes. These include neuronal growth, growth cone formation, and synaptic function (Zhu et al., 2011). DSCAM is overexpressed in postmortem tissue and murine models (Saito et al., 2000; Jia et al., 2011; Pérez-Núñez et al., 2016).

Down syndrome is also the largest cause of genetic-related intellectual disability (ID). The overexpression of DSCAM is being studied as a potential target for the development of therapeutic interventions, as it has effects on cognitive function and can ultimately impact intellectual disability. DSCAM is one of many genes implicated with the triplication of HSA21, the smallest human autosomal chromosome. The long (q) arm of the chromosome has been completely sequenced by Hattori et al. in 2000, but the shorter (p) arm is still in the process of being sequenced (Patterson, 2009). The function of all the possible genes and gene models is not completely known. Even with those whose functions have been identified, the mechanisms by which the phenotype of intellectual disability arises are not well understood. There are many confounding variables, as Down syndrome also presents across a spectrum of severity, making it

harder to determine the effect of gene dosage, alternative splicing, and other mechanisms that make an individual's genotype and phenotype unique to their condition.

DSCAM, specifically, has been linked with abnormalities in synapse formation and maturation (Sachse et al., 2019). Synaptic alterations are often the culprit in the development and progression of cognitive deficits in neurodevelopmental disorders such as Down syndrome. DSCAM has also been found to affect dendritic morphology, which is a crucial component of synaptic plasticity and connectivity (Maynard & Stein, 2012). DSCAM has effects on downstream pathways and is associated with changes in gene expression patterns and cell signaling pathways that are directly affected in neurodevelopment. These could contribute to the impairments common to individuals with DS, including intellectual disability. Thus, DSCAM is a good gene of interest to target in developing treatments because it is widely known to be overexpressed, and impacts mechanisms known to potentially contribute to ID. Herein, we find that DSCAM expression is increased in Down syndrome hiPSC-derived cortical neurons (**Figure 3**). This is important, as it establishes this particular cell line as a viable and effective model that can be used to develop treatments and study pathophysiology and underlying molecular mechanisms related to DSCAM. However, we were surprised to find that our treatment with DSCAM siRNA was unsuccessful and did not result in a knockdown in DSCAM protein expression, as observed by unchanged levels of DSCAM between the DS Scrambled siRNA and Sham groups as compared to the DS DSCAM siRNA group (**Figure 3**). It may also be considered that compensatory mechanisms may be maintaining high DSCAM protein levels, such as alternative splicing, post-transcriptional modifications, or differences in cellular metabolism and other factors that may affect the efficacy of siRNA knockdowns. Nevertheless, increased DSCAM and TUBB3 expression in these cell lines verify previous results in neurons

derived from other Down syndrome iPSC lines (Manasi Agrawal, unpublished results), and establishes that this line is an effective model that can be used to study these gene expression pathways.

Synaptic deficits and morphology rescue

It has previously been established that DSCAM overexpression leads to altered synaptic formation and neurite growth. A variety of papers have observed a relationship between the overexpression of DSCAM and altered dendritic arborization, as well as the gene's influence on synaptic density, specificity, and plasticity in a *Drosophila* model (Yamagata et al., 2003; Chen et al., 2006). The current experiment supported the generalization that a change in neuronal morphology is influenced by the expression of DSCAM. DS neurons showed increased axon length and total neurite length relative to the control group. These changes may be attributed to fluctuating levels of microtubules present in the growing neuron's cytoskeleton.

Cortical neurogenesis among individuals with Down syndrome is generally downregulated, and there is also a slower differentiation rate of neurons (Contestabile et al., 2007; Guidi et al., 2008). This mechanism contributes to the DS-associated cognitive defects and neurological complications (Korenberg et al., 1994; Lott & Dierssen, 2010). Neurons in earlier stages of differentiation exhibit shorter axon length and less branching, as previously mentioned (Shi et al., 2012). Therefore, we find it puzzling that we had longer axons and neurite length in the current experiment. This also leads to another identifiable limitation of this experiment. The small sample size may have hindered the ability to draw a significant conclusion from the limited pattern present. Specifically, it was difficult to identify isolated pyramidal neurons in the control line, leading to a lesser number of neurons to analyze, which may have had an effect on the data.

It is also worth noting that creating and maintaining hiPSC lines can be a challenging, resource-intensive, and time-consuming process, which limits the number of samples available for research. Additionally, there may be inherent variability between lines, as DS presents with high inter-individual variability, meaning that individuals with DS exhibit a large spectrum of phenotypes.

TUBB3, or beta tubulin 3, is a protein expressed primarily in neurons. It is involved in the formation and stabilization of microtubules in the axon, as well as dendrite development and maintenance (Radwitz et al., 2022). Therefore, there is a correlation between morphological attributes of neurites and the expression of TUBB3 in these hiPSC-derived neurons. There is limited research on the direct interaction between DSCAM and TUBB3. The indirect impact of DSCAM overexpression on microtubule dynamics may be causing a compensatory effect of TUBB3. For example, a paper by Simmons et al. suggests that DSCAM and TUBB3 interactions regulate dendrite and axon arborization. This interaction may be important for establishing connectivity and synaptic plasticity, which may be integral to understanding DS-associated cognitive defects (Simmons et al., 2017). The question still remains: to what extent can knocking down DSCAM (and thereby modulation of TUBB3 expression) rescue intellectual disability?

DSCAM siRNA knockdown and other mechanisms

DSCAM has a crucial role in the development of the nervous system, including neural development, synaptic connectivity, and axon guidance, as previously described (Yamakawa et al., 1998). It has specifically been shown to be important in dendrite arborization and spine formation in cortical pyramidal neurons, such as those studied in this experiment (Maynard & Stein, 2012). In addition to this, DSCAM has been implicated in several other cellular processes

in the developing brain. For example, DSCAM has been shown to play a role in cell survival, proliferation, and migration (Mitsogiannis et al., 2020). DSCAM has also been implicated in other diseases and disorders, such as autism spectrum disorder and Alzheimer's disease, highlighting the importance of this gene in Down syndrome and beyond (Viard et al., 2022). Therefore, it is a promising target for therapy development.

One potential approach is using small interfering RNA molecules, or siRNAs, to “knock down” the DSCAM gene. siRNAs target and degrade messenger RNA (mRNA), leading to decreased expression of a desired protein (Haiyong 2018). This technique is very useful, as it has a high specificity and potency to almost any gene of interest (Semizarov et al., 2003). siRNAs are synthesized relatively quickly and inexpensively. However, a major limitation of this mechanism is the chance of unintended off-target effects, which can have unknown consequences and serve as a confounding variable in models (Sudbery et al., 2010). This is why using a scrambled control siRNA alongside a sham group is important. The scrambled control siRNA is used by researchers to distinguish between effects of knockdown and the aforementioned off-target effects. A scrambled control is typically a treatment of siRNA that does not target any specific gene or using a treatment that has no known biological effect. The scrambled control siRNA can be compared to the specific siRNA knockdown (e.g. DSCAM) to ensure the validity and reliability of the results and enables researchers to draw accurate conclusions about the role of the target gene. One more limitation of siRNAs is their variable efficacy, which depends on the target gene and the cell type they are used in. In the case of DSCAM, siRNA knockdown may be an effective approach due to the high expression of DSCAM in neurons. However, it is important to consider the potential for off-target effects and alternative mechanisms for targeting DSCAM, as well. For example, in this experiment, siRNA

knockdown was not successful, therefore it cannot be established based on its results alone that siRNAs are the most effective treatment.

Although the siRNA knockdown was unsuccessful, there are still various avenues for further exploration. This may include testing the efficacy of other siRNAs to target different regions of the DSCAM gene. The particular siRNA may not be as effective on this cell line, as individual variability impacts gene expression as well. Additionally, alternatives include examining alternative mechanisms, such as CRISPR-Cas9, antisense oligonucleotides, or small inhibitory molecules. These are other targeting mechanisms which have been studied in the context of controlling gene expression across a wide variety of disorders and diseases and could be used to examine the disease phenotype in DS, and more specifically, in the DS1 / DS2U hiPSC cell lines.

CRISPR-Cas9 (Hsu et al., 2014) is a new, evolving technique for genome editing which is used to target and cut specific DNA sequences within a gene, generating the “knockout” or “knockdown” of a gene. It is a highly favored gene editing tool in recent times for its high specificity and precision, but also has certain shortcomings. Its limitations include the potential for off-target effects and the difficulty of delivering Cas9 protein and guide RNA to more specialized cell types. Antisense oligonucleotides can also be utilized as another technique to knockdown genes. They are short synthetic molecules of genetic material, either DNA or RNA, which hybridize with complementary sequences to interfere with function. The power of these oligonucleotides has been used successfully in several preclinical and clinical research settings. This technique is often not as efficient and can vary in its design and delivery in particular tissues and cells. Lastly, small molecules are yet another potential approach for targeting specific genes of interest. They are designed to inhibit protein function or interfere with gene expression

through inhibition or destruction of transcript sequences, destabilizing mRNA, and translation of the protein. This technique is most useful when targeting genes that are more difficult to access using other techniques. Much like other techniques, this one also has its limitations. It requires careful optimization of molecular properties and delivery method, as there is a potential of toxicity and off-target effects. These techniques are relevant to not only DSCAM, but also various other genes implicated in this neurodevelopmental disorder.

Other genes implicated in the DS intellectual disability phenotype

Identification and understanding of the genes present on HSA21 and their roles in neurodevelopmental processes is crucial in advancing an understanding of DS itself and the development of effective treatments. Intellectual disability is one of the most prominent, but not the only, effect of altered gene expression in DS. Individuals with Down syndrome have distinct physical features (small head, flat facial profile, almond-shaped eyes, etc.), higher risk of developing medical conditions (including Alzheimer's disease, congenital heart defects, respiratory infections, hearing and vision problems, and more), delays in development (not just intellectually, but also in motor skills, speech, and language). The severity and impact of these comorbidities varies but are all associated with the triplication of HSA21. The genes studied herein, DSCAM and TUBB3, must be studied in context of the entire disorder, as they interact with other genes and proteins to produce a personal phenotype for each individual with the syndrome. TUBB3 is located on chromosome 16, which is not triplicated in DS, but expression of this gene is upregulated in DS neurons. There are about 225 protein-coding genes located on chromosome 21 and triplicated in individuals with Down syndrome. Overall, this triplication of chromosome 21 in individuals with Down syndrome is thought to result in genome-wide

dysregulation. Some of these genes interact with DSCAM and TUBB3, which were found to be upregulated in hiPSC-derived glutamatergic neurons from individuals with Down syndrome.

Anywhere between 40 to 60 percent of individuals with Down syndrome develop Alzheimer's disease in later life (Salehi et al., 2016). Amyloid beta precursor protein (APP) is a transmembrane protein involved in the formation of amyloid beta plaques, pathological hallmarks of Alzheimer's disease (Zheng & Koo, 2011). The APP gene is of interest, as individuals with Down syndrome have three copies of APP and therefore an elevated expression, which may be related to their increased risk of developing Alzheimer's (Salehi et al., 2016). APP has also been shown to interact with DSCAM in regulating axon guidance and synaptic function. There is currently limited research to demonstrate that APP may regulate the expression and function of DSCAM, and potentially affect its role in synapse formation and axon guidance. Although studies suggest that APP and DSCAM have a related expression pattern (Jia et al., 2017), it is still largely unknown how exactly DSCAM overexpression relates to APP and Alzheimer's. Therefore, this is a potential avenue hinting towards future studies, especially in hiPSC models, as much of the existing literature revolves around murine models. One study found that APP deficiency led to decreased spine number and dendritic branching, and impaired synaptic function (Tyan et al., 2012), suggesting that APP might also be contributing to this. APP may be the most widely studied gene in Down syndrome pathology, and therefore it is important to examine its interactions with DSCAM and its greater implications for neuronal morphology.

Dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) is a protein kinase also known to be overexpressed in individuals with DS. It is involved in several cellular processes, including cell cycle regulation, learning, and memory. It is also crucial to neural

development and interacts with DSCAM to regulate this process (Sachse et al., 2019). Disruption of DYRK1A has also been linked to autism spectrum disorder (ASD) and has been speculated to be important in other neurological disorders as well (Earl et al., 2017). A recent study by Viard et al., 2022 found that the intracellular domain of DSCAM binds DLGs, (multimeric scaffolds comprising receptors), ion channels and associated signaling proteins, or DYRK1A. This binding forms a distinct complex, which the researchers coined as the "DSCAM-DYRK1A interaction domain." The pathways that are affected by DYRK1A triplication impairs are not completely understood, but the discovery of this interaction domain suggests that DYRK1A and DSCAM have a complex link to not only Down syndrome, but also other neurological diseases with a genetic component. The studies mentioned in the introduction by Guedj et al. (2009) and De Toma et al. (2019) investigated the potential therapeutic benefits for targeting the DYRK1A-DSCAM pathway with epigallocatechin gallate (EGCG), a natural compound found in green tea. The studies found that the treatment reduced the overexpression of DYRK1A and improved cognitive function in mice. Overexpression of DYRK1A has been linked to cognitive deficits and other features of Down syndrome. Additionally, one study found that inhibition of DYRK1A led to a lack of differentiation in human embryonic stem cells (Bellmaine et al., 2017). This is interesting when compared to the findings of this experiment, as the triplication of DYRK1A in the DS line may have resulted in premature differentiation. However, this is not conclusive, as individual gene interactions are highly variable between individuals and also between cell lines. Therefore, the DYRK1A and DSCAM interaction is relevant to studying cognitive deficits in relation to neuronal morphology in DS.

Super oxidase dismutase 1 (SOD1) is an antioxidant enzyme that protects cells from oxidative stress by catalyzing the conversion of superoxide radicals into hydrogen peroxide and

molecular oxygen (Xu et al., 2022). However, the relationship between SOD1 and oxidative stress is not always linear and has been demonstrated to be complex. In some cases, overexpression of SOD1 decreases oxidative stress (Dimayuga et al., 2007). In other studies, knockdown of SOD1 leads to an observed increase in oxidative stress (Xu et al., 2022). However, an overexpression of SOD1 has been linked to increased cellular oxidative stress and pathology of DS (Nabarra et al., 1996; Lee et al., 2001). Overexpression of SOD1 has been linked to learning and memory deficits (Shin et al., 2004), which suggests it may have a role to play in cognitive defects present in disorders like Down syndrome. SOD1 has been demonstrated to be upregulated in DS brains (Brooksbank & Balázs, 1983). Another study found that in animals with SOD1 mutations, DCC expression is also altered (Liu et al., 2020). As DCC is a DSCAM-interacting gene, it could be affected in SOD1 mutants and may be a potential opportunity for further research. Irregularities in TUBB3 are known to disrupt netrin signaling (Shao et al., 2019), hinting towards a relationship between all of these components of neural development. One study generated motor neurons from hiPSCs and observed that mutations in SOD1 caused significant differences in cell morphology (Dash et al., 2022). Although this was not studied in the context of Down syndrome, this may suggest that studying the effects of altered SOD1 in DS neurons is an interesting way to observe changes in cell morphology, and ultimately their ability to establish strong connections. Overall, these findings establish SOD1 as an interesting candidate in the study of gene interactions impacting the intellectual disability phenotype associated with Down syndrome.

Potassium inwardly-rectifying channel, subfamily J, member 6 (KCNJ6) is a potassium channel known to regulate membrane potential and neuronal excitability by regulating potassium flow into the cell (Horvath et al., 2018). It is triplicated in DS, and its overexpression alters

synaptic plasticity and causes learning and memory deficits in mouse models of DS (Cooper et al., 2012). Rescue of deficits using fluoxetine was successful, demonstrating that gene dosage of KCNJ6 are relevant in these anomalies (Kleschevnikov et al., 2017). This demonstrates a relationship to the abnormal neurological phenotypes characteristic of DS. One paper found that a KCNJ6 haplotype led to an observed increase in the number of neurites, their area, and amount of branching (Popova et al., 2023). This is important in the context that the experiments performed as the basis of this paper are studying excitatory cortical neurons. Therefore, alterations in potassium channels can also impact the ability of neurons to extend neurites, form synapses, and be stimulated for a signaling function.

Interferon signaling, oxidative stress, and cellular senescence in relation to pathways altered in various phenotypes of Down syndrome

Interferons are signaling proteins released in response to the detection of viruses to heighten antiviral defense (Kline & Kitagaki, 2006). Interferon signaling is a complex pathway that contributes to the innate immune system by binding to specific receptors and triggering a signaling cascade to express interferon-stimulated genes (ISGs). One very important ISG known to contribute to the weakened immune response and increased susceptibility to respiratory infections observed in individuals with Down syndrome is myxovirus resistance 1 (MX1), an interferon-inducible antiviral defense protein. It is known to be triplicated in Down syndrome and linked to cognitive impairment (Blank et al., 2016), presenting an interesting case for potential targeting mechanisms as well. MX1 interferon signaling is involved in the immune response and inflammatory reaction (Hadjadj et al., 2020), both of which are known to be altered in DS (Ram & Chinen, 2011). The increased risk of autoimmune disorders, infections, and

cancers in individuals with DS has been suggested to be caused by dysregulation in interferon signaling (Chung et al., 2021). Transcriptional analysis and further research have shown that upregulation of IFN-related factors not encoded on chromosome 21 contribute extensively to this dysregulation (Krivega et al., 2021; Waugh et al., 2019).

Correction of interferon receptor gene dosage has been shown to rescue multiple key phenotypes in a mouse model of trisomy 21 by increasing antiviral responses, preventing malformations of the heart, decreasing developmental delays, improving cognition and correction of craniofacial abnormalities (Waugh et al., 2022). MX1 is likely therefore involved in the regulation of neuronal function and plasticity.

Oxidative stress, among other genotoxic factors, is known to impair the innate immune response, especially in human cells containing extra chromosomes, such as in individuals with DS (Krivega et al., 2021). Upregulation of interferon signaling in DS has been suggested to be a result of upregulated IFN-related factors and the low-grade inflammation they induce (Krivega et al., 2021). This increased inflammation feeds into increased oxidative stress, further illustrating a relationship between intellectual disability and interferon signaling in DS. Therefore, targeting MX1 interferon signaling could be a possible therapeutic strategy, although it may prove to be challenging with the numerous pathways and functions in which MX1 participates. Interferon signaling is thereby determined to have a major role in oxidative stress, which impacts neurodevelopmental cellular senescence (Frisch & MacFawn, 2020; Jin et al., 2022).

In neurodevelopment, cellular senescence is a process in which neurons enter a stage of growth arrest which is irreversible and forces them to stop dividing (Klein et al., 2023). This can occur in embryonic development, impacting crucial stages in brain formation and other pre- and postnatal growth (Huang et al., 2022). Senescence is a stress response, resulting from an

imbalance between reactive oxygen species (ROS) and antioxidant defense mechanisms. Increased ROS damages DNA and other macromolecules and increases neuroinflammation via release of cytokines and chemokines (Perluigi & Butterfield, 2011). It is associated with accelerated cellular aging (Hayflick & Moorhead, 1961), relating to the preexisting knowledge that individuals with DS experience premature aging, including that of their immune system (Marcovecchio et al., 2021). Therefore, it is important to consider the role of pathways in cellular senescence and oxidative stress when studying the etiology and pathogenesis of neurodevelopmental disorders with phenotypes of intellectual disability, such as Down syndrome.

One interesting hypothesis regarding this proposes that despite the known detrimental effects of oxidative stress and cellular senescence on brain function, the brain can adapt to respond via compensatory mechanisms (Perluigi & Butterfield, 2011). As the developing brain faces greater levels of ROS and stress at the start of pathogenesis, the surviving cells learn to proliferate by using defense mechanisms to compensate. This proposition was supported by one study which examined oxidative stress in lymphocytes, a type of white blood cell crucial to immune response (Zana et al., 2006). This, taken with the importance of interferon signaling, demonstrates a need to study the effects of the microenvironment of the neurons growing in developing individuals with Down syndrome. One study showed that compensatory mechanisms may include an increased metabolic rate and activation of plasticity mechanisms (Head et al., 2007). Genes such as APP, DYRK1A and SOD1, known to regulate neuronal growth, may also promote compensatory mechanisms as the cells age prematurely (Head et al., 2007), and the total effects of their overexpression require further understanding.

To further support the role of interferon signaling, an upregulation of MX1 is known to promote the senescence-associated secretory phenotype (SASP) (Nagaraj et al., 2022), which can contribute to neuroinflammation and cognitive decline in association with neurodegenerative diseases, including DS (Martínez-Cué & Rueda, 2020). MX1 has also been shown to play a role in regulating cellular senescence in various cell types, including neurons. Similarly, the activation of pathways involved in cellular senescence can lead to changes in gene expression and cellular metabolism that promote survival and maintain cellular function. It has been recently discovered that in DS cells, genome-wide disruptions caused by the trisomy of chromosome 21 lead to alterations in chromosome configuration, DNA accessibility in neural progenitor cells, and a senescence state (Meharena et al., 2022). This study establishes senescence as a potential target for studying rescue effects.

The expression of SOD1 and other antioxidant enzymes may be upregulated in response to this stress, which can reduce the amount of ROS and protect the cells from further damage. However, as examined before, SOD1's mechanism has not been studied sufficiently to determine that its relationship with neuron morphology and neurite extension is not a result of downstream effects. An increased expression of SOD1 results in greater breakdown of ROS into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). However, a large accumulation of H_2O_2 can result in increased oxidative stress, further exacerbating the issue. Despite this, SOD1 is known to be upregulated in various tissues from individuals with DS (de Haan et al., 1995), suggesting that the regulation of oxidative stress is variable, and therefore may contribute to the variability in cognitive phenotypes observed in individuals with DS. The APP gene is also known to increase levels of oxidative stress via an increased accumulation of beta amyloids, but also has some protective effects with co-overexpression with BACE2 (Azkona et al., 2010), which is a protein

that cleaves amyloid precursor. As mentioned before, a variety of genes known to also regulate neuronal growth and morphological attributes have been found contributing to this compensatory effect, and thereby require more research and review of the existing literature. The role of DSCAM is not widely understood in this context, and the knowledge of its function as a cell adhesion molecule, role in neurogenesis and neural development pose it as a candidate gene for further understanding.

In summary, a hypothesis that can be made is that the genes we know to regulate neuronal development may be contributing to compensatory mechanisms that contribute to the variability in phenotypes observed in individuals with DS, as the cellular processes in neurodevelopment may better adapt some to oxidative stress and cellular senescence than others. A schematic of this hypothesis is illustrated below (Figure 6). Further research aimed at elucidating the precise mechanisms of DSCAM and its interacting genes may provide insight on interferon signaling, the accumulation of oxidative stress, and the proposed pathway by which cognitive function is determined. This would be effective to observe in murine models, hiPSCs, and organoid models, as each offer a different level of insight into behavioral and biochemical effects.

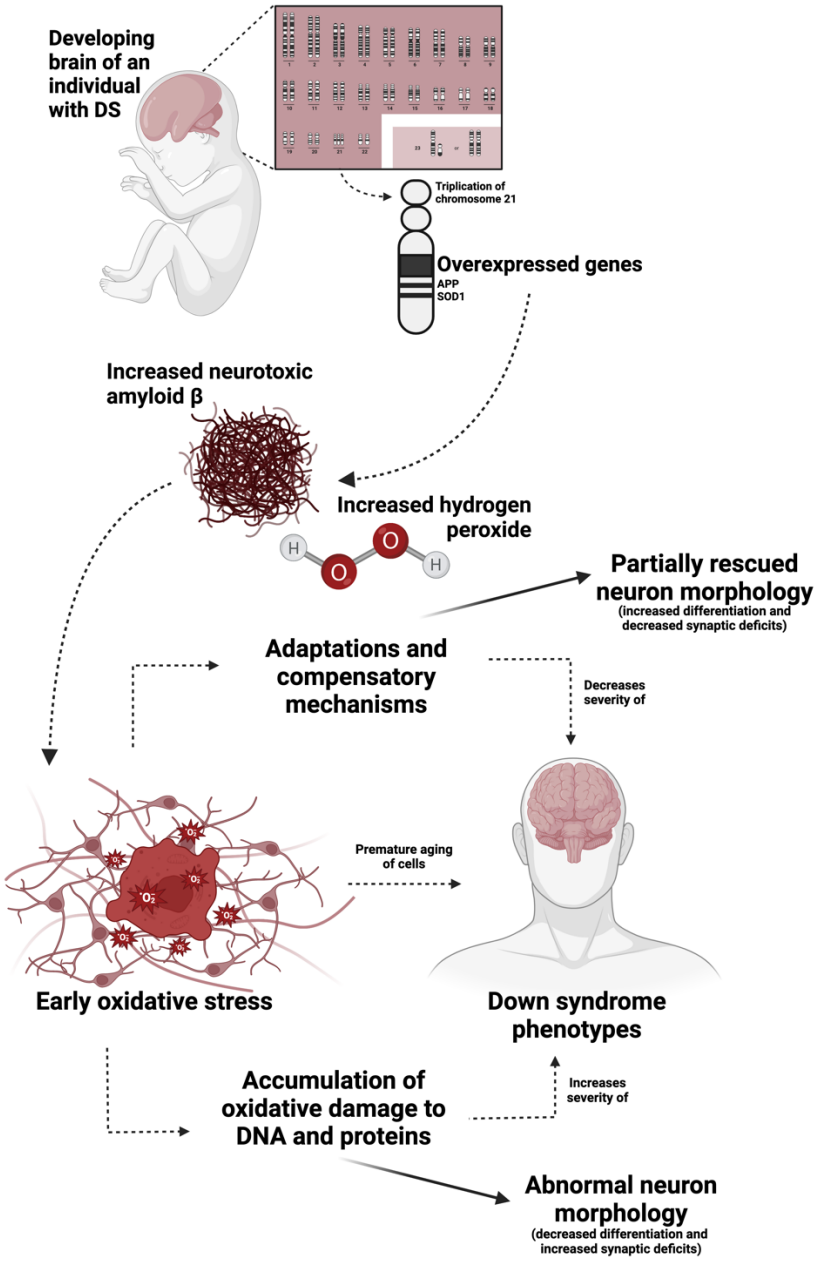

Figure 6. Based on literature review, a proposed mechanism by which Down syndrome hiPSC-derived neurons may exhibit more complex morphologies. Compensatory mechanisms and gene interactions in response to increased oxidative stress and triplication of chromosome 21 may cause premature differentiation and more complex morphologies. Adapted from Perluigi and Butterfield, 2011.

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References

Alcamo, E. A., Chirivella, L., Dautzenberg, M., Dobreva, G., Fariñas, I., Grosschedl, R., & McConnell, S. K. (2008). Satb2 Regulates Callosal Projection Neuron Identity in the Developing Cerebral Cortex. *Neuron*, *57*(3), 364–377. https://doi.org/10.1016/j.neuron.2007.12.012

Azkona, G., Levannon, D., Groner, Y., & Dierssen, M. (2010). In vivo effects of APP are not exacerbated by BACE2 co-overexpression: Behavioural characterization of a double transgenic mouse model. *Amino Acids*, *39*(5), 1571–1580. https://doi.org/10.1007/s00726-010-0662-8

Bansal, P., Banda, E. C., Glatt-Deeley, H. R., Stoddard, C. E., Ahern, D. T., Kondaveeti, Y., Nicouleau, M., & Pinter, S. F. (2022). *"A dynamic in vitro model of Down Syndrome neurogenesis with Trisomy 21 gene dosage correction"* (p. 2022.05.11.491519). bioRxiv. https://doi.org/10.1101/2022.05.11.491519

Barlow, G. M., Micales, B., Lyons, G. E., & Korenberg, J. R. (2001). Down syndrome cell adhesion molecule is conserved in mouse and highly expressed in the adult mouse brain. *Cytogenetics and Cell Genetics*, *94*(3–4), 155–162. https://doi.org/10.1159/000048808

Bellmaine, S. F., Ovchinnikov, D. A., Manallack, D. T., Cuddy, C. E., Elefanty, A. G., Stanley, E. G., Wolvetang, E. J., Williams, S. J., & Pera, M. (2017). Inhibition of DYRK1A disrupts neural lineage specificationin human pluripotent stem cells. *ELife*, *6*, e24502. https://doi.org/10.7554/eLife.24502

Benavides-Piccione, R., Ballesteros-Yáñez, I., Martínez de Lagrán, M., Elston, G., Estivill, X., Fillat, C., DeFelipe, J., & Dierssen, M. (2004). On dendrites in Down syndrome and DS murine models: A spiny way to learn. *Progress in Neurobiology*, *74*(2), 111–126. https://doi.org/10.1016/j.pneurobio.2004.08.001

Bianchi, P., Ciani, E., Guidi, S., Trazzi, S., Felice, D., Grossi, G., Fernandez, M., Giuliani, A., Calzà, L., & Bartesaghi, R. (2010). Early Pharmacotherapy Restores Neurogenesis and Cognitive Performance in the Ts65Dn Mouse Model for Down Syndrome. *The Journal of Neuroscience*, *30*(26), 8769–8779. https://doi.org/10.1523/JNEUROSCI.0534-10.2010

Blank, T., Detje, C. N., Spieß, A., Hagemeyer, N., Brendecke, S. M., Wolfart, J., Staszewski, O., Zöller, T., Papageorgiou, I., Schneider, J., Paricio-Montesinos, R., Eisel, U. L. M., Manahan-Vaughan, D., Jansen, S., Lienenklaus, S., Lu, B., Imai, Y., Müller, M., Goelz, S. E., … Prinz, M. (2016). Brain Endothelial- and Epithelial-Specific Interferon Receptor Chain 1 Drives Virus-Induced Sickness Behavior and Cognitive Impairment. *Immunity*, *44*(4), 901–912. https://doi.org/10.1016/j.immuni.2016.04.005

Bletsch, A., Mann, C., Andrews, D. S., Daly, E., Tan, G. M. Y., Murphy, D. G. M., & Ecker, C. (2018). Down syndrome is accompanied by significantly reduced cortical grey–white matter tissue contrast. *Human Brain Mapping*, *39*(10), 4043–4054. https://doi.org/10.1002/hbm.24230

Boyer, N. P., & Gupton, S. L. (2018). Revisiting Netrin-1: One Who Guides (Axons). *Frontiers in Cellular Neuroscience*, *12*. https://www.frontiersin.org/articles/10.3389/fncel.2018.00221

Brooksbank, B. W., & Balázs, R. (1983). Superoxide dismutase and lipoperoxidation in Down's syndrome fetal brain. *Lancet (London, England)*, *1*(8329), 881–882. https://doi.org/10.1016/s0140-6736(83)91427-7

Buck, K. B., & Zheng, J. Q. (2002). Growth Cone Turning Induced by Direct Local Modification of Microtubule Dynamics. *The Journal of Neuroscience*, *22*(21), 9358–9367. https://doi.org/10.1523/JNEUROSCI.22-21-09358.2002

Carcea, I., & Froemke, R. C. (2013). Cortical Plasticity, Excitatory–Inhibitory Balance, and Sensory Perception. *Progress in Brain Research*, *207*, 65–90. https://doi.org/10.1016/B978-0-444-63327-9.00003-5

Chen, B. E., Kondo, M., Garnier, A., Watson, F. L., Püettmann-Holgado, R., Lamar, D. R., & Schmucker, D. (2006). The Molecular Diversity of Dscam Is Functionally Required for Neuronal Wiring Specificity in Drosophila. *Cell*, *125*(3), 607–620. https://doi.org/10.1016/j.cell.2006.03.034

Chen, X.-Q., Salehi, A., Pearn, M. L., Overk, C., Nguyen, P. D., Kleschevnikov, A. M., Maccecchini, M., & Mobley, W. C. (2021). Targeting increased levels of APP in Down syndrome: Posiphen-mediated reductions in APP and its products reverse endosomal phenotypes in the Ts65Dn mouse model. *Alzheimer's & Dementia*, *17*(2), 271–292. https://doi.org/10.1002/alz.12185

Chiotto, A. M. A., Migliorero, M., Pallavicini, G., Bianchi, F. T., Gai, M., Di Cunto, F., & Berto, G. E. (2019). Neuronal Cell-Intrinsic Defects in Mouse Models of Down Syndrome. *Frontiers in Neuroscience*, *13*. https://www.frontiersin.org/article/10.3389/fnins.2019.01081

Chung, H., Green, P. H. R., Wang, T. C., & Kong, X.-F. (2021). Interferon-Driven Immune Dysregulation in Down Syndrome: A Review of the Evidence. *Journal of Inflammation Research*, *14*, 5187–5200. https://doi.org/10.2147/JIR.S280953

Clark, S., Schwalbe, J., Stasko, M. R., Yarowsky, P. J., & Costa, A. C. S. (2006). Fluoxetine rescues deficient neurogenesis in hippocampus of the Ts65Dn mouse model for Down syndrome. *Experimental Neurology*, *200*(1), 256–261. https://doi.org/10.1016/j.expneurol.2006.02.005

Contestabile, A., Benfenati, F., & Gasparini, L. (2010). Communication breaks-Down: From neurodevelopment defects to cognitive disabilities in Down syndrome. *Progress in Neurobiology*, *91*(1), 1–22. https://doi.org/10.1016/j.pneurobio.2010.01.003

Contestabile, A., Fila, T., Ceccarelli, C., Bonasoni, P., Bonapace, L., Santini, D., Bartesaghi, R., & Ciani, E. (2007). Cell cycle alteration and decreased cell proliferation in the hippocampal dentate gyrus and in the neocortical germinal matrix of fetuses with Down syndrome and in Ts65Dn mice. *Hippocampus*, *17*(8), 665–678. https://doi.org/10.1002/hipo.20308

Cooper, A., Grigoryan, G., Guy-David, L., Tsoory, M. M., Chen, A., & Reuveny, E. (2012). Trisomy of the G protein-coupled K+ channel gene, Kcnj6, affects reward mechanisms, cognitive functions, and synaptic plasticity in mice. *Proceedings of the National Academy of Sciences*, *109*(7), 2642–2647. https://doi.org/10.1073/pnas.1109099109

Czermiński, J. T., & Lawrence, J. B. (2020). Silencing Trisomy 21 with XIST in Neural Stem Cells Promotes Neuronal Differentiation. *Developmental Cell*, *52*(3), 294-308.e3. https://doi.org/10.1016/j.devcel.2019.12.015

Dash, B. P., Freischmidt, A., Weishaupt, J. H., & Hermann, A. (2022). Downstream Effects of Mutations in SOD1 and TARDBP Converge on Gene Expression Impairment in Patient-Derived Motor Neurons. *International Journal of Molecular Sciences*, *23*(17), Article 17. https://doi.org/10.3390/ijms23179652

de Haan, J., Cristiano, F., Iannello, R. C., & Kola, I. (1995). Cu/Zn-superoxide dismutase and glutathione peroxidase during aging. *Biochemistry and Molecular Biology International*, *35*(6), 1281–1297.

De Toma, I., Ortega, M., Aloy, P., Sabidó, E., & Dierssen, M. (2019). DYRK1A Overexpression Alters Cognition and Neural-Related Proteomic Pathways in the Hippocampus That Are Rescued by Green Tea Extract and/or Environmental Enrichment. *Frontiers in Molecular Neuroscience*, *12*. https://www.frontiersin.org/articles/10.3389/fnmol.2019.00272

Dimayuga, F. O., Wang, C., Clark, J. M., Dimayuga, E. R., Dimayuga, V. M., & Bruce-Keller, A. J. (2007). SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglial cells. *Journal of Neuroimmunology*, *182*(1–2), 89–99. https://doi.org/10.1016/j.jneuroim.2006.10.003

Dowjat, W. K., Adayev, T., Kuchna, I., Nowicki, K., Palminiello, S., Hwang, Y. W., & Wegiel, J. (2007). Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome. *Neuroscience Letters*, *413*(1), 77–81. https://doi.org/10.1016/j.neulet.2006.11.02

Earl, R. K., Turner, T. N., Mefford, H. C., Hudac, C. M., Gerdts, J., Eichler, E. E., & Bernier, R. A. (2017). Clinical phenotype of ASD-associated DYRK1A haploinsufficiency. *Molecular Autism*, *8*, 54. https://doi.org/10.1186/s13229-017-0173-5

Feki, A., & Hibaoui, Y. (2018). DYRK1A Protein, A Promising Therapeutic Target to Improve Cognitive Deficits in Down Syndrome. *Brain Sciences*, *8*(10), 187. https://doi.org/10.3390/brainsci8100187

Franco, S. J., Martinez-Garay, I., Gil-Sanz, C., Harkins-Perry, S. R., & Müller, U. (2011). Reelin regulates cadherin function via Dab1/Rap1 to control neuronal migration and lamination in the neocortex. *Neuron*, *69*(3), 482–497. https://doi.org/10.1016/j.neuron.2011.01.003

Frisch, S. M., & MacFawn, I. P. (2020). Type I interferons and related pathways in cell senescence. *Aging Cell*, *19*(10), e13234. https://doi.org/10.1111/acel.13234

Froemke, R. C. (2015). Plasticity of cortical excitatory-inhibitory balance. *Annual Review of Neuroscience*, *38*, 195–219. https://doi.org/10.1146/annurev-neuro-071714-034002

Guedj, F., Sébrié, C., Rivals, I., Ledru, A., Paly, E., Bizot, J. C., Smith, D., Rubin, E., Gillet, B., Arbones, M., & Delabar, J. M. (2009). Green Tea Polyphenols Rescue of Brain Defects Induced

by Overexpression of DYRK1A. *PLoS ONE*, *4*(2), e4606. https://doi.org/10.1371/journal.pone.0004606

Guidi, S., Bonasoni, P., Ceccarelli, C., Santini, D., Gualtieri, F., Ciani, E., & Bartesaghi, R. (2008). Neurogenesis impairment and increased cell death reduce total neuron number in the hippocampal region of fetuses with Down syndrome. *Brain Pathology (Zurich, Switzerland)*, *18*(2), 180–197. https://doi.org/10.1111/j.1750-3639.2007.00113.x

Guthrie, S. (1997). Axon guidance: Netrin receptors are revealed. *Current Biology*, *7*(1), R6–R9. https://doi.org/10.1016/S0960-9822(06)00007-8

Hadjadj, J., Yatim, N., Barnabei, L., Corneau, A., Boussier, J., Smith, N., Péré, H., Charbit, B., Bondet, V., Chenevier-Gobeaux, C., Breillat, P., Carlier, N., Gauzit, R., Morbieu, C., Pène, F., Marin, N., Roche, N., Szwebel, T.-A., Merkling, S. H., … Terrier, B. (2020). Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. *Science (New York, N.y.)*, *369*(6504), 718–724. https://doi.org/10.1126/science.abc6027

Haiyong, H. (2018). RNA Interference to Knock Down Gene Expression. *Methods in Molecular Biology (Clifton, N.J.)*, *1706*, 293–302. https://doi.org/10.1007/978-1-4939-7471-9_16

Hattori, M., Fujiyama, A., Taylor, T. D., Watanabe, H., Yada, T., Park, H.-S., Toyoda, A., Ishii, K., Totoki, Y., Choi, D.-K., Soeda, E., Ohki, M., Takagi, T., Sakaki, Y., Taudien, S., Blechschmidt, K., Polley, A., Menzel, U., Delabar, J., … Yaspo, M.-L. (2000). The DNA sequence of human chromosome 21. *Nature*, *405*(6784), Article 6784. https://doi.org/10.1038/35012518

Hayflick, L., & Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Experimental Cell Research*, *25*, 585–621. https://doi.org/10.1016/0014-4827(61)90192-6

Head, E., Lott, I. T., Patterson, D., Doran, E., & Haier, R. J. (2007). Possible Compensatory Events in Adult Down Syndrome Brain Prior to the Development of Alzheimer Disease Neuropathology:

Targets for Nonpharmacological Intervention. *Journal of Alzheimer's Disease*, *11*(1), 61–76. https://doi.org/10.3233/JAD-2007-11110

Horvath, G. A., Zhao, Y., Tarailo-Graovac, M., Boelman, C., Gill, H., Shyr, C., Lee, J., Blydt-Hansen, I., Drögemöller, B. I., Moreland, J., Ross, C. J., Wasserman, W. W., Masotti, A., Slesinger, P. A., & van Karnebeek, C. D. M. (2018). Gain-of-function KCNJ6 mutation in a severe hyperkinetic movement disorder phenotype. *Neuroscience*, *384*, 152–164. https://doi.org/10.1016/j.neuroscience.2018.05.031

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell*, *157*(6), 1262–1278. https://doi.org/10.1016/j.cell.2014.05.010

Huang, W., Hickson, L. J., Eirin, A., Kirkland, J. L., & Lerman, L. O. (2022). Cellular senescence: The good, the bad and the unknown. *Nature Reviews Nephrology*, *18*(10), Article 10. https://doi.org/10.1038/s41581-022-00601-z

Illouz, T., Biragyn, A., Iulita, M. F., Flores-Aguilar, L., Dierssen, M., De Toma, I., Antonarakis, S. E., Yu, E., Herault, Y., Potier, M.-C., Botté, A., Roper, R., Sredni, B., London, J., Mobley, W., Strydom, A., & Okun, E. (2021). Immune Dysregulation and the Increased Risk of Complications and Mortality Following Respiratory Tract Infections in Adults With Down Syndrome. *Frontiers in Immunology*, *12*, 621440. https://doi.org/10.3389/fimmu.2021.621440

Jain, S., & Welshhans, K. (2016). Netrin-1 induces local translation of down syndrome cell adhesion molecule in axonal growth cones. *Developmental Neurobiology*, *76*(7), 799–816. https://doi.org/10.1002/dneu.22360

Jia, Y., Jing, L., Li, J., Lu, J., Han, R., Wang, S., Peng, T., & Jia, Y. (2011). Expression and significance of DSCAM in the cerebral cortex of APP transgenic mice. *Neuroscience Letters*, *491*(2), 153–157. https://doi.org/10.1016/j.neulet.2011.01.028

Jia, Y. L., Fu, Z. X., Zhang, B. H., & Jia, Y. J. (2017). Hippocampal overexpression of Down syndrome cell adhesion molecule in amyloid precursor protein transgenic mice. *Brazilian Journal of Medical and Biological Research = Revista Brasileira De Pesquisas Medicas E Biologicas*, *50*(6), e6049. https://doi.org/10.1590/1414-431X20176049

Jin, M., Xu, R., Wang, L., Alam, M. M., Ma, Z., Zhu, S., Martini, A. C., Jadali, A., Bernabucci, M., Xie, P., Kwan, K., Pang, Z. P., Head, E., Liu, Y., Hart, R. P., & Jiang, P. (2022). *Type I Interferon Signaling Drives Microglial Dysfunction and Senescence in Human iPSC Models of Down Syndrome and Alzheimer's Disease* (p. 2021.12.22.473858). bioRxiv. https://doi.org/10.1101/2021.12.22.473858

Juric-Sekhar, G., & Hevner, R. F. (2019). Malformations of Cerebral Cortex Development: Molecules and Mechanisms. *Annual Review of Pathology*, *14*, 293–318. https://doi.org/10.1146/annurev-pathmechdis-012418-012927

Keihan Falsafi, S., Dierssen, M., Ghafari, M., Pollak, A., & Lubec, G. (2016). Reduced cortical neurotransmitter receptor complex levels in fetal Down syndrome brain. *Amino Acids*, *48*(1), 103–116. https://doi.org/10.1007/s00726-015-2062-6

Klein, A., Rhinn, M., & Keyes, W. M. (2023). Cellular senescence and developmental defects. *The FEBS Journal*, *290*(5), 1303–1313. https://doi.org/10.1111/febs.16731

Kleschevnikov, A. M., Yu, J., Kim, J., Lysenko, L. V., Zhen, Z., Yu, Y. E., & Mobley, W. C. (2017). Evidence that increased Kcnj6 gene dose is necessary for deficits in behavior and dentate gyrus synaptic plasticity in the Ts65Dn mouse model of Down syndrome. *Neurobiology of Disease*, *103*, 1–10. https://doi.org/10.1016/j.nbd.2017.03.009

Kline, J. N., & Kitagaki, K. (2006). Interferons. In S. M. Janes (Ed.), *Encyclopedia of Respiratory Medicine (Second Edition)* (pp. 271–274). Academic Press. https://doi.org/10.1016/B978-0-08-102723-3.10573-6

Krivega, M., Stiefel, C. M., Karbassi, S., Andersen, L. L., Chunduri, N. K., Donnelly, N., Pichlmair, A., & Storchová, Z. (2021). Genotoxic stress in constitutive trisomies induces autophagy and the innate immune response via the cGAS-STING pathway. *Communications Biology*, *4*(1), Article 1. https://doi.org/10.1038/s42003-021-02278-9

Lai, T., Jabaudon, D., Molyneaux, B. J., Azim, E., Arlotta, P., Menezes, J. R. L., & Macklis, J. D. (2008). SOX5 Controls the Sequential Generation of Distinct Corticofugal Neuron Subtypes. *Neuron*, *57*(2), 232–247. https://doi.org/10.1016/j.neuron.2007.12.023

Lasser, M., Tiber, J., & Lowery, L. A. (2018). The Role of the Microtubule Cytoskeleton in Neurodevelopmental Disorders. *Frontiers in Cellular Neuroscience*, *12*. https://www.frontiersin.org/articles/10.3389/fncel.2018.00165

Lee, M., Hyun, D., Jenner, P., & Halliwell, B. (2001). Effect of overexpression of wild-type and mutant Cu/Zn-superoxide dismutases on oxidative damage and antioxidant defences: Relevance to Down's syndrome and familial amyotrophic lateral sclerosis. *Journal of Neurochemistry*, *76*(4), 957–965. https://doi.org/10.1046/j.1471-4159.2001.00107.x

Lee, S. E., Duran-Martinez, M., Khantsis, S., Bianchi, D. W., & Guedj, F. (2020). Challenges and Opportunities for Translation of Therapies to Improve Cognition in Down Syndrome. *Trends in Molecular Medicine*, *26*(2), 150–169. https://doi.org/10.1016/j.molmed.2019.10.001

Li, Z., Klein, J. A., Rampam, S., Kurzion, R., Campbell, N. B., Patel, Y., Haydar, T. F., & Zeldich, E. (2022). Asynchronous excitatory neuron development in an isogenic cortical spheroid model of

Down syndrome. *Frontiers in Neuroscience*, *16*, 932384. https://doi.org/10.3389/fnins.2022.932384

Liu, G., Li, W., Wang, L., Kar, A., Guan, K.-L., Rao, Y., & Wu, J. Y. (2009). DSCAM functions as a netrin receptor in commissural axon pathfinding. *Proceedings of the National Academy of Sciences*, *106*(8), 2951–2956. https://doi.org/10.1073/pnas.0811083106

Liu, W., Venugopal, S., Majid, S., Ahn, I. S., Diamante, G., Hong, J., Yang, X., & Chandler, S. H. (2020). Single-cell RNA-seq analysis of the brainstem of mutant SOD1 mice reveals perturbed cell types and pathways of amyotrophic lateral sclerosis. *Neurobiology of Disease*, *141*, 104877. https://doi.org/10.1016/j.nbd.2020.104877

Lott, I. T., & Dierssen, M. (2010). Cognitive deficits and associated neurological complications in individuals with Down's syndrome. *The Lancet. Neurology*, *9*(6), 623–633. https://doi.org/10.1016/S1474-4422(10)70112-5

Ly, A., Nikolaev, A., Suresh, G., Zheng, Y., Tessier-Lavigne, M., & Stein, E. (2008). DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1. *Cell*, *133*(7), 1241–1254. https://doi.org/10.1016/j.cell.2008.05.030

Marcovecchio, G. E., Ferrua, F., Fontana, E., Beretta, S., Genua, M., Bortolomai, I., Conti, A., Montin, D., Cascarano, M. T., Bergante, S., D'Oria, V., Giamberti, A., Amodio, D., Cancrini, C., Carotti, A., Di Micco, R., Merelli, I., Bosticardo, M., & Villa, A. (2021). Premature Senescence and Increased Oxidative Stress in the Thymus of Down Syndrome Patients. *Frontiers in Immunology*, *12*. https://www.frontiersin.org/articles/10.3389/fimmu.2021.669893

Martínez Cué, C., & Dierssen, M. (2020). Chapter 9—Plasticity as a therapeutic target for improving cognition and behavior in Down syndrome. In M. Dierssen (Ed.), *Progress in Brain Research* (Vol. 251, pp. 269–302). Elsevier. https://doi.org/10.1016/bs.pbr.2019.11.001

Martínez-Cerdeño, V. (2017). Dendrite and spine modifications in autism and related neurodevelopmental disorders in patients and animal models. *Developmental Neurobiology*, *77*(4), 393–404. https://doi.org/10.1002/dneu.22417

Martínez-Cué, C., & Rueda, N. (2020). Cellular Senescence in Neurodegenerative Diseases. *Frontiers in Cellular Neuroscience*, *14*, 16. https://doi.org/10.3389/fncel.2020.00016

Maynard, K. R., & Stein, E. (2012). DSCAM Contributes to Dendrite Arborization and Spine Formation in the Developing Cerebral Cortex. *Journal of Neuroscience*, *32*(47), 16637–16650. https://doi.org/10.1523/JNEUROSCI.2811-12.2012

Meharena, H. S., Marco, A., Dileep, V., Lockshin, E. R., Akatsu, G. Y., Mullahoo, J., Watson, L. A., Ko, T., Guerin, L. N., Abdurrob, F., Rengarajan, S., Papanastasiou, M., Jaffe, J. D., & Tsai, L.-H. (2022). Down-syndrome-induced senescence disrupts the nuclear architecture of neural progenitors. *Cell Stem Cell*, *29*(1), 116-130.e7. https://doi.org/10.1016/j.stem.2021.12.002

Mitra, A., Blank, M., & Madison, D. V. (2012). Developmentally altered inhibition in Ts65Dn, a mouse model of Down syndrome. *Brain Research*, *1440*, 1–8. https://doi.org/10.1016/j.brainres.2011.12.034

Mitsogiannis, M. D., Pancho, A., Aerts, T., Sachse, S. M., Vanlaer, R., Noterdaeme, L., Schmucker, D., & Seuntjens, E. (2021). Subtle Roles of Down Syndrome Cell Adhesion Molecules in Embryonic Forebrain Development and Neuronal Migration. *Frontiers in Cell and Developmental Biology*, *8*. https://www.frontiersin.org/articles/10.3389/fcell.2020.624181

Montesinos, M. L. (2014). Roles for DSCAM and DSCAML1 in central nervous system development and disease. *Advances in Neurobiology*, *8*, 249–270. https://doi.org/10.1007/978-1-4614-8090-7_11

Nabarra, B., Casanova, M., Paris, D., Nicole, A., Toyama, K., Sinet, P. M., Ceballos, I., & London, J. (1996). Transgenic mice overexpressing the human Cu/Zn-SOD gene: Ultrastructural studies of a premature thymic involution model of Down's syndrome (trisomy 21). *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *74*(3), 617–626.

Nagaraj, K., Sarfstein, R., Laron, Z., & Werner, H. (2022). Long-Term IGF1 Stimulation Leads to Cellular Senescence via Functional Interaction with the Thioredoxin-Interacting Protein, TXNIP. *Cells*, *11*(20), 3260. https://doi.org/10.3390/cells11203260

Newpher, T. M., & Ehlers, M. D. (2009). Spine microdomains for postsynaptic signaling and plasticity. *Trends in Cell Biology*, *19*(5), 218–227. https://doi.org/10.1016/j.tcb.2009.02.004

Ovchinnikov, D. A., Korn, O., Virshup, I., Wells, C. A., & Wolvetang, E. J. (2018). The Impact of APP on Alzheimer-like Pathogenesis and Gene Expression in Down Syndrome iPSC-Derived Neurons. *Stem Cell Reports*, *11*(1), 32–42. https://doi.org/10.1016/j.stemcr.2018.05.004

Pan, X., Chang, X., Leung, C., Zhou, Z., Cao, F., Xie, W., & Jia, Z. (2015). PAK1 regulates cortical development via promoting neuronal migration and progenitor cell proliferation. *Molecular Brain*, *8*, 36. https://doi.org/10.1186/s13041-015-0124-z

Patterson, D. (2009). Molecular genetic analysis of Down syndrome. *Human Genetics*, *126*(1), 195–214. https://doi.org/10.1007/s00439-009-0696-8

Pérez-Núñez, R., Barraza, N., Gonzalez-Jamett, A., Cárdenas, A. M., Barnier, J.-V., & Caviedes, P. (2016). Overexpressed Down Syndrome Cell Adhesion Molecule (DSCAM) Deregulates P21-Activated Kinase (PAK) Activity in an In Vitro Neuronal Model of Down Syndrome: Consequences on Cell Process Formation and Extension. *Neurotoxicity Research*, *30*(1), 76–87. https://doi.org/10.1007/s12640-016-9613-9

Perluigi, M., & Butterfield, D. A. (2011). Oxidative Stress and Down Syndrome: A Route toward Alzheimer-Like Dementia. *Current Gerontology and Geriatrics Research*, *2012*, e724904. https://doi.org/10.1155/2012/724904

Popova, D., Gameiro-Ros, I., Youssef, M. M., Zalamea, P., Morris, A. D., Prytkova, I., Jadali, A., Kwan, K. Y., Kamarajan, C., Salvatore, J. E., Xuei, X., Chorlian, D. B., Porjesz, B., Kuperman, S., Dick, D. M., Goate, A., Edenberg, H. J., Tischfield, J. A., Pang, Z. P., … Hart, R. P. (2023). Alcohol reverses the effects of KCNJ6 (GIRK2) noncoding variants on excitability of human glutamatergic neurons. *Molecular Psychiatry*, *28*(2), Article 2. https://doi.org/10.1038/s41380-022-01818-x

Pritchard, M. A., & Kola, I. (1999). The "gene dosage effect" hypothesis versus the "amplified developmental instability" hypothesis in Down syndrome. In G. Lubec (Ed.), *The Molecular Biology of Down Syndrome* (pp. 293–303). Springer. https://doi.org/10.1007/978-3-7091-6380-1_20

Pujol, J., del Hoyo, L., Blanco-Hinojo, L., de Sola, S., Macià, D., Martínez-Vilavella, G., Amor, M., Deus, J., Rodríguez, J., Farré, M., Dierssen, M., & de la Torre, R. (2015). Anomalous brain functional connectivity contributing to poor adaptive behavior in Down syndrome. *Cortex*, *64*, 148–156. https://doi.org/10.1016/j.cortex.2014.10.012

Purohit, A. A., Li, W., Qu, C., Dwyer, T., Shao, Q., Guan, K.-L., & Liu, G. (2012). Down Syndrome Cell Adhesion Molecule (DSCAM) Associates with Uncoordinated-5C (UNC5C) in Netrin-1-mediated Growth Cone Collapse. *The Journal of Biological Chemistry*, *287*(32), 27126–27138. https://doi.org/10.1074/jbc.M112.340174

Radwitz, J., Hausrat, T. J., Heisler, F. F., Janiesch, P. C., Pechmann, Y., Rübhausen, M., & Kneussel, M. (2022). Tubb3 expression levels are sensitive to neuronal activity changes and determine

microtubule growth and kinesin-mediated transport. *Cellular and Molecular Life Sciences*, *79*(11), 575. https://doi.org/10.1007/s00018-022-04607-5

Ram, G., & Chinen, J. (2011). Infections and immunodeficiency in Down syndrome. *Clinical and Experimental Immunology*, *164*(1), 9–16. https://doi.org/10.1111/j.1365-2249.2011.04335.x

Sachse, S. M., Lievens, S., Ribeiro, L. F., Dascenco, D., Masschaele, D., Horré, K., Misbaer, A., Vanderroost, N., De Smet, A. S., Salta, E., Erfurth, M., Kise, Y., Nebel, S., Van Delm, W., Plaisance, S., Tavernier, J., De Strooper, B., De Wit, J., & Schmucker, D. (2019). Nuclear import of the DSCAM-cytoplasmic domain drives signaling capable of inhibiting synapse formation. *The EMBO Journal*, *38*(6), e99669. https://doi.org/10.15252/embj.201899669

Saito, Y., Oka, A., Mizuguchi, M., Motonaga, K., Mori, Y., Becker, L. E., Arima, K., Miyauchi, J., & Takashima, S. (2000). The developmental and aging changes of Down's syndrome cell adhesion molecule expression in normal and Down's syndrome brains. *Acta Neuropathologica*, *100*(6), 654–664. https://doi.org/10.1007/s004010000230

Salehi, A., Ashford, J. W., & Mufson, E. J. (2016). The Link between Alzheimer's Disease and Down Syndrome. A Historical Perspective. *Current Alzheimer Research*, *13*(1), 2–6.

Schopperle, W. M., & DeWolf, W. C. (2007). The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. *Stem Cells (Dayton, Ohio)*, *25*(3), 723–730. https://doi.org/10.1634/stemcells.2005-0597

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D. N., & Fesik, S. W. (2003). Specificity of short interfering RNA determined through gene expression signatures. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(11), 6347–6352. https://doi.org/10.1073/pnas.1131959100

Shao, Q., Yang, T., Huang, H., Majumder, T., Khot, B. A., Khouzani, M. M., Alarmanazi, F., Gore, Y. K., & Liu, G. (2019). Disease-associated mutations in human TUBB3 disturb netrin repulsive signaling. *PLOS ONE*, *14*(6), e0218811. https://doi.org/10.1371/journal.pone.0218811

Shi, Y., Kirwan, P., Smith, J., Robinson, H. P. C., & Livesey, F. J. (2012). Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nature Neuroscience*, *15*(3), Article 3. https://doi.org/10.1038/nn.3041

Shin, J.-H., London, J., Le Pecheur, M., Höger, H., Pollak, D., & Lubec, G. (2004). Aberrant Neuronal and Mitochondrial Proteins in Hippocampus of Transgenic Mice Overexpressing Human Cu/Zn Superoxide Dismutase 1. *Free Radical Biology and Medicine*, *37*(5), 643–653. https://doi.org/10.1016/j.freeradbiomed.2004.05.019

Simmons, A. B., Bloomsburg, S. J., Sukeena, J. M., Miller, C. J., Ortega-Burgos, Y., Borghuis, B. G., & Fuerst, P. G. (2017). DSCAM-mediated control of dendritic and axonal arbor outgrowth enforces tiling and inhibits synaptic plasticity. *Proceedings of the National Academy of Sciences*, *114*(47), E10224–E10233. https://doi.org/10.1073/pnas.1713548114

Simon, R., Wiegreffe, C., & Britsch, S. (2020). Bcl11 Transcription Factors Regulate Cortical Development and Function. *Frontiers in Molecular Neuroscience*, *13*. https://www.frontiersin.org/articles/10.3389/fnmol.2020.00051

Sudbery, I., Enright, A. J., Fraser, A. G., & Dunham, I. (2010). Systematic analysis of off-target effects in an RNAi screen reveals microRNAs affecting sensitivity to TRAIL-induced apoptosis. *BMC Genomics*, *11*(1), 175. https://doi.org/10.1186/1471-2164-11-175

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*, *131*(5), 861–872. https://doi.org/10.1016/j.cell.2007.11.019

Takashima, S., Becker, L. E., Armstrong, D. L., & Chan, F. (1981). Abnormal neuronal development in the visual cortex of the human fetus and infant with down's syndrome. A quantitative and qualitative golgi study. *Brain Research*, *225*(1), 1–21. https://doi.org/10.1016/0006-8993(81)90314-0

Takashima, S., Ieshima, A., Nakamura, H., & Becker, L. E. (1989). Dendrites, dementia and the down syndrome. *Brain and Development*, *11*(2), 131–133. https://doi.org/10.1016/S0387-7604(89)80082-8

Tang, X.-Y., Xu, L., Wang, J., Hong, Y., Wang, Y., Zhu, Q., Wang, D., Zhang, X.-Y., Liu, C.-Y., Fang, K.-H., Han, X., Wang, S., Wang, X., Xu, M., Bhattacharyya, A., Guo, X., Lin, M., & Liu, Y. (2021). DSCAM/PAK1 pathway suppression reverses neurogenesis deficits in iPSC-derived cerebral organoids from patients with Down syndrome. *The Journal of Clinical Investigation*, *131*(12), 135763. https://doi.org/10.1172/JCI135763

Tyan, S.-H., Shih, A. Y.-J., Walsh, J. J., Murayama, H., Sarsoza, F., Ku, L., Eggert, S., Hof, P. R., Koo, E. H., & Dickstein, D. L. (2012). Amyloid precursor protein (APP) regulates synaptic structure and function. *Molecular and Cellular Neurosciences*, *51*(1–2), 43–52. https://doi.org/10.1016/j.mcn.2012.07.009

Viard, J., Loe-Mie, Y., Daudin, R., Khelfaoui, M., Plancon, C., Boland, A., Tejedor, F., Huganir, R. L., Kim, E., Kinoshita, M., Liu, G., Haucke, V., Moncion, T., Yu, E., Hindie, V., Bléhaut, H., Mircher, C., Herault, Y., Deleuze, J.-F., … Lepagnol-Bestel, A.-M. (2022). Chr21 protein–protein interactions: Enrichment in proteins involved in intellectual disability, autism, and late-onset Alzheimer's disease. *Life Science Alliance*, *5*(12), e202101205. https://doi.org/10.26508/lsa.202101205

Wang, W., Rai, A., Hur, E.-M., Smilansky, Z., Chang, K. T., & Min, K.-T. (2016). DSCR1 is required for both axonal growth cone extension and steering. *Journal of Cell Biology*, *213*(4), 451–462. https://doi.org/10.1083/jcb.201510107

Waugh, K. A., Araya, P., Pandey, A., Jordan, K. R., Smith, K. P., Granrath, R. E., Khanal, S., Butcher, E. T., Estrada, B. E., Rachubinski, A. L., McWilliams, J. A., Minter, R., Dimasi, T., Colvin, K. L., Baturin, D., Pham, A. T., Galbraith, M. D., Bartsch, K. W., Yeager, M. E., … Espinosa, J. M. (2019). Mass Cytometry Reveals Global Immune Remodeling with Multi-lineage Hypersensitivity to Type I Interferon in Down Syndrome. *Cell Reports*, *29*(7), 1893-1908.e4. https://doi.org/10.1016/j.celrep.2019.10.038

Waugh, K. A., Minter, R., Baxter, J., Chi, C., Tuttle, K. D., Eduthan, N. P., Galbraith, M. D., Kinning, K. T., Andrysik, Z., Araya, P., Dougherty, H., Dunn, L. N., Ludwig, M., Schade, K. A., Tracy, D., Smith, K. P., Granrath, R. E., Busquet, N., Khanal, S., … Espinosa, J. M. (2022). *Interferon receptor gene dosage determines diverse hallmarks of Down syndrome* (p. 2022.02.03.478982). bioRxiv. https://doi.org/10.1101/2022.02.03.478982

Wu, Y., West, N. R., Bhattacharyya, A., & Wiseman, F. K. (2022). Cell models for Down syndrome-Alzheimer's disease research. *Neuronal Signaling*, *6*(1), NS20210054. https://doi.org/10.1042/NS20210054

Xu, J., Su, X., Burley, S. K., & Zheng, X. F. S. (2022). Nuclear SOD1 in Growth Control, Oxidative Stress Response, Amyotrophic Lateral Sclerosis, and Cancer. *Antioxidants*, *11*(2), 427. https://doi.org/10.3390/antiox11020427

Xu, R., Brawner, A. T., Li, S., Liu, J.-J., Kim, H., Xue, H., Pang, Z. P., Kim, W.-Y., Hart, R. P., Liu, Y., & Jiang, P. (2019). OLIG2 Drives Abnormal Neurodevelopmental Phenotypes in Human

iPSC-Based Organoid and Chimeric Mouse Models of Down Syndrome. *Cell Stem Cell*, *24*(6), 908-926.e8. https://doi.org/10.1016/j.stem.2019.04.014

Xu, S.-Y., Lu, F.-M., Wang, M.-Y., Hu, Z.-S., Zhang, J., Chen, Z.-Y., Armada-da-Silva, P. A. S., & Yuan, Z. (2020). Altered Functional Connectivity in the Motor and Prefrontal Cortex for Children With Down's Syndrome: An fNIRS Study. *Frontiers in Human Neuroscience*, *14*, 6. https://doi.org/10.3389/fnhum.2020.00006

Yamagata, M., Sanes, J. R., & Weiner, J. A. (2003). Synaptic adhesion molecules. *Current Opinion in Cell Biology*, *15*(5), 621–632. https://doi.org/10.1016/S0955-0674(03)00107-8

Yamakawa, K., Huo, Y.-K., Haendel, M. A., Hubert, R., Chen, X.-N., Lyons, G. E., & Korenberg, J. R. (1998). DSCAM: A Novel Member of the Immunoglobulin Superfamily Maps in a Down Syndrome Region and is Involved in the Development of the Nervous System. *Human Molecular Genetics*, *7*(2), 227–237. https://doi.org/10.1093/hmg/7.2.227

Zana, M., Szécsényi, A., Czibula, Á., Bjelik, A., Juhász, A., Rimanóczy, Á., Szabó, K., Vetró, Á., Szűcs, P., Várkonyi, Á., Pákáski, M., Boda, K., Raskó, I., Janka, Z., & Kálmán, J. (2006). Age-dependent oxidative stress-induced DNA damage in Down's lymphocytes. *Biochemical and Biophysical Research Communications*, *345*(2), 726–733. https://doi.org/10.1016/j.bbrc.2006.04.167

Zheng, H., & Koo, E. H. (2011). Biology and pathophysiology of the amyloid precursor protein. *Molecular Neurodegeneration*, *6*(1), 27. https://doi.org/10.1186/1750-1326-6-27

Zhu, K., Xu, Y., Liu, J., Xu, Q., & Ye, H. (2011). Down syndrome cell adhesion molecule and its functions in neural development. *Neuroscience Bulletin*, *27*(1), 45–52. https://doi.org/10.1007/s12264-011-1045-1