University of Massachusetts Medical School

eScholarship@UMMS

Modeling Down Syndrome Neurodevelopment with Dosage Compensation

Jan T. Czerminski
University of Massachusetts Medical School

Let us know how access to this document benefits you.

MODELING DOWN SYNDROME NEURODEVELOPMENT WITH

DOSAGE COMPENSATION

A Dissertation Presented

By

JAN TOMASZ CZERMIŃSKI

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

JULY 11TH, 2019

CELL BIOLOGY

MODELING DOWN SYNDROME NEURODEVELOPMENT WITH DOSAGE COMPENSATION

A Dissertation Presented

By

JAN TOMASZ CZERMIŃSKI

This work was undertaken in the Graduate School of Biomedical Sciences

M.D./Ph.D. Program

Under the mentorship of

Jeanne B. Lawrence, Ph.D., Thesis Advisor

Daryl Bosco, Ph.D., Member of Committee

Hong Zhang, Ph.D., Member of Committee

Ingolf Bach, Ph.D., Member of Committee

Eric Morrow, M.D., Ph.D., External Member of Committee

Jaime Rivera, Ph.D., Chair of Committee

Mary Ellen Lane, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

JULY 11TH, 2019

Acknowledgements

The years of work represented by this thesis would not have been possible without the help, large and small, of many people along the way. First, I would like to thank my mentor, Dr. Jeanne Lawrence, for her unwavering support of this project and my career development. Your perpetual optimism even in the face of disheartening results allowed me to keep pushing forward when it seemed hopeless. You offered me an incredible degree of freedom to explore any scientific avenues that piqued my interest, and I have become a significantly better scientist because of it.

I'd also like to thank all the members of the Lawrence lab for creating a supportive and positive environment in which to grow as a scientist (and for feeding my cells when I was away). I want to thank J Eunmi Moon and Dr. Kevin Creamer for their bioinformatic and experimental help and for much needed comedic relief. Dr. Melvys Valledor for struggling with me through endless organoid culture and ELISA failures with a relentlessly positive attitude. Meg Byron for teaching me everything from FISH to cell culture, and then being willing to learn obnoxious organoid culture in return. Laurie Lizotte for her invaluable administrative support and last-minute room reservations. And former lab members Drs. Jun Jiang and Jen-Chieh Chiang for teaching me everything about iPSC culture and differentiation.

I'd like to thank my QEC and TRAC for helping to guide my experiments and pointing me in the right direction from the start. I'd like to thank Dr. Su-Chun Zhang, Dr. Anita Bhattacharyya, and members of their lab for hosting me and teaching me the nuances of neuron differentiation. I'd also like to thank Dr. Oliver King for his invaluable

statistical and bioinformatic support, and for always being willing to repeatedly explain these concepts to me.

I'd like to thank my MD/PhD long lunch crew: Peter Lee, Eric Schmidt, Zack Milstone, Barry Kriegsman, and Pat Lowe (*in absentia*). We may or may not have still squeaked by without one another, but it would certainly have been a whole lot more painful.

I would also like to thank my parents for instilling upon me a sense of scientific wonder from a young age that made all of this possible. Also, for countless dinners and their steadfast support throughout the Ph.D. years, even when I refused to discuss my thesis work with them (sorry).

Finally, I'd like to thank my fiancée Emily, who entered my life just when I was starting my thesis work. I can't imagine doing this without you by my side. Thank you for putting up with endless cell feedings and impossibly long "quick" trips to the lab. I can't wait to move on to the next chapter of our lives together.

Abstract

Due to their underlying genetic complexity, chromosomal disorders such as Down syndrome (DS), which is caused by trisomy 21, have long been understudied and continue to lack effective treatments. With over 200 genes on the extra chromosome, even the specific cell pathologies and pathways impacted in DS are not known, and it has not been considered a viable target for the burgeoning field of gene therapy. Recently, our lab demonstrated that the natural mechanism of dosage compensation can be harnessed to silence the trisomic chromosome in pluripotent cells. Using an inducible *XIST* transgene allows us to study the effects of trisomy in a tightly controlled system by comparing the same cells with either two or three active copies of chromosome 21. In addition, it raises the prospect that insertion of a single gene into a trisomic chromosome could potentially be developed in the future for "chromosome therapy".

This thesis aims to utilize this inducible system for dosage compensation to study the neurodevelopmental effects of trisomy 21 *in vitro,* and to answer basic epigenetic questions critical to the viability of chromosome silencing as a therapeutic approach. Foremost, for *XIST* to have any prospect as a therapeutic, and to strengthen its experimental utility, it must be able to initiate chromosome silencing beyond its natural context of pluripotency. Here I demonstrate that, contrary to the current literature, *XIST* is capable of initiating chromosome silencing in differentiated cells and producing fully dosage compensated DS neurons. Additionally, I show that silencing of the trisomic chromosome in neural stem cells enhances their terminal differentiation to neurons, and transcriptome analysis provides evidence of a specific pathway involved. Separate

experiments utilize novel three-dimensional organoid technology and transcriptome analysis to model DS neurodevelopment in relation to isogenic euploid cells. Overall, this work demonstrates that dosage compensation provides a powerful experimental tool to examine early DS neurodevelopment, and establishes that *XIST* function does not require pluripotency, thereby overcoming a perceived obstacle to the potential of *XIST* as a therapeutic strategy for trisomy.

Table of contents

List of figures

List of tables

CHAPTER I : Introduction

Unlike many genetic conditions for which a causative mutation in one gene can be identified, Down syndrome (DS) is caused by the presence of an extra copy of an entire chromosome, chromosome 21 (chr21). This means that individuals with DS have an extra copy of ~250 protein-coding genes (including 16 transcription factors), at least 5 functional microRNAs, and hundreds of potential non-coding RNAs. This presents a significant challenge to understanding the precise genetic causes underlying each of the myriad physiological abnormalities present in individuals with DS. An incomplete understanding of the causative genes in DS, as well as normal variation between individuals, has hindered progress in our understanding of the molecular and cellular basis for disease. Because of this, there are currently no effective treatments for the most prevalent finding in individuals with DS, cognitive disability.

Recently, our lab has introduced a novel approach to studying DS pathogenesis, which also has the potential to one day serve as the basis for a comprehensive therapy for DS. This "trisomy silencing" system utilizes a natural mechanism of chromosome silencing using the gene *XIST*, in this case inserted into one copy of chr21 in a DS patient-derived induced pluripotent stem cell (iPSC) line. In this thesis, I utilize this powerful system to study the effects that trisomy has on early neurodevelopment and to investigate whether *XIST*-mediated trisomy silencing can correct these deficits. I will investigate whether *XIST* is capable of functioning outside of its natural developmental context of pluripotency, contrary to the current literature. Additionally, I utilize newly-developed three-dimensional cell culture techniques to further investigate the

neurodevelopmental effects of trisomy 21. This introduction will provide the necessary background on the subjects covered in this thesis, including dosage sensitivity, *XIST* biology, normal human neurodevelopment, DS neurobiology and its modelling *in vitro*, and the use of *XIST* to advance translational research in DS.

The importance of dosage balance

'Sola dosis facit venenum'

(Latin: only the dose makes a thing not a poison)

-Paracelsus (1493–1541)

It has been appreciated for hundreds if not thousands of years that the toxicity of a substance depends entirely on its dosage. This idea also applies to gene dosage. In fact, it has been hypothesized that the major phenotypic differences between humans and our closest living relatives, chimpanzees, depends largely on regulatory differences that influence expression levels rather than differences in protein composition (King and Wilson, 1975). Thus, it could be the collective dosages of the building blocks of life, rather than the structure of the blocks themselves, that makes us human.

Recent work has identified hominid-specific *NOTCH2* paralogs as potentially dosage-sensitive regulators of the Notch pathway, which will be described in more detail in a later section, that may be partially responsible for the evolution of larger brains in humans (Fiddes et al., 2018). Strikingly, duplications of these paralogs have been linked to macrocephaly, and deletions have been associated with microcephaly. In the case of these paralogs, the building blocks are present only in hominids, yet they serve to tweak the degree of Notch pathway signaling, leading to our uniquely powerful cognitive capacities.

In addition to potentially shaping our species, gene dosage also plays an important role in health and disease, yet this is an area of genome biology that is not well understood. In fact, it is not well-known what fraction of genes in the genome are dosage sensitive. While many genetic disorders are caused by mutations in genes that render them nonfunctional or toxic, there are genes for which the presence of an extra non-mutant copy is known to be pathogenic. Perhaps the most well-known and striking example also happens to be located on chr21. As will be discussed in more detail in a later section, the amyloid precursor protein (APP) gene is the only gene on chr21 that has been strongly linked to a specific phenotype in DS: the near-universal development of Alzheimer's disease (AD) (Olson and Shaw, 1969). The mapping of the APP gene to chromosome 21, in concert with the findings of prevalent AD in DS patients, led to the development of the amyloid hypothesis, the dominant theory in the pathogenesis of AD (Hardy and Selkoe, 2002). Further supporting that the dosage of this single gene can be sufficient for AD pathogenesis, euploid individuals with a duplication of the APP gene develop early-onset autosomal dominant AD (Rovelet-Lecrux et al., 2006).

It is important to note that many, if not most, genes are not dosage sensitive, as evidenced by the prevalence of copy number variations (CNV) covering 12% of the human genome (Redon et al., 2006). Additionally, there are genes for which an increased copy number can be advantageous in certain scenarios, as exemplified by the gene encoding amylase, which is present in higher copy numbers in cultures that consume a high-starch diet (Perry et al., 2007), and presumably gives a competitive advantage to individuals who can more thoroughly digest starches.

While there are individual genes which are not dosage sensitive, the presence or absence of an entire autosomal chromosome is nearly always incompatible with life. Recent estimates suggest that up to 80% of spontaneous abortions are caused by chromosomal abnormalities (Hardy et al., 2016). A plurality of these cases is caused by trisomy, a trend that has been exacerbated recently by increasing average maternal age. The few trisomies that are compatible with life tend to be on the smaller chromosomes, which contain fewer genes, such as chromosome 21. Notable exceptions to these trends involve the large X chromosome; monosomy and trisomy X are both compatible with life and cause mild phenotypes compared to other chromosomal abnormalities. This is largely due to the unique process of dosage compensation.

Concomitant with the evolution of heterogametic sex chromosomes came systems for dosage compensation, which ensure gene dosage balance between the sexes (Charlesworth, 1996). In drosophila, males (XY) *upregulate* expression from one X chromosome by the redundant action of two non-coding RNAs, roX1 and roX2, in order to equilibrate X chromosome expression between males and females (Franke and Baker, 1999). In contrast, female (XX) mammals *downregulate* expression from one X chromosome. This phenomenon is orchestrated by another non-coding RNA, the X-inactive specific transcript (*XIST*). *XIST* RNA normally coats one X chromosome, eventually leading to its transcriptional silencing. In females with trisomy X, *XIST* is expressed from two copies of the X chromosome, ensuring the presence of just one transcriptionally active X chromosome and explaining viability of these trisomies. The mild phenotypes that are seen in trisomy X, and become more severe in the rare cases

of tetrasomy X, are thought to be caused by overexpression a subset of genes that escape silencing by *XIST* (Tartaglia et al., 2010). However, as will become evident throughout this thesis and is the case with most trisomies, the specific genes that lead to any potential phenotypes in cases of trisomy X have not been identified. One notable exception to this is the *SHOX* gene, which escapes X inactivation and has been linked to the short stature of women with Turner's syndrome (XO) and the tall stature of both men and women with supernumerary copies of the X chromosome (Ottesen et al., 2010).

Case studies of specific dosage-sensitive genes, the high prevalence of chromosomal abnormalities in spontaneous abortions, and the potential role of gene expression changes in the evolution of our species make a clear case for the importance of gene dosage throughout biology. The natural phenomenon of X chromosome inactivation (XCI) provides an important window into understanding how perturbation of transcriptional gene dosage affects development, and the ability to harness this unique phenomenon to model and potentially treat disorders of dosage imbalance opens a new path for these previously untreatable diseases.

XIST and X chromosome inactivation

The discovery of X chromosome inactivation and *XIST*

Mary Lyon first put forward a comprehensive theory of X inactivation that has survived decades of scientific inquiry (Lyon, 1961). Her theory built on the then-recent discovery that the "sex chromatin" in female cells previously identified by Barr and Bertram (1949) was in fact one X chromosome (Ohno and Hauschka, 1960), and that monosomy X was compatible with a healthy and fertile female phenotype in mice

(Welshons and Russell, 1959). Additionally, work in mouse genetics had identified an interesting finding where heterozygous mutations in X-linked coat color genes led to the patchy appearance of female mice, suggestive of a mosaic phenotype (Fraser et al., 1953). In this scenario, one X chromosome is randomly inactivated in the early female embryo. If the chromosome containing the nonmutant coat color allele is inactivated, then pigmented cells arising from this early progenitor will not produce pigment. On the other hand, cells derived from the progenitor that silences the other X chromosome will produce pigment, ultimately leading to the mottled phenotype seen in female animals of certain mouse mutants as well as in the common house cat. Putting these pieces together, Lyon's hypothesis stated that the heteropyknotic (condensed) X chromosome can be either maternal or paternal in origin in different cells of the same animal, that this chromosome is genetically inactivated, and that this inactivation occurs early in development (Lyon, 1961).

Soon after proposing this theory, evidence from X;autosome translocations indicated that a certain portion of the X chromosome was required for chromosomal silencing to take place (Russell, 1963). This led to the idea that there exists a portion of the X chromosome called the X inactivation center (XIC) from which the inactivation of the X chromosome spreads (Cattanach, 1975). It was not until 30 years after Mary Lyon proposed the theory of X inactivation that the gene located in the XIC and associated with aberrant XCI was discovered (Brown et al., 1991a, 1991b). *XIST* was unique in that it was the only gene known to be expressed exclusively from the *inactive* X chromosome. Prior genes had been discovered that were expressed only from the active X (silenced genes) and genes expressed from both copies of X (genes that

escape silencing). Importantly, Brown et al. (1991a) also found that *XIST* expression

increased with the number of inactive X chromosomes present, indicating that *XIST* is

capable of effectively silencing multiple X chromosomes and rescuing these individuals

from trisomy, as mentioned in the previous section, which is an important finding for any

potential therapeutic applications of this unique gene. Soon after, once the entire 17kb

sequence of *XIST* was determined (Brown et al., 1992), it became clear that this gene

does not encode a protein, but instead is transcribed into a long RNA that is retained in

the nucleus and spatially overlaps nearly perfectly with the inactive X chromosome

territory, or Barr body (Clemson et al., 1996). In fact, *XIST* RNA established the

precedent for a large non-coding RNA (lncRNA) which functions in chromatin. *XIST* was

also determined to contain several well-conserved repeat elements known as repeats

A-F (Brown et al., 1992). At about the same time, mouse mutant studies found that *Xist*

was indeed required for X chromosome silencing. These studies showed that in mice

with one mutant allele of *Xist*, the silenced chromosome in every cell was always the

one containing the intact allele (Penny et al., 1996), indicating lethality of cells which

attempted XCI using a non-functional mutant allele of *Xist*.

Mechanisms of *XIST*-mediated chromosome silencing

In the nearly 30 years since the discovery of the *XIST* gene, numerous

researchers have tried to understand how this RNA mediates transcriptional silencing of

an entire chromosome. While there is much that remains unknown about this process,

significant progress has been made in understanding the underlying mechanisms. It has

long been understood that *XIST* recruits many factors in order to effectively and

redundantly render the X chromosome transcriptionally silenced (Migeon, 1994), and

some of the mechanisms employed to this end, which have largely been discovered in the mouse, will be reviewed here and are summarized in Figure I-1.

XIST RNA functions by triggering multiple repressive chromatin modifications that contribute to the silent state, such as polycomb protein repressive complexes PRC1 and PRC2, which induce the canonical heterochromatin hallmarks: monoubiquitination of lysine 117 on histone 2A (H2AK119ub1) and trimethylation of lysine 27 on histone 3 (H3K27me3), respectively (Cao et al., 2002; Fang et al., 2004; de Napoles et al., 2004; Plath et al., 2003). Initial experiments suggested that Xist RNA directly recruited PRC2 to the X chromosome via the A-repeat segment of the RNA (Zhao et al., 2008). In this model, PCR2 would first lay down the H3K27me3 heterochromatin mark, which would then be bound by the CBX component of PCR1, allowing for H2AK119ub1 enrichment (Brockdorff, 2017). Subsequent studies, however, have come to the conclusion that it is in fact a non-canonical version of PRC1 that is first recruited to the X chromosome (Tavares et al., 2012), and that PRC2 recruitment depends on prior deposition of H2AK119ub1 (Almeida et al., 2017).

The polycomb complexes and their respective histone modifications have long been associated with the inactive X chromosome, yet their role in transcriptional silencing of the chromosome has been debated. While there is evidence in the mouse of some extraembryonic cells requiring polycomb for maintenance of XCI (Wang et al., 2001), one study found that these complexes did not seem to be required for initiation of
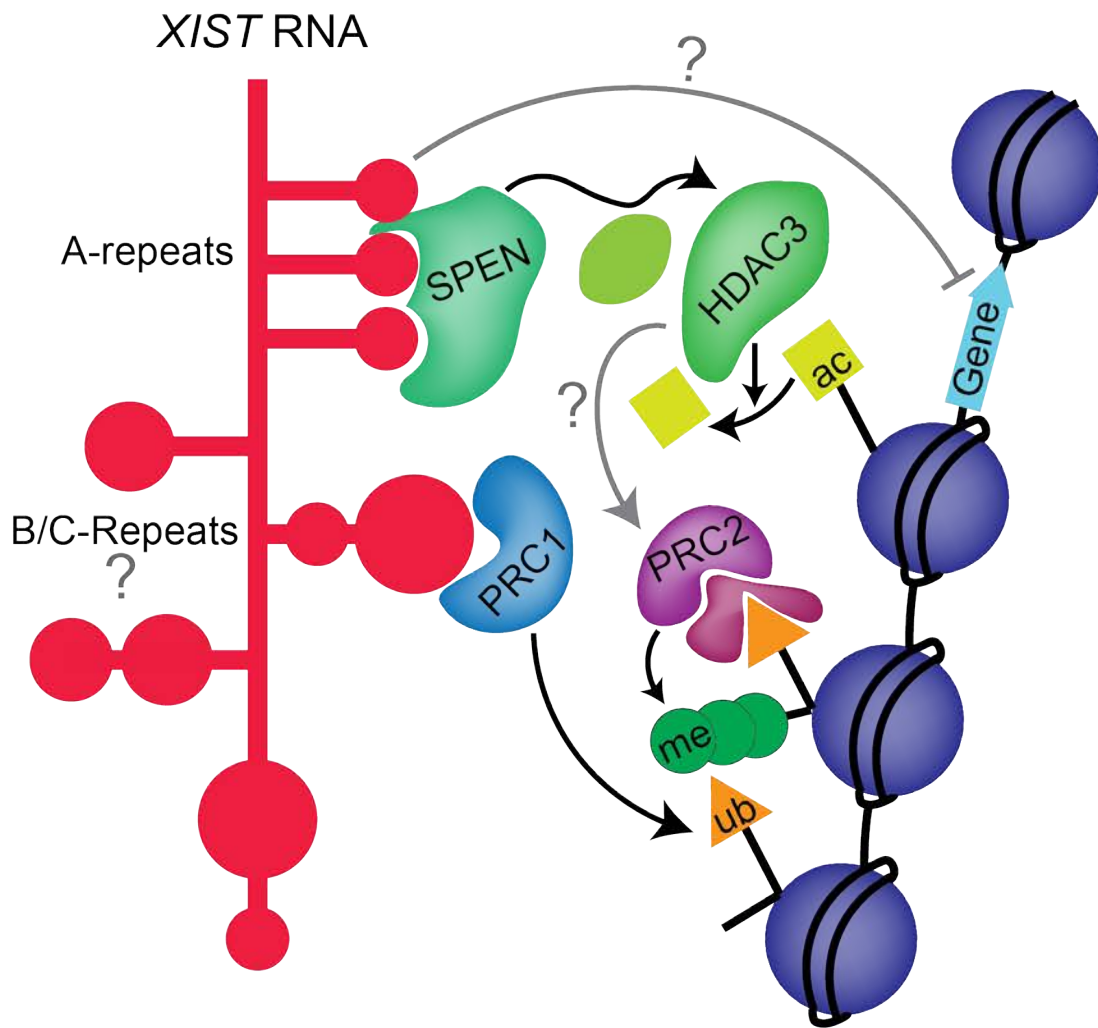
Figure I-1: Summary of *XIST* mechanisms for heterochromatin recruitment.

XIST RNA (red lines and circles) has a complicated 3D structure including several hairpins. Genomic DNA (black lines) is wrapped in nucleosomes (blue circles) containing histones which can undergo modifications that make the DNA more or less accessible to transcriptional machinery. The A-repeat is known to directly interact with SPEN, which recruits HDAC3 through intermediate partners. The A-repeat may also act via other mechanisms to enact gene silencing. Other elements of *XIST* recruit PRC1, which lays down H2AK119ub1. This subsequently leads to the recruitment of PRC2, which lays down H3K27me3. HDAC3 may also affect PRC2 recruitment. Figure partially adapted from Brockdorff (2017).

XCI in the embryo itself (Kalantry and Magnuson, 2006). There is also early evidence for *XIST* mutants that do not recruit PRC1/2 to undergo effective silencing, although the extent of silencing or the requirement of these complexes for the maintenance of the silenced state could not be assessed (Bousard et al., 2018). However, other studies have shown that the embryonic lethality in PRC1 knockout mice may not be totally attributable to the failure of extraembryonic cells to complete XCI, and that PRC1 mutant mouse embryonic stem cells (ESC) show impaired XCI (Almeida et al., 2017). Ultimately, further experiments, particularly in human cells, are required to determine to what extent the polycomb complexes are required for the initiation and maintenance of XCI.

The A-repeat sequence of *XIST* has been most tightly linked to the silencing function of this RNA. An *Xist* mutant in this portion of the gene leads to total abrogation of its silencing capability (Wutz et al., 2002), with no effect on the ability to recruit polycomb complexes and related heterochromatin modifications (Plath et al., 2003). The human A-repeat sequence alone was shown to be capable of silencing a reporter gene in the same transgenic construct (Minks et al., 2013), and may also be capable of silencing endogenous genes, potentially megabases away (Valledor et al., in preparation). Due to its importance in the role of silencing and its apparent uncoupling from the deposition of heterochromatin marks, there has been a concerted effort to determine how the A-repeat sequence induces transcriptional silencing. Through these efforts it was discovered that the RNA binding protein Spen was required for *Xist*-mediated silencing (Moindrot et al., 2015; Monfort et al., 2015). Further, novel techniques for identifying RNA binding proteins, such as comprehensive identification of

RNA binding proteins by mass spectrometry (ChIRP-MS), revealed that Spen interacts directly with the A-repeat of *Xist* RNA (Chu et al., 2015). Spen was also shown to activate histone deacetylase 3 (HDAC3) which is required for both exclusion of RNA polymerase II from the inactive X chromosome and recruitment of PRC2 (McHugh et al., 2015; Żylicz et al., 2019). Interestingly, while the A-repeat mutant studies demonstrate decoupling of this sequence from PRC2 recruitment, it has been shown to be required for Spen binding, which in turn is necessary for recruitment of PRC2 to the X chromosome. This suggests a complex interaction between the *XIST* RNA, RNA binding proteins, and polycomb complexes that is still poorly understood.

In addition to polycomb-mediated heterochromatin marks and Spen-mediated histone deacetylation, a number of other chromatin modifications have been linked to XCI. For example, H3K9me was one of the earliest reported chromatin changes associated with XCI (Heard et al., 2001), and the histone variant macroH2A is highly enriched on the inactive X, but its functional significance for XCI is questionable (Pehrson et al., 2014). Recent work has identified adenosine methylation of *XIST* RNA as a requirement for transcriptional repression (Patil et al., 2016), yet the mechanism behind its action is not yet understood. DNA methylation is one of the final steps in the XCI process that is thought to be required for maintenance of the inactive state, for which *XIST* is dispensable (Brown and Willard, 1994). Another factor, SMCHD1, has been shown to be enriched on the XCI and to play a role in DNA hypermethylation on the inactive X chromosome (Blewitt et al., 2008). While the focus of most studies have been on histone modifications and DNA methylation, *XIST* RNA also interacts with architectural proteins, such as SAF-A, and may act directly at the level of chromatin

architecture (Creamer and Lawrence, 2017). Overall, the plethora of heterochromatin modifications and mechanisms involved in the induction and maintenance of the inactive X chromosome state highlight the complementary and redundant nature of XCI. Many of the factors involved have been shown to be insufficient for gene silencing on their own, but knockout of multiple factors can lead to erosion of the silenced state (Csankovszki et al., 2001).

Once XCI has taken place, it is thought to be stable throughout the rest of development. Indeed, *XIST* RNA is largely dispensable for maintenance of XCI after it has been initiated (Brown and Willard, 1994). However, there is evidence that deletion of *XIST* in hematopoietic cells leads to inevitable hematological malignancy in mice (Yildirim et al., 2013), suggesting a possible role of *XIST* in fully maintaining long-term silencing. The importance of some factors for the induction of XCI, but not the long-term maintenance of silencing and vice-versa further complicates the identification of mechanisms involved, and it remains unclear what directly silences transcription as a result of *XIST* expression. Novel strategies to identify proteins that directly interact with *XIST* RNA have identified several pathways that may be crucial for gene silencing and bring us ever closer to a complete picture of the X inactivation process.

Induction of chromosome silencing in development

Very soon after the discovery of the Barr body, the temporal dynamics of its formation in development began to be studied. It was quickly realized that this body was not present in human or macaque zygotes, but appeared several days later in both cells of the embryo and extraembryonic tissues (Park, 1957). Since these early studies, much progress has been made in understanding the onset of XCI in both mouse and

human development. Additionally, the ability of *XIST* to induce chromosome silencing outside its normal developmental window has been an important area of research and will be a major area of study in this thesis.

The differences in mouse and human XCI are most significant in the preimplantation embryo. Beginning in the four-cell embryo in mice, *Xist* expression is initiated from the paternally inherited X chromosome (Marahrens et al., 1997), a process known as imprinted XCI. In extraembryonic tissues this pattern of XCI continues throughout pregnancy, with exclusive silencing of the paternally-derived X chromosome being vital for extraembryonic tissue survival in female animals (Mugford et al., 2012). In contrast, the inner cell mass (from which the embryo is derived) reactivates the paternally derived X chromosome in late stage blastocysts before random XCI takes place (Mak et al., 2004).

The XCI process in early human preimplantation embryos is just beginning to be explored, yet it is clear that imprinted XCI does not occur. Instead, beginning in the morula, *XIST* is expressed from both copies of the X chromosome (Okamoto et al., 2011; Petropoulos et al., 2016a). Recent evidence indicates that at the same time, X chromosome gene expression is biallelically repressed in a process called X chromosome dampening (XCD) that has also been observed in naïve iPSCs (Sahakyan et al., 2017). The mechanism behind *XIST*-mediated XCD is not completely understood, but a recently discovered X-linked lncRNA called *XACT* (Vallot et al., 2013), which is also transcribed from both X chromosomes in the early human embryo (Vallot et al., 2017), may play a role in attenuating the silencing action of *XIST*.

Post-implantation XCI in humans mimics that of embryonic mouse cells, where one X chromosome is randomly inactivated in each cell by *XIST*. The exact developmental timeline of the transition from XCD to random XCI in the human embryo is not well defined. This transition likely occurs in the blastocyst, where evidence of a single *XIST* paint and heterochromatin markers indicating XCI has been seen (van den Berg et al., 2009).

Because XCI occurs very early in development, it is perhaps logical that this developmental context is ideal for the silencing initiation function of *XIST*. In fact, it was reported that inducing *Xist* expression in mouse ES cells that were differentiated for 48-hours no longer leads to the induction of chromosome silencing (Wutz and Jaenisch, 2000). Since then, it has largely been accepted that *XIST* is *only* capable of inducing silencing in the pluripotent state (or in the hours immediately following). This has led to research in search of factors present in the pluripotent state that allow for *XIST*-mediated silencing. While one such factor, Satb1, was proposed as a "silencing factor" that allows for XCI outside of the normal developmental context (Agrelo et al., 2009), this finding has been challenged by a study that found Satb1 and Satb2 knockout animals to successfully undergo XCI (Nechanitzky et al., 2012). Other studies have demonstrated effective induction of XCI in neoplastic somatic cell lines (Chow et al., 2007; Hall et al., 2002a), but the human fibrosarcoma cell lines used in these studies likely possess a particularly malleable epigenetic state that is common in cancers (Jones et al., 2016). Other work has shown that certain hematopoietic precursor cells are also capable of initiating XCI (Savarese et al., 2006), which the authors interpreted as a "transient re-establishment" of competence to initiate the XCI process. The authors

noted, however, that the ROSA26 locus, which contained the transactivator transgene required for *Xist* transgene expression, was known to be expressed in hematopoietic cells. Given that transgene silencing is a common occurrence, it is possible that other cell types did not express *Xist* in this transgenic system, and thus the potential of these cells to initiate chromosome silencing could not be fully assessed.

There certainly could be factors that are present at high levels in pluripotent cells that allow for rapid and effective XCI. However, it is possible that these factors are also present at lower levels in differentiated cells which may support XCI, potentially at a slower rate. Certain cell types may lack key players, rendering them incapable of initiating XCI, but proper expression and localization of *XIST* must be assessed before such conclusions can be reached (Clemson et al., 1998). Currently, the ability of normal differentiated human cells to initiate XCI has not been evaluated. Work in this thesis will directly test this key question which has relevance for basic epigenetics and developmental biology but is also important for the potential therapeutic applications of *XIST* or derived sequences.

Early human brain development and *in vitro* modeling

The human brain is the most complicated and least understood organ in the body. The transformation of a small group of embryonic stem cells to the cognitive powerhouse within our skulls is an immensely complex developmental process that is only beginning to be unraveled. Due to the potential breadth of this topic, I will limit discussions here to those areas most relevant to the goals and results of the present thesis work. This will include discussion of the early induction of neural lineage cells in the embryo and the onset of neurogenesis, major differences between human and

mouse brain development, selected signaling pathways involved in cell fate specification and differentiation, postnatal brain development, and attempts at *in vitro* modeling of neurodevelopment.

Neural lineage commitment and neurogenesis

Cells committed to the neural lineage in human embryos arise following gastrulation, when the embryo transforms from a single-layer blastula to the gastrula containing three germ layers: ectoderm, mesoderm, and endoderm. Ectoderm gives rise to both the epidermal ectoderm (i.e. skin) and neuroectoderm which gives rise to neurons and macroglia (astrocytes and oligodendrocytes). Derivation of neuroectoderm (also known as neuroepithelium) is thought to be the "default" cell fate in the absence of other signals, such as bone morphogenetic proteins (BMP) (Khokha et al., 2005; Stern, 2005; Zimmerman et al., 1996). Starting at about three weeks of human development, neuroepithelial cells at the neural plate begin folding to form the neural tube. The hollow cavity inside the tube will eventually become the ventricular system of the brain, and the progenitors surrounding this tube form a region known as the ventricular zone (VZ) and will give rise to the majority of cells in the brain. By embryonic day 28, there is clear separation of the neural tube into three primary vesicles: the prosencephalon, mesencephalon, and rhombencephalon, which will give rise to the forebrain, midbrain, and hindbrain, respectively.

The neuroepithelial cells of the neural tube form a single layer of pseudostratified epithelium and exhibit interkinetic nuclear migration, a process whereby the cell nuclei undergo mitosis at the apical surface of the VZ and then migrate basally during S phase (Huttner and Brand, 1997). Once the neural tube is closed, neuroepithelial cells switch

from symmetric proliferation, which expands the neuroepithelial cell pool, to asymmetric divisions, which mark the start of neurogenesis. At this point, the cell cycle of the neuroepithelial cells is lengthened, due to a four-fold lengthening of G1 phase (Takahashi et al., 1995), a change that is believed to be able to trigger neurogenesis in and of itself (Calegari and Huttner, 2003). These neurogenic asymmetric divisions form two classes of cells, epithelial and non-epithelial. The non-epithelial cells, which have lost their attachment to the apical surface of the ventricle, can be further divided into non-dividing cells (i.e. neurons and glia) and dividing cells (i.e. intermediate progenitors) which can divide symmetrically to produce two neurons (Englund et al., 2005; Huttner and Brand, 1997). The epithelial cells, which retain their apical attachment site, are known as a specialized type of neuroepithelial cell called radial glial cells (RGCs). Radial glia have long been known to serve a structural function in the developing brain, whereby their apical processes form a scaffold to guide newly born neurons to migrate apically to their final destination in the cortical plate (Rakic, 1971, 1972). Additionally, RGCs are now known to serve a vital function as neuronal progenitors through successive rounds of asymmetric divisions and as astrocyte progenitors after neurogenesis is complete (Malatesta et al., 2000; Noctor et al., 2001). In fact, direct or indirect progeny of RGCs make up the majority of neurons in the brain (Anthony et al., 2004). A summary of early cortical neurogenesis is provided in Figure I-2.

Human-specific neocortical development

A longstanding question in developmental neurobiology has been the cellular mechanism of the greatly expanded human neocortex. Mouse studies have shown that

most neurons are directly born through symmetric divisions of intermediate progenitors, which reside in a region just basal to the VZ known as the subventricular zone (SVZ) (Kowalczyk et al., 2009). The SVZ region is greatly expanded in the primate cortex (Lukaszewicz et al., 2005), which, unlike in the mouse, can be subdivided into an inner and outer SVZ and, in addition to containing non-epithelial intermediate progenitors like the mouse SVZ, also contains radially organized epithelial-like cells (Fish et al., 2008; Smart et al., 2002). These cells were recently shown to be another class of RGCs whose numbers are greatly increased in the outer SVZ, known as outer radial glia (oRG) (Hansen et al., 2010). These cells have the ability to divide asymmetrically into another oRG cell and an intermediate progenitor (IP), in contrast to mouse RGCs which generally only divide symmetrically to produce two neurons (Noctor et al., 2008). This ability to self-renew and enlarge the progenitor pool allows for a much larger number of progenitors that leads to the greatly increased neuron cell number in the primate brain. Additionally, many of these transit-amplifying cells retain pial contacts that allow for proper migration of the increased number of neurons.

The neurogenic events covered above are best described for the birth of excitatory neurons in the cortex. Inhibitory neurons in the mouse are known to originate from the ventral portion of the telencephalon (known as the ganglionic eminences) and migrate dorsally to integrate with excitatory neurons born from the VZ and SVZ of the dorsal telencephalon in order to form functional neuronal networks (Anderson et al., 1997). The origin of human and primate cortical interneurons has been contentiously debated. Ever since it was suggested that a majority of human cortical interneurons originate in the dorsal telencephalon, based on classical markers of interneuron
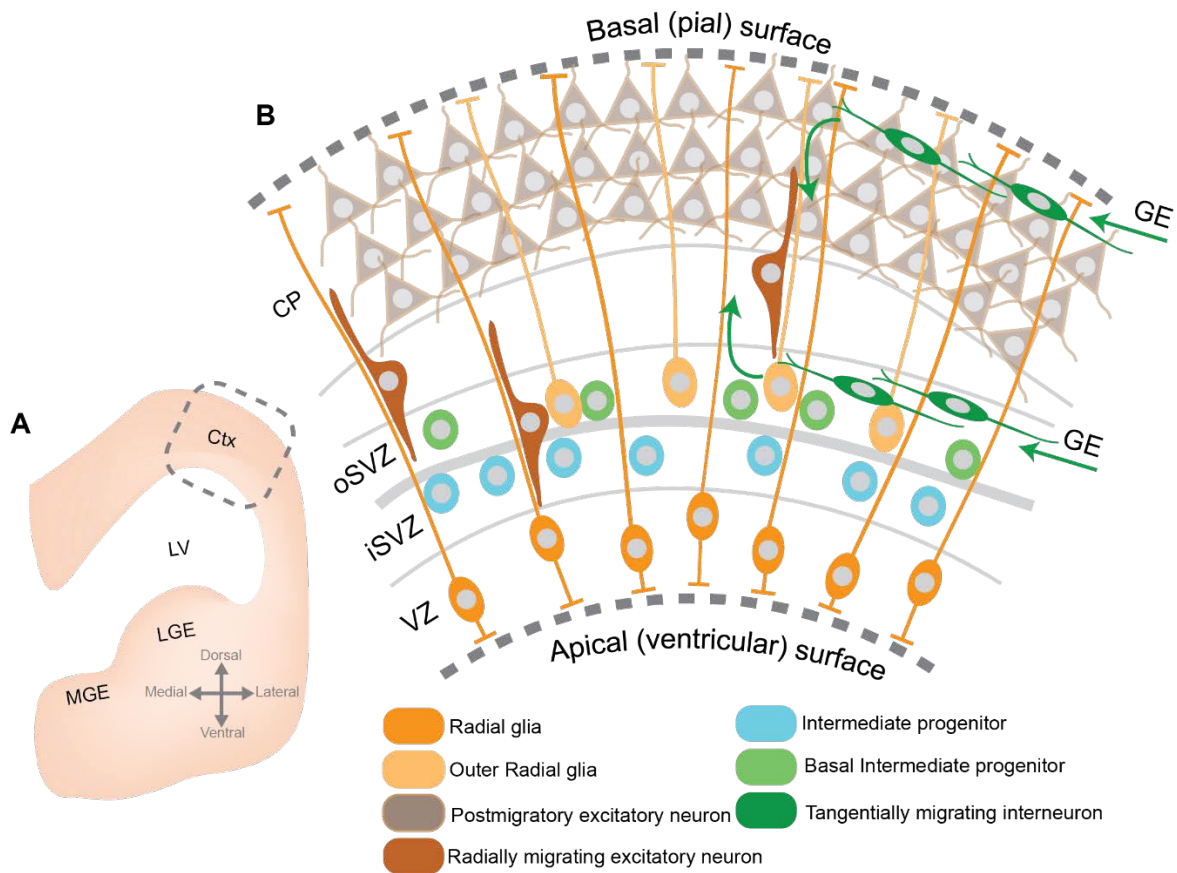
Figure I-2: Summary of early cortical neurogenesis

A) Schematic of a coronal section of one anterior telencephalic hemisphere during early human development. Ctx, cortex; LV, lateral ventricle; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. B) Schematic of cellular composition of developing cortex, enhancement of dashed box from (A). Radial glia form apical-basal scaffold for radially migrating excitatory neurons. Interneurons from the ganglionic eminences enter the cortex tangentially and then switch to radial migration within the dorsal cortex. CP, cortical plate; oSVZ, outer subventricular zone; iSVZ, inner subventricular zone; VZ, ventricular zone; GE, ganglionic eminences. Figure adapted from Buchsbaum and Cappello (2019).

progenitors like DLX1/2 and Mash1 (Letinic et al., 2002), numerous studies have supported these findings (Petanjek et al., 2009; Yu and Zecevic, 2011; Zecevic et al., 2011). However, more recent studies using embryonic human tissues have found that the purported dorsal interneuron progenitors did not incorporate BrdU, suggesting that they are not proliferative (Hansen et al., 2013), and that interneuron-specific transcription factor expression patterns in the human telencephalon could be more similar to mice than previously appreciated (Ma et al., 2013). These studies did suggest that the SVZ of the ventral telencephalon, as well as the caudal ganglionic eminence in humans, is greatly expanded compared to mice. Together with another study which found that human interneuron neurogenesis takes place over a much longer period extending into the third trimester of gestation (Arshad et al., 2016) this provides a potential explanation for the greatly increased number and diversity of interneurons in the human brain. The growing evidence implicating interneuron function in a range of psychiatric and neurodevelopmental disorders due to their vital modulatory role in cortical neural networks (Chattopadhyaya and Cristo, 2012; Marín, 2012) necessitates further study into the developmental origins of interneurons in humans.

Notch signaling in neurodevelopment

Notch is one of several signaling pathways that are necessary for proper brain development and plays a key role in several aspects of neurodevelopment. Here, I provide an overview of this pathway in particular detail due to our findings in chapter II that unexpectedly revealed a possible role for Notch in DS neurogenesis.

Notch is vital for maintaining a proper balance between progenitors and differentiated neurons and also plays a role in a number of other key steps during

neurodevelopment. The canonical Notch signaling pathway is composed of single-pass heterodimeric notch receptors and single-pass ligands in neighboring cells that bind to the extracellular domain of these receptors. Each component of this pathway is subject to extensive post-translational modifications and proteolytic events (Kovall et al., 2017). Ligand-receptor binding leads to intracellular cleavage of the Notch receptor by the presenilin/γ-secretase complex (Selkoe and Kopan, 2003), releasing the Notch intracellular domain (NICD) which translocates to the nucleus and acts in a complex to drive target gene expression.

Notch plays a central role in the coordination of neurogenesis. Upon neuronal differentiation, neuronal genes induce expression of Notch ligands, which in turn activate Notch receptors on adjacent cells. Notch target genes, such as Hes1 and Hes5 then repress proneuronal genes, which maintains these adjacent cells as neural progenitors (Gaiano and Fishell, 2002). This system of lateral inhibition prevents all cells from differentiating simultaneously into neurons and promotes cellular diversity in the developing brain. Experimental inactivation of Hes1 and Hes5 causes premature differentiation and depletion of late-born neuronal subtypes (Hatakeyama et al., 2004; Ishibashi et al., 1995), and Notch signaling has been shown to be absolutely required for the maintenance of neural stem cells in both embryonic and adult brains (Imayoshi et al., 2010).

An interesting question arises when considering the role of Notch signaling in the asymmetric divisions of progenitors such as radial glial cells, which divide into another progenitor and a differentiated neuron. In asymmetric divisions, the cell divides during periods of high proneuronal gene levels, which are cyclically regulated (Hirata et al.,

2002). One daughter cell then inherits the basal fiber of the radial glial cell that reaches into the SVZ, an area of high Notch ligand activity (Miyata et al., 2004). As a result, Notch receptors are activated in this daughter cell leading to downstream Notch signaling which maintains the progenitor state, while the cell without basal fiber is primed to differentiate into a neuron (Lui et al., 2011).

In addition to its important role in regulated neurogenesis, Notch has also been shown to be important in astrogliogenesis. Neurons and astrocytes are sequentially derived from common progenitors, a process regulated by increased gliogenic signals in the postnatal brain (Morrow et al., 2001). While an indirect role of Notch signaling in maintaining the progenitor pool and allowing for later astrogliogenesis to take place has long been inferred, a direct role of Notch in astroglial differentiation has also been identified. Notch ligand expression from neuronal precursors activates Notch signaling in nearby radial glial cells leading to demethylation of STAT3 binding sites on the glial fibrillary acidic protein (GFAP) gene, leading to astroglial differentiation (Namihira et al., 2009; Takizawa et al., 2001). This finding potentially explains the sequential differentiation of neurons followed by astrocytes due to the important role that neuron-to-progenitor signaling plays in this process.

Beyond influencing neuron and astrocyte differentiation, Notch signaling also plays a vital role in the maturation, morphology, and function of neurons. The first hint that Notch may still play an important role in differentiated neurons came from the finding of robust nuclear Notch expression in postmitotic neurons (Ahmad et al., 1995). Indeed, Notch signaling has been shown to inhibit dendritic growth (Redmond et al., 2000) and influence axon guidance (Song and Giniger, 2011). Additionally, mice with

reduced levels of Notch expression, which develop normally and have grossly normal brain morphology, demonstrate impaired hippocampal long term potentiation (LTP) and exhibit learning deficits (Costa et al., 2003; Wang et al., 2004). Together, these findings indicate that Notch plays an important role in both the development of the proper balance of cell types in the brain as well as the long-term maintenance of neuronal circuitry.

Postnatal brain development

Humans are born with nearly all the neurons they will ever have and, apart from some interneurons that continue migrating several months after birth (Paredes et al., 2016), neuronal migration is also complete at birth (Sidman and Rakic, 1973). However, there may be ongoing neurogenesis in the external granule layer of the cerebellum for up to a year after birth (Walton, 2012). Additionally, although debated (Sorrells et al., 2018), recent work suggests that there may be limited ongoing neurogenesis in the dentate gyrus of the hippocampus throughout human life (Boldrini et al., 2018), which could play an important role in memory formation and has been shown to be decreased in patients with AD (Moreno-Jiménez et al., 2019). Beyond neurogenesis, however, there is enormous ongoing change that occurs to the brain from infancy through childhood and adolescence that allows for its development to the adult form (Figure I-3). These postnatal changes would be most amenable to any potential therapeutic approaches, including traditional pharmaceuticals or *XIST*-mediated chromosome silencing.

As alluded to above, gliogenesis follows neurogenesis and is incomplete at birth. Astrogliogenesis continues postnatally (Roessmann and Gambetti, 1986) and

astrocytes are known to maintain proliferative capacity throughout the lifespan, particularly in response to injury (Burda and Sofroniew, 2014). Another type of macroglial cell, oligodendrocytes, which play a central role in myelination of axons in the central nervous system, arise after astroglia and actively myelinate axons starting in the second half of pregnancy through early adolescence (Barnea-Goraly et al., 2005; Jakovcevski and Zecevic, 2005; Miller et al., 2012).

Both astroglia and oligodendrocytes play a critical role in neuronal development (Zuchero and Barres, 2015), thus it is of no surprise that maturation of neurons also continues long after birth. Synaptogenesis, which is the process of forming functional connections between neurons, is rampant after birth. This leads to an overproduction of synapses that peaks at about two years of age. Synapses are subsequently pruned away over the next few decades until a stable adult level is reached (Huttenlocher, 1979; Petanjek et al., 2011). Similarly, the dendritic fields of neurons in the prefrontal cortex have been shown to grow dramatically postnatally and stabilize at about one year of age (Koenderink et al., 1994). These cellular events all work in harmony to develop the neonatal brain through childhood and beyond, including maturation of motor, sensory, and cognitive circuits. While some measurable cellular properties may stabilize with adulthood, the adult neural circuitry remains plastic, allowing for both learning and forgetting throughout the lifespan.

In vitro modeling of human neurodevelopment

The relative inaccessibility and scarcity of accessible human fetal brain tissue has hindered our understanding of human brain development, especially compared to animal models. Early work with embryonic stem cells (ESC) began to reveal the
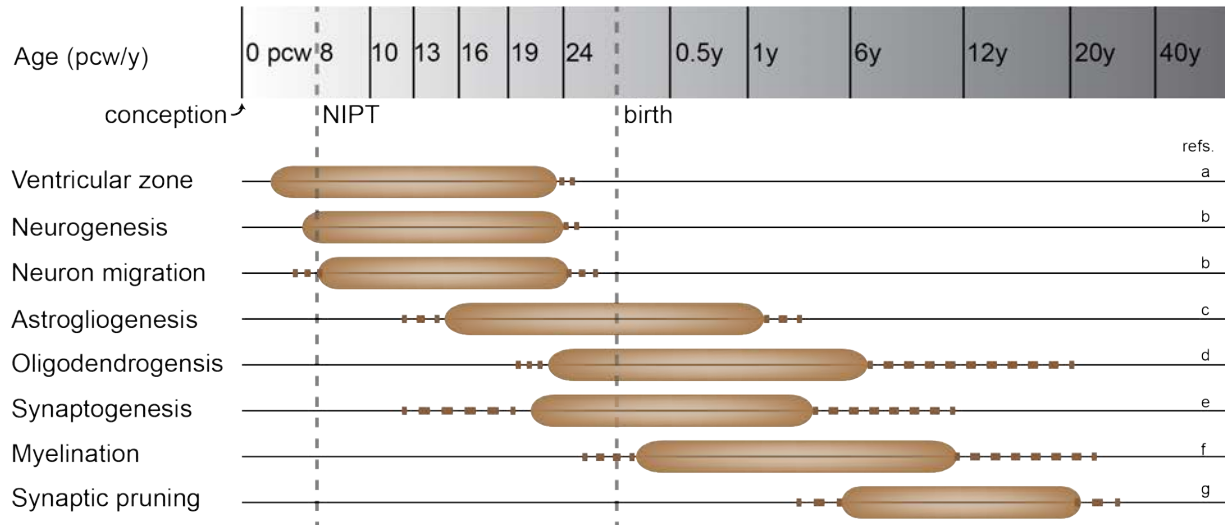
Figure I-3: Timeline of major cellular processes during human neurodevelopment

Summary of major cellular processes and events that occur in the developing brain and their estimated time course. The top panel describes the time course of human development in post-conception weeks (pcw) and post-natal years (y). Dashed lines indicate time of earliest currently available non-invasive prenatal testing (NIPT) for diagnosis of trisomies, as well as time of birth. The lower panel outlines estimated timing of cellular events in the developing brain. Rounded bars indicate peak developmental period and dashed lines indicate minor degree of feature development. Relevant references for each process are provided on the right: a) (Gould et al., 1990; Malik et al., 2013), b) (Bystron et al., 2006; Meyer, 2007; Workman et al., 2013), c) (Choi and Lapham, 1978; deAzevedo et al., 2003; Kang et al., 2011), d) (Kang et al., 2011; Yeung et al., 2014), e) (Huttenlocher, 1979; Kwan et al., 2012; Molliver et al., 1973; Petanjek et al., 2011), f) (Miller et al., 2012; Yakovlev, 1967), g) (Huttenlocher, 1979; Petanjek et al., 2011). This figure has been adapted from Silbereis, et al. (2016).

potential therapeutic applications and developmental insights that pluripotent stem cells have to offer. The advent of iPSC technology (Takahashi and Yamanaka, 2006; Takahashi et al., 2007) allowed for an infinite supply of normal and patient-derived pluripotent stem cells, which has greatly accelerated research into early human neurodevelopment and its various pathologies. While iPSCs can theoretically be differentiated into any cell type in the body, the utility of iPSCs to model development relies on robust differentiation protocols to reproducibly generate the cell type(s) of interest.

As early evidence of the utility of PSCs to model early neurodevelopment, studies have found that differentiating ESCs follow developmental principles. For example, ESC and iPSC-derived neuroepithelia form neural-tube like structures that mimic early neurodevelopment (Shi et al., 2012a; Zhang et al., 2001). Additionally, *in vitro* differentiation follows a similar time course to *in vivo* neurodevelopment (Hu et al., 2010; Tao and Zhang, 2016). These indications of the accurate modeling of *in vivo* development using *in vitro* methods has encouraged insights that can carry over from one to the other, and vice-versa. Knowledge of patterning in the early embryo has aided in the development of protocols to form a wide variety of region-specific neural cell types (Tao and Zhang, 2016). For example, by varying the concentration of sonic hedgehog (SHH) or SHH agonists, iPSCs can be induced to form more dorsal or ventral neural progenitors, which eventually differentiate to either more excitatory or more inhibitory neuron types, respectively (Li et al., 2009; Liu et al., 2013; Maroof et al., 2013). Similarly, retinoic acid has been shown to direct progenitors towards a more caudal fate, which, in the presence of SHH, can lead to efficient production of motor

neurons (Li et al., 2005). The use of individual morphogens can lead to imprecise

patterning that can have low yields of the specific cell types of interest. This efficiency

can be improved with precise titration of opposing morphogens, for example

coordinated SHH and Wnt signaling in addition to retinoic acid treatment allows for

extremely pure generation of motor neurons (Du et al., 2015). Similar patterning

techniques can also generate region-specific astrocytes (Krencik et al., 2011), allowing

for differentiation of a variety of region-specific neural cell types. Generation of pure

neural subtypes holds promise both for potential cell replacement therapeutic

opportunities as well as for a more detailed molecular characterization of specific cell

types.

Generally, *in vitro* differentiation protocols aim to form a pure population of a

specific neural cell type of interest. However, *in vivo* neurodevelopment involves many

interacting diverse cell types, which has led to the development of co-culture techniques

that aim to mimic these complex interactions. Various techniques have been developed,

including embedding neural stem cells in extracellular matrix to allow for extracellular

plaque development in a model of AD (Choi et al., 2014), co-culture of astrocytes and

neurons on a microfluidic platform (Majumdar et al., 2011), and a tri-culture system that

can examine migration of microglia in the context of neuron/astrocyte co-culture (Park et

al., 2018).

In addition to these engineered systems, other methods have capitalized on the

self-organizing capacity of cells *in vitro* to form complex three-dimensional structures

known as organoids (Eiraku et al., 2008; Lancaster et al., 2013; Mariani et al., 2015;

Paşca et al., 2015; Qian et al., 2018). These organoid techniques vary in their degree of

complexity and self-organization, but all include numerous cell types including neural progenitors, radial glia, early- and late-born neurons, and astrocytes. Additionally, these systems have been used to model numerous developmental disorders, such as microcephaly (Lancaster et al., 2013), Zika virus infection (Qian et al., 2016), autism (Mariani et al., 2015), and Timothy syndrome (Birey et al., 2017). Three dimensional culture provides a more natural developmental tissue context, but also allows for analysis of spatial events, such as the orientation of progenitor cell division relative to the ventricular plane, as was shown to be skewed towards perpendicular symmetric divisions in a model of microcephaly (Lancaster et al., 2013). While most protocols are currently limited to cells from the ectodermal lineage, from which the majority of the brain parenchyma is derived, others have also included mesodermal precursors (Quadrato et al., 2017), functional microglia (Ormel et al., 2018), and endothelial cells that form primitive vascular networks (Pham et al., 2018). These recent advances have allowed for visualization and dissection of embryonic human brain development *in vitro* in a complex three-dimensional environment that includes a variety of cell types involved in this intricate process.

Of course, even contemporary organoid protocols still fall far short of modeling the full complexity of human brain development. While organoids demonstrate a capacity for self-organization at the level of neural-tube like structures, they lack organization at a macroscopic level. Protocols have been developed to generate organoids that model differentiation of individual brain regions (Qian et al., 2016), and other labs have even gone as far as merging together dorsal and ventral patterned organoids in order to model interneuron migration (Bagley et al., 2017; Birey et al.,

2017). Yet, there is still a long way to go towards modeling development of the human CNS. Currently, one must choose between directed protocols, which utilize high degrees of patterning, and less directed methods. Directed methods can accurately model a specific brain region but lack some of the cell diversity and tissue complexity of less directed methods which, on the other hand, tend to have a much higher degree of organoid-to-organoid variability (Kelava and Lancaster, 2016). Reproducibility is an important limitation of current neural differentiation protocols (Young-Pearse and Morrow, 2016), and while some groups claim to have consistent organoid differentiation protocols (Yoon et al., 2019), there is still considerable room for improvement.

In addition to variability between differentiations, iPSC modeling in general is affected by genetic variability between individuals, variability between cell lines from the same individual, and variability of a cell clone over time (Koyanagi-Aoi et al., 2013; Liang and Zhang, 2013; Soldner and Jaenisch, 2012; Young-Pearse and Morrow, 2016), although some of these limitations can be minimized by improved differentiation protocols, iPSC culturing techniques, and quality control measures. Additionally, recent advances in genome engineering have made production of isogenic control lines from mutant cells and insertion of specific mutations into control lines easier than ever. Finally, while current iPSC differentiation protocols closely mimic very early *in vivo* neurodevelopment, their ability to model neurodegenerative diseases is complicated by their fetal phenotype and transcriptomic profile (Abeliovich and Doege, 2009). Overall, human pluripotent stem cells, particularly iPSCs, have provided insight into a previously inaccessible tissue and, despite their limitations, have potential to drastically change our

approach towards studying neurodevelopment and treating neurodevelopmental diseases.

The neurobiology of Down syndrome

Over 90 years after John Langdon Down described the features of the disorder that would come to bear his name (Down, 1867), Marthe Gauthier, Jérôme Lejeune, and Raymond Turpin (1959) discovered what is likely the first known cause of a genetic disorder: an extra copy of chromosome 21 in individuals with DS. In general, despite the hundreds of extra genes that these individuals have in three copies instead of the usual two, most lead happy and relatively healthy lives. Still, DS individuals suffer from a number of medical problems, and the specific genetic etiology of these various symptoms remains poorly understood. Apart from the *APP* gene which has been strongly linked to the development of Alzheimer's disease in DS, no single genes have been linked to other DS phenotypes. In addition to single dosage-sensitive genes causing specific phenotypes, it is also possible that the presence of an entire extra chromosome, along with the associated transcriptional, translational, and proteomic burden, may be responsible for some DS phenotypes. In fact, studies in yeast and mice have shown that aneuploidy in general causes cellular stress, which may impact several cellular phenotypes such as metabolism and proliferation (Bonney et al., 2015; Oromendia et al., 2012; Williams et al., 2008). Importantly, the physical presence of an extra chromosome is known to not cause symptoms on its own, as demonstrated by trisomy X patients.

Despite the unknown etiology of many DS symptoms, advances in prenatal testing now allow for the diagnosis of DS and other trisomies as early as 8 weeks of

gestation, meaning that newborns with DS are more likely to receive treatments for their specific needs, particularly for serious congenital heart defects that affect nearly half of DS individuals (Freeman et al., 1998). Children with DS are at an increased risk for several types of leukemia such as acute megakaryoblastic leukemia (AMKL), which is 500 times more common in DS than in the general population (Zipursky et al., 1992). DS individuals are also prone to other endocrine, gastrointestinal, metabolic, and morphological abnormalities (Roizen and Patterson, 2003). Interestingly, DS individuals are less likely than the general population to develop solid tumors (Hasle et al., 2016; Satgé et al., 1998), a phenomenon which is not well understood but may relate to impaired angiogenesis (Baek et al., 2009). The most prevalent and perhaps least understood feature of DS, and one for which no effective treatments exist, is intellectual disability (ID).

Intellectual disability in DS individuals and its neurological correlates

Individuals with DS exhibit a range of cognitive abilities, with IQs ranging from 30 to 70 and averaging 50 (Chapman and Hesketh, 2000), which corresponds to mild or moderate ID. Learning delays are present from birth, and accelerate at ages 2-4. Communication skills are often particularly impaired from as early as two years of age (Dykens and Kasari, 1997) and include nonverbal communication (Mundy et al., 1995), suggesting that the language delay seen in older children with DS may not be limited to speech production difficulties. While there is no delay in the onset of babbling compared to typically developing children (Smith and Oller, 1981), DS children show a delay in first word acquisition and delayed use of multi-word phrases (Levy and Eilam, 2013). In contrast with language production, comprehension is relatively spared particularly with

regard to vocabulary (Rosin et al., 1988). Beyond communication, children with DS also have deficits in attention that persist in adulthood (Rowe et al., 2006), as well as deficits in working memory, again with particular impairment of verbal compared to visuospatial aspects of working memory (Jarrold and Baddeley, 1997; Yang et al., 2014). Explicit long-term memory is also severely impaired in DS individuals, even when compared with individuals with ID of another etiology, and this is thought to stem from deficits in both encoding and retrieval abilities (Carlesimo et al., 1997). Additionally, children with DS have a much higher incidence of comorbidities such as autism spectrum disorder (ASD) and attention-deficit-hyperactivity disorder (ADHD) compared to typically developing children (DiGuiseppi et al., 2010; Godfrey et al., 2019).

Given how common DS is, it is remarkable that precise neurobiological correlates of the cognitive deficits are so poorly understood and remain to be identified. However, there are several brain anomalies at both the macrostructural and cellular levels that have been reported. On average, individuals with DS have mild overall microcephaly, a difference which appears as early as mid-gestation and persists through adulthood, with disproportionately small frontal and temporal lobes (Guihard-Costa et al., 2006; Schmidt-Sidor et al., 1990). The hippocampus, which is vital for certain explicit memory tasks that are impaired in DS individuals, is smaller in the DS population and its size is negatively correlated with general intelligence and mastery of linguistic concepts (Raz et al., 1995). The cerebellum is also disproportionately small (Guihard-Costa et al., 2006), which could be associated with the hypotonia, motor dysfunction, and perceptual-motor coordination seen in DS individuals (Charlton et al., 2000; Savelsbergh et al., 2000), but may also impact a wide variety of cognitive abilities (Stoodley, 2016).

At the cellular level, DS fetuses have been reported to have decreased neuron numbers in the hippocampus, neocortex, and cerebellum, with some studies finding differences as drastic as a 34% reduction in total cell number (Guidi et al., 2008, 2011; Larsen et al., 2008). Other studies have shown decreased cell proliferation in the hippocampus of mid-gestation DS fetuses (Contestabile et al., 2007), which may be responsible for the decreased neuron numbers. There have also been reports that decreased neuron numbers are specific to small, presumably inhibitory, granular neurons of the neocortex (Ross et al., 1984). In addition to decreased neuron numbers, the dendritic processes of DS neurons in the cortex and hippocampus have decreased branching and dendritic spine numbers (Becker et al., 1986; Ferrer and Gullotta, 1990; Suetsugu and Mehraein, 1980). Interestingly, unlike the decreased neuron numbers that were seen in fetal DS brains, dendritic morphology and spine numbers were considered normal at the fetal stage and only started showing pathological features in early childhood (Becker et al., 1986; Takashima et al., 1981; Vuksić et al., 2002). In addition to reduced dendritic spine numbers, the morphology of dendritic spines has also been reported to be altered in children with DS, showing both unusually long and unusually short spines (Marin-Padilla, 1976), which may reflect impaired spine maturation. A spine-related protein, drebrin, was reported to be consistently decreased in DS cortical tissue (Shim and Lubec, 2002).

Apart from neurons, DS brains may also show alterations in astrocyte and oligodendrocyte populations. While some studies have shown no change in absolute astrocyte number in DS brains in the context of decreased neuron numbers (and thus an increased percentage of astrocytes) (Guidi et al., 2008), others have reported

increased absolute non-reactive astrocyte number in DS fetal brains and increased

reactive astrocytes in adult DS brains (Mito and Becker, 1993; Zdaniuk et al., 2011).

However, increased astrogliosis in adult DS brains may also be related to the

development of AD pathology (Griffin et al., 1989; Jørgensen et al., 1990), as will be

discussed in a later section. Myelination by oligodendrocytes has also been reported to

be impaired in DS brains, with both histological and transcriptional markers of delayed

myelination in the frontal and temporal lobes as well as the hippocampus (Ábrahám et

al., 2012; Olmos-Serrano et al., 2016; Wisniewski, 1990; Wisniewski and Schmidt-Sidor,

1989).

Overall, there have been numerous reported macro and microstructural

alterations in the brains of individuals with DS involving many cell types and brain

regions. However, analysis of human brains is limited by small sample sizes and

differences in age, pathological status, and sample preparation. While some histological

findings may correlate with clinical phenotypes, there are few direct links between

precise morphological changes and behavioral or cognitive findings in individuals.

Studies in DS mouse models have worked towards making such connections.

Mouse models of DS neurobiology

Unlike the human genome, which is comprised of 23 pairs of chromosomes, the

mouse genome contains only 20 pairs. This of course means that the syntenic regions

of human chr21 are not all neatly arranged on one mouse chromosome. Instead, the

syntenic genes are spread across three mouse chromosomes, with the majority residing

on mouse chromosome 16 (Mmu16) and the rest on Mmu17 and Mmu10. Clearly, this

complicates the creation of an accurate mouse model of DS. Despite these difficulties,

numerous mouse models have been generated. The best studied mouse model of DS

contains a freely segregating marker chromosome that contains the centromere of

Mmu17 and the distal segment of Mmu16 (Reeves et al., 1995). This model, named

Ts65Dn, contains 125 genes syntenic to Hsa21, but also contains 35 protein-coding

genes syntenic to Hsa6 (Gupta et al., 2016). Other models have improved on this by

eliminating Mmu16 genes not syntenic to Hsa21 (Li et al., 2007) and including Hsa21

syntenic genes from other mouse chromosomes (Yu et al., 2010). A different approach

led to the creation of a mosaic mouse model in which some cells contain a single copy

of human chromosome 21 (O'Doherty et al., 2005). While this model has substantial

utility for studying the overexpression of human chr21 genes in the mouse, subsequent

analysis has identified significant duplications and deletions within this chromosome,

including for the important *APP* gene (Gribble et al., 2013). Many of these models

present with wide-ranging phenotypes, some of which may correlate with defects seen

in DS individuals, and others that may be model-specific. For example, a recent

transcriptome study found little overlap in differentially expressed genes between three

widely used mouse models of DS (Aziz et al., 2018).

  In agreement with the previously described findings of decreased neuron number

and cellular proliferation in DS brains, several mouse models show decreased numbers

of radial glia in the cerebral cortex and hippocampus, including the dentate gyrus

(Chakrabarti et al., 2007; Contestabile et al., 2007; Ishihara et al., 2010). These findings

may be responsible for altered hippocampus-dependent learning and memory tasks

(Belichenko et al., 2009, 2007; Reeves et al., 1995) and potentially broader cognitive

impairment as well, such as in the novel object recognition task (Belichenko et al., 2009;

Fernandez et al., 2007). Also in line with findings in DS, several mouse models have significantly decreased cerebellar size and cell density, particularly in the granule cell layer (Baxter et al., 2000; Olson et al., 2004). Interestingly, evidence indicates that this is caused by a defective response to SHH signaling (Roper et al., 2006), and can be totally prevented in the Ts65Dn mouse by a single injection of SHH agonist at birth (Das et al., 2013). DS mouse models also exhibit altered dendritic spine morphology, potentially consistent with alterations seen in DS individuals. Specifically, projection neurons in Ts65Dn mice have reduced spine numbers and shorter dendrites (Dierssen et al., 2003). This is also true for pyramidal neurons in the hippocampus of several, but not all mouse models (Belichenko et al., 2009, 2004, 2007).

While several findings in mouse models are consistent with DS pathology, there are also findings in certain mouse models that conflict with the human condition and/or with other mouse models. For example, the Ts65Dn mouse has the well-described phenotype of increased GABAergic neuron number along with decreased excitatory neuron number leading to an over-inhibited state that impairs LTP in the hippocampus and can be ameliorated with GABA receptor antagonists (Belichenko et al., 2004; Fernandez et al., 2007; Kleschevnikov et al., 2004; Pérez-Cremades et al., 2010). The increase in GABAergic interneurons has been reported to be corrected by normalization of the gene dosage of *Olig1* and *Olig2* (Chakrabarti et al., 2010). However, a more genetically accurate mouse model of DS that does not include extra copies of non-chr21 syntenic genes has the opposite finding, with a decrease in the number of interneurons (Goodliffe et al., 2016). This result is more in line with findings in human cells, as will be discussed in the next section.

Overall, mouse models of DS can provide a useful system for unveiling the effects of trisomy on early brain development, a process that is difficult to study in human samples. However, caution must be exercised in translating findings from imperfect mouse models to the human condition. The failure of a recent DS clinical trial of the GABAergic inverse agonist RG1662, despite promising pre-clinical results, has many potential explanations. However, a lack of shared genetic defect and physiology between mouse models and individuals must be taken into consideration as a possible cause.

Human cellular models of DS neurobiology

Despite not having the *in vivo* relevance of mouse models, human cellular models of DS have the advantage of fully recapitulating the root cause of the syndrome, namely trisomy for chr21 in human cells. Neuro-cellular models come in two broad categories, ES or iPS-derived neural cells and primary fetal neural stem cells. Despite the difficulty in obtaining fetal brain tissue samples, several studies have examined the proliferative and differentiation capacity of primary neural progenitor cells from DS fetuses. Three such studies have found decreased neurogenesis in DS samples compared to controls (Bahn et al., 2002; Bhattacharyya et al., 2009; Esposito et al., 2008). One of these studies described a critical window in neurogenesis whereby early passages of NSCs led to an equivalent degree of neurogenesis in DS and control samples, but later passages had decreased neurogenesis in the DS samples ,which was ascribed to a defect in interneuron formation (Bhattacharyya et al., 2009). In addition to a defect in neurogenesis, another study found upregulation of glial progenitor markers in DS samples (Esposito et al., 2008), while a third study found no such

differences in glial cell proportions (Bahn et al., 2002). These discrepancies could arise from differences in source tissue, gestational age, the anatomic location from which the cells were derived, and *in vitro* culturing conditions.

Circumventing these difficulties and allowing for an infinite number of cells available for experimental perturbation, advances in ES and iPS neuron differentiation protocols have led to many published reports investigating the effects of DS on specific neural subtypes. Several studies that have used "default pathway" neuron differentiation to generate mostly excitatory forebrain neurons have seen no difference in the propensity to form neurons when comparing DS to control cell lines (Gonzales et al., 2018; Shi et al., 2012b; Weick et al., 2013), while one study did report decreased neurogenesis in DS cells (Lu et al., 2013). Additionally, while one report found no difference in either the number of synaptic punctae or the magnitude of synaptic currents (Shi et al., 2012b), another found both decreased synaptic punctae and decreased spontaneous postsynaptic currents (Weick et al., 2013), potentially due to the formation of more mature neural networks that also included GABAergic neurons. Differences in cell culture methodologies can also have significant impact on findings, as evidenced by the identification of shortened neurite length in DS cell lines grown for 120 days (Ovchinnikov et al., 2018), but no such finding in the same cell lines grown for only 60 days (Briggs et al., 2013). Other studies have focused on specific neuron subtypes that are suspected of being pathological in DS. For example, one study found defective *in vitro* and *in vivo* migratory capacity of DS GABAergic interneurons as well as a change in the proportions of interneuron subtypes generated (Huo et al., 2018).

In addition to potential deficits in stem cell derived neurons, *in vitro* studies have also investigated the astrogliogenic potential of DS cells. As described in a previous section, neural precursor cells undergo a gliogenic switch after most of neurogenesis is complete. Using primary neural stem cells from DS fetuses, it was reported that DS cells undergo a gliocentric shift that leads to increase glial cell production at the cost of neuronal production (Lu et al., 2011). Others have replicated this finding in iPSCs and have suggested that DS astroglia, potentially due to the extra copy of the chr21 gene, *S100B*, could negatively affect surrounding neurons through a secretory mechanism (Briggs et al., 2013; Chen et al., 2014). Other reports, in some cases using the same cell lines, have not seen a difference in astroglia production (Bahn et al., 2002; Ovchinnikov et al., 2018), suggesting that this finding may again be highly dependent on protocol specifics. The field of iPSC disease modeling has made significant progress over the course of its short existence. However, several technical and biological challenges, including difficult differentiation procedures and many poorly defined sources of variability, present significant obstacles towards accurate and reproducible disease modeling.

Alzheimer's disease in Down syndrome

The discussion above has focused on the neurodevelopmental aspects of DS, yet as mentioned in the first section of this introduction, AD pathology is a nearly-universal finding in DS individuals. It is also perhaps the only DS neurological phenotype that has been unequivocally linked to a specific gene on chr21, *APP*. There are several interesting cases that solidify the role of *APP* in AD-DS, and which have led to the development of the amyloid hypothesis, a leading theory in non-DS AD. Notably,

several studies have identified multiple families with familial early-onset AD caused by duplications involving the *APP* gene (Cabrejo et al., 2006; Kasuga et al., 2009; Rovelet-Lecrux et al., 2006, 2007; Sleegers et al., 2006), which, along with other lines of evidence, have solidified the role of *APP* in AD and, crucially, the importance of *APP* gene dosage in early-onset AD in DS. Additionally, two case studies of older DS individuals without any evidence of AD and only two copies of *APP* (due to partial trisomy for chromosome 21) further support the evidence that this single gene is sufficient for the AD phenotype in DS (Doran et al., 2017; Prasher et al., 1998). The evidence is clear that *APP* plays a central role in AD-DS pathogenesis, yet despite the near-universal prevalence of AD neuropathology in DS individuals above age 30, only ~80% of these individuals develop dementia (Mann and Esiri, 1989; Wisniewski et al., 1985a; Zigman et al., 1996). This raises the possibility that there may potentially be moderating factors on chr21 (Wiseman et al., 2015) in addition to well-known mitigators in the rest of the genome, like the *APOE* allele *ε2* (Corder et al., 1994).

AD dementia in DS has both similarities and unique features when compared to the general AD population. Particular impairment of recent memory with sparing of distant memory and confusion are present in both populations, while impairments generally associated with frontal lobe degeneration, such as apathy, depression, and communication decline, are present at an earlier stage of disease in the DS population (Deb et al., 2007). There is evidence that this discrepancy may arise from pre-existing frontal cortex hypoplasia in DS individuals (Holland et al., 1998, 2000; Rowe et al., 2006), which highlights the difficulty in assessing cognitive decline related to dementia in a population with pre-existing ID.

Many of the pathological changes seen in AD-DS brains mimic those seen in AD, including the pathognomonic Aβ42-containing extracellular plaques and hyperphosphorylated tau intracellular tangles (Goedert et al., 1992; Wisniewski et al., 1994). These lesions are present in many brain regions, and their number, particularly that of tau tangles, is correlated with the severity of dementia (Ropper and Williams, 1980; Wisniewski et al., 1985b). Notably, there is a degeneration of basal forebrain cholinergic neurons (BFCNs) in both AD-DS and AD individuals which is not present at birth in DS (Godridge et al., 1987; Kish et al., 1989), emphasizing the distinction between developmental defects caused by DS and neurodegeneration related to AD.

While mouse models of DS do not exhibit the classical lesions of plaques or tangles (Reeves et al., 1995), there is marked degeneration of BFCNs beginning from 6 months of age (Granholm et al., 2000; Holtzman et al., 1996), modeling the human syndrome. Human cellular models of AD-DS have also been developed, and have demonstrated both increased secretion of Aβ peptides in DS neural cultures as well as the reliance of this increase on trisomic *APP* gene dosage (Ovchinnikov et al., 2018; Shi et al., 2012b). Additionally, 3D human cellular models of AD have also managed to produce amyloid aggregates as well as tau inclusions, albeit in cells greatly overexpressing multiple mutant proteins (Choi et al., 2014; Raja et al., 2016). These systems provide useful models for drug testing in human neural cells and allow for modeling of early AD pathogenesis.

The pathology of AD in DS has been well described and its root cause in the *APP* gene is generally accepted. However, several recent clinical trials aimed at removing amyloid aggregation from AD patient brains have not succeeded in preventing

cognitive decline (Doody et al., 2014; Salloway et al., 2014). This suggests that either a new strategy must be undertaken and/or patients must be identified and started on treatment early in the disease course before symptoms arise (Mullard, 2016). To this end, DS individuals present a unique patient population at a high risk of developing AD that can be identified long before any symptom onset. Additionally, the AD aspect of DS is one that offers a much larger therapeutic window for intervention, particularly when compared to early neurodevelopmental defects, both for conventional therapeutics and potentially for gene/chromosomal therapy approaches.

Finally, it is interesting to note that the biological roles of *APP* are poorly understood, as much research has focused on its complex processing and role in AD. Since we and others find that *APP* is highly expressed in human iPSCs and throughout neurodevelopment, it is intriguing to consider that this dosage-sensitive gene could also play a role in neurodevelopment, and its potential impact in DS (van der Kant and Goldstein, 2015). While not detailed in this thesis, I have been involved in other ongoing work by others in the Lawrence lab examining the role of *APP* dosage in DS cells, including its impact on Aβ pathology and initial studies on neurogenesis and organoid formation.

Using dosage compensation to advance translational research in Down syndrome

Therapeutic approaches generally involve gaining a thorough understanding of disease mechanism through the use of cellular and/or animal models, followed by drug discovery aimed at modulating the disrupted pathways. This is a long and arduous process for even the simplest of genetic disorders, i.e. those known to be caused by

mutations in a single gene. While DS has the advantage of a well-established root cause, understanding the disease mechanism is complicated by the hundreds of potentially causative dosage sensitive genes on chr21. Thus, just like modern gene therapy aims to bypass the difficult task of gaining a complete understanding of disease pathogenesis by correcting the causative mutation, our lab has pioneered a strategy to harness the natural mechanism of dosage compensation to correct the root cause of DS in a cellular model. This provides an advantageous experimental approach to manipulate chr21 gene expression dosage in an otherwise identical population of cells. In addition, this innovative strategy may one day also provide the basis for a therapeutic approach to trisomy.

We demonstrated that targeted insertion of an *XIST* transgene into an intronic *DYRK1A* locus on one copy of chr21 is capable of robust chromosome-wide dosage correction of chr21 transcription in DS patient-derived iPSCs (Jiang et al., 2013). This provided strong evidence for robust chromosome-wide silencing of a human autosome by a targeted *XIST* transgene. In addition, this created a needed model to study the basic biology of human *XIST*. As an inducible non-lethal autosomal silencing system this can be utilized to study the timing and kinetics of the human chromosome silencing process. It can also be used to reveal any defects in the development of a wide variety of cell types derived from DS iPSCs by directly linking a given phenotype to the expression status of chr21. While this system has not yet been used for in-depth study of neurogenesis, our lab has provided initial evidence for an effect on the kinetics of neural rosette formation (Jiang et al., 2013). Additionally, we have demonstrated that transcriptional silencing of one chr21 beginning in iPSCs prevents development of the

hyperproliferative phenotype during hematopoietic differentiation *in vitro* (Chiang et al.,

2018). Despite this exciting progress, several questions in both the *XIST* and DS fields

remain unanswered.

In order to have any potential as a therapeutic for any aspect of the syndrome,

XIST must be able to initiate silencing in differentiated cells as DS can currently be

diagnosed only as early as 8 gestational weeks (Bianchi et al., 2012). Early *XIST*

literature suggests that the cell's capacity to initiate silencing is no longer present 48

hours after inducing ESC differentiation, which is many weeks before the diagnosis of

DS is even known. Evidence from mouse and human carcinoma cell lines suggested

that this initiation window might not be as narrow as previously described (Hall et al.,

2002a; Savarese et al., 2006), encouraging the studies in chapter II which examine the

ability of normal differentiated human cells (iPSC-derived NSCs and neurons) to initiate

chromosome silencing.

As described in the previous section, the cellular mechanisms behind ID in DS

are still poorly understood, and many findings from *in vitro* models are contradictory. We

believe that our isogenic and isoepigenetic cellular system, where the same cells in

either the trisomic or disomic state can be studied in parallel, provides a uniquely

powerful method for discerning the effects of trisomy on neurodevelopment. In the latter

half of chapter II, I will utilize the chromosome silencing system to study the effects of

trisomy on early neurogenesis. In chapter III, I will apply modern three-dimensional cell

culture techniques to gain an understanding of the effects of trisomy 21 on early

neurodevelopment. While we had initially hoped to combine organoid models with our

dosage compensation system, difficulties with transgene expression, as discussed in

the appendix, led us to focus our exploration of organoid models to isogenic comparisons of trisomic and disomic lines, which is the current standard in the field. Ultimately, this thesis aims to provide insight into the biology of dosage compensation and early DS neurodevelopment in order to advance translational research for DS, including the prospect of chromosome therapy (further considered in chapter V) and to one day improve the lives of patients with chromosomal abnormalities.

CHAPTER II : Silencing trisomy 21 with *XIST* in neural stem cells promotes neuronal differentiation

Preface

I performed all of the experiments described in this chapter, as well as all of the analysis, with helpful suggestions from Dr. Oliver King. This chapter was written by me and revised by me and Dr. Jeanne Lawrence. This work was submitted for publication and is currently in revision.

Jan T. Czerminski and Jeanne B. Lawrence. **Silencing Trisomy 21 with *XIST* in Neural Stem Cells Promotes Neuronal Differentiation**. (2019)

Introduction

Chromosomal abnormalities are surprisingly common – detected in about 0.6% of newborns (Shaffer and Lupski, 2000) – yet because they involve a dosage imbalance for many genes, this major component of the human genetic disease burden has remained largely outside the hopeful advances in genetics research. Research into the most common chromosomal disorder, Down Syndrome (DS), has received more attention in recent years, with most studies attempting to identify chromosome 21 genes that cause specific phenotypic features which involve various physiological systems. It has been difficult to establish which cell-types and pathways are impacted in Down

syndrome, hence better experimental strategies to determine how trisomy 21 impacts cell function and development are needed. As with any developmental disorder, it is particularly challenging to determine when a deficit arises, and when in development it may remain correctible. Here, we further develop and apply an approach using epigenetics to advance translational research for chromosomal imbalances.

Previously, our lab demonstrated that a natural epigenetic phenomenon could be harnessed to repress gene expression across one chromosome 21 in trisomic DS patient-derived iPS cells by targeted insertion of a single gene, *XIST* (Jiang et al., 2013). *XIST* is a long non-coding RNA that functions in *cis* to silence one X chromosome in female cells and dosage compensate X-linked genes between female (XX) and male (XY) cells. The expression and accumulation of *XIST* transcripts across the nuclear chromosome territory is essential to initiate chromosome silencing (Brown et al., 1992; Clemson et al., 1996; Lee and Jaenisch, 1997; Penny et al., 1996). Although there are differences between mouse and human X-inactivation, in both organisms *XIST* RNA initiates random X-chromosome silencing in pluripotent cells of the inner cell mass (van den Berg et al., 2009; Payer and Lee, 2008; Petropoulos et al., 2016b; Sahakyan et al., 2018). Given that epigenetic changes are especially rapid and widespread as naïve cells begin to form facultative heterochromatin, it is logical that this developmental context is optimal for *XIST* function. The chromosome silencing process requires not only *XIST* RNA, but multiple responses of the cell to this RNA, hence it is of fundamental interest to understand whether the epigenetic plasticity of cells in this special developmental window is required for *XIST* RNA to enact chromatin modifications which stably repress chromosome-wide transcription.

Based on prior studies in mouse ESCs, the initiation of *XIST*-mediated chromosome silencing has long been thought to be stringently limited to within 48-hours of ESC differentiation (Wutz and Jaenisch, 2000). Cells of a fibrosarcoma tumor line showed partial chromosome silencing, although this potentially reflects a more malleable epigenetic state in cancer cells (Chow et al., 2007; Hall et al., 2002a). Savarese et al. (2006) studied *Xist* transgenic mice in which chromosome silencing would create cell lethality and concluded that some mouse hematopoietic cell types were unusual in that they "transiently reestablish permissiveness for X inactivation". However, the authors mentioned that hematopoietic cells were known to avoid transgene silencing of the transactivator required for *Xist* expression in their doxycycline (dox) inducible system. Therefore, the silencing potential in other tissues in which *Xist* RNA might not have been expressed could not be assessed. Other studies suggest a role of SATB1 in the "pluripotency machinery" that supports chromosome silencing in mouse ES cells (Agrelo et al., 2009; Nechanitzky et al., 2012), but to our knowledge the capacity of normal human differentiated cells to respond to human *XIST* RNA has not been directly investigated.

The extent to which cells retain epigenetic plasticity beyond pluripotency is of fundamental interest for developmental biology, but whether or not *XIST* can induce chromosome silencing in later-stage somatic cells is also critical to the broader translational potential of this remarkable RNA. For this reason, the first priority of this study was to address this pivotal question, which we did using an inducible *XIST* transgene on one chromosome 21 in DS patient derived iPS cells, undergoing neural differentiation *in vitro*. Recently we demonstrated that *XIST* expression from one

chromosome 21, begun in pluripotent cells prior to differentiation, prevents development

of well-known DS hematopoietic cell pathologies *in vitro* (Chiang et al., 2018). While this

study did not attempt to induce post-differentiation chromosome silencing, the results

support inducible chromosome silencing as a valid way to identify differences between

trisomic and functionally euploid cells.

Unlike the well-established effects of trisomy 21 on hematopoietic cells, specific

cell pathologies that underlie other clinical phenotypes, including cognitive disabilities,

remain unclear (Haydar and Reeves, 2012; Mégarbané et al., 2009; Roper and Reeves,

2006). Some studies indicate trisomy 21 impacts post-natal neurodevelopment, such as

myelination (Olmos-Serrano et al., 2016) or cerebellar growth (Das et al., 2013), and it

is now recognized that there is often progressive cognitive decline in DS adults, as well

as nearly-ubiquitous early-onset Alzheimer Disease. Importantly, variable results have

been reported regarding the impact of trisomy 21 on early *in vitro* neural differentiation

(Bhattacharyya et al., 2009; Briggs et al., 2013; Gonzales et al., 2018; Jiang et al.,

2013; Lu et al., 2013; Shi et al., 2012b; Weick et al., 2013), with most studies reporting

no difference in the ability of DS stem cells to form neurons. However, studies have

mostly compared the differentiation capacity of separate iPS lines, which may be limited

by the fact that even iPSC lines from the same individual can demonstrate

transcriptional heterogeneity (Liang and Zhang, 2013; Soldner and Jaenisch, 2012) and

can show differences in their capacity for neural differentiation (Koyanagi-Aoi et al.,

2013).

Here, we first set out to test whether *XIST* can induce chromosomal silencing in

differentiated neural cells, which led us to examine kinetic differences in the epigenetic

steps of chromosome silencing in differentiated cells. Our second major goal was to utilize this tightly controlled inducible chromosomal silencing system to compare the trisomic and disomic states without variability between cell lines or samples, to investigate the effects of trisomic chromosome 21 expression on *in vitro* DS neurodevelopment.

Results

The panel of isogenic DS iPSCs studied includes multiple transgenic clones carrying a dox-inducible *XIST* cDNA on one chromosome 21 (Figure II-1A), which were created and characterized as previously described (Jiang et al., 2013; and Methods). Since prior analysis of chromosome 21 transcriptional repression was only done in undifferentiated pluripotent cells (Jiang et al., 2013), we first determined the extent of gene silencing in cells induced to express *XIST* RNA beginning in pluripotency just prior to inducing neural differentiation, the natural developmental context for optimal *XIST* RNA function. If *XIST* can produce neural cells with robust dosage compensation for chromosome 21, this itself would be valuable to investigate potential effects of trisomy 21 on neurogenesis. However, it would be even more advantageous if trisomy could be dosage corrected at later steps in cell development, which we tested by inducing *XIST* RNA later during *in vitro* neural differentiation.

XIST RNA induces heterochromatin hallmarks in differentiated somatic cells

To test whether *XIST*-mediated silencing is possible in differentiated cells, DS-patient derived iPSCs were differentiated using established protocols which mirror

cortical neurogenesis (Cao et al., 2017; Chambers et al., 2009), with dox added to

induce *XIST* RNA expression at different time points. These experiments used a 28-day

neural differentiation time-course, with dox introduced at either day 0, 14, or 21 (Figure

II-1B). By day 14 no OCT4+ cells are detected, while nearly all cells were SOX1+ and

SOX2+, indicating efficient neural differentiation with no remaining pluripotent cells

(Figure II-1C). By day 28 the cells are a mixture of SOX2+ neural stem cells (NSC) and

TUBB3+ post-mitotic neurons (Figure II-1D).

     XIST RNA functions by triggering multiple repressive chromatin modifications

that contribute to the silent state, such as polycomb protein repressive complexes PRC1

and PRC2, which induce canonical heterochromatin hallmarks H2AK119ub1 and

H3K27me3, respectively (Cao et al., 2002; Fang et al., 2004; de Napoles et al., 2004;

Plath et al., 2003). Cells with *XIST* induced from day 0 showed well-localized *XIST* RNA

paints associated consistently with these two major repressive histone modifications

(Figure II-2A, II-2B, and II-2D). This indicated that inducing *XIST* just before

differentiation could produce differentiated DS neural cells with one chromosome 21

silenced. In parallel samples we investigated whether *XIST* could trigger these

heterochromatin hallmarks if induced at day 14 or 21 of differentiation, well outside the

window of pluripotency. Most cells from each day of dox initiation accumulated

characteristic *XIST* RNA paints, indicating proper localization to the nuclear

chromosome territory. At later time points of differentiation there is some reduction in

the proportion of cells in which dox can induce *XIST* transcription (Figure II-2C), likely

reflecting transgene silencing, as commonly seen for the dox-inducible system during

differentiation (Gödecke et al., 2017). Importantly, over 90% of *XIST*+ cells in samples

Figure II-1: Experimental design and neural differentiation of DS iPSCs

A) Schematic of *XIST*-mediated chromosome 21 silencing system. B) Outline of neural differentiation protocol. Dox initiation days marked by red arrows. Analysis timepoints marked by blue arrows. C) IF at days 0 and 14 of differentiation for SOX2, OCT4, and SOX1. DAPI counterstain shown in blue. D) IF at day 28 of differentiation for SOX2 and TUBB3. Insets are single channel images. Scale bars are 50μm.
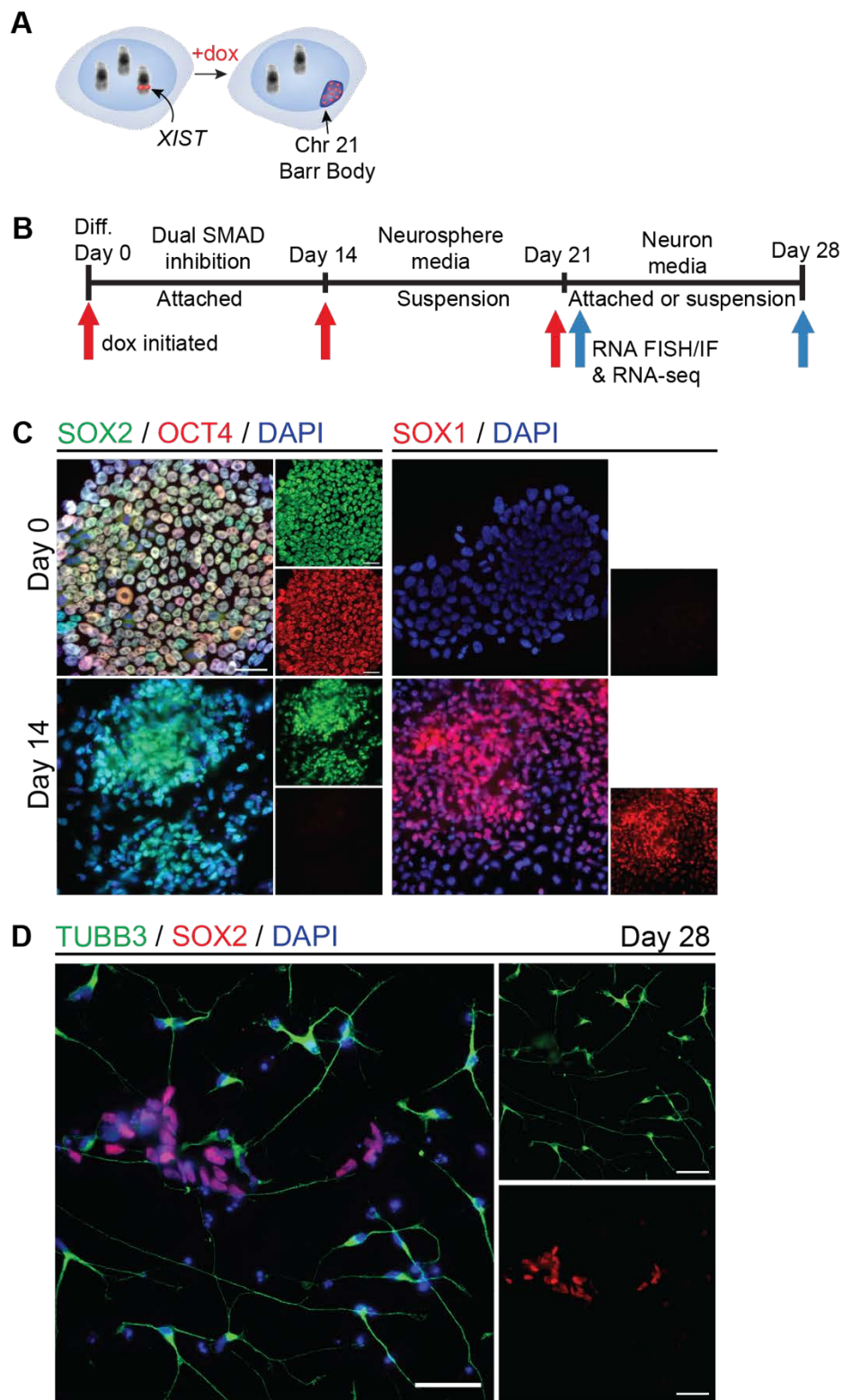
Figure II-1: Experimental design and neural differentiation of DS iPSCs

Figure II-2: Recruitment of heterochromatin hallmarks by *XIST* initiated in NSCs

A-B) Combined RNA FISH for XIST RNA and IF staining for H2AK119ub1 (A) and H3K27me3 (B) in transgenic cells at day 28 of differentiation. Arrows indicate associated signals, and arrowheads indicate *XIST* paint without associated H3K27me3 signal. Insets are magnified single channel images of outlined area. Schematics of experimental timelines illustrate dox addition (red) and analysis timepoints (blue). C) Quantification of *XIST*+ cells at day 28 of differentiation with different dox addition timepoints. D) Quantification of association of heterochromatin markers with *XIST*+ cells. Data are represented as mean ± SEM (n=3 differentiations). 309-926 cells were examined for each sample (median=556). *p = ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; one-way ANOVA followed by Tukey's multiple comparisons test. E) Combined RNA FISH for *XIST* with IF for H3K27me3 in day 21 sectioned neurospheres treated with dox beginning at day 0 or day 14. Micrographs are maximal intensity projections of 3D z-stacks. Insets are magnified single channel images of outlined area. Arrows point to H3K27me3 enrichment associated with *XIST* paint. Arrowheads point to *XIST* paint without associated H3K27me3 enrichment. Scale bars are 5μm.
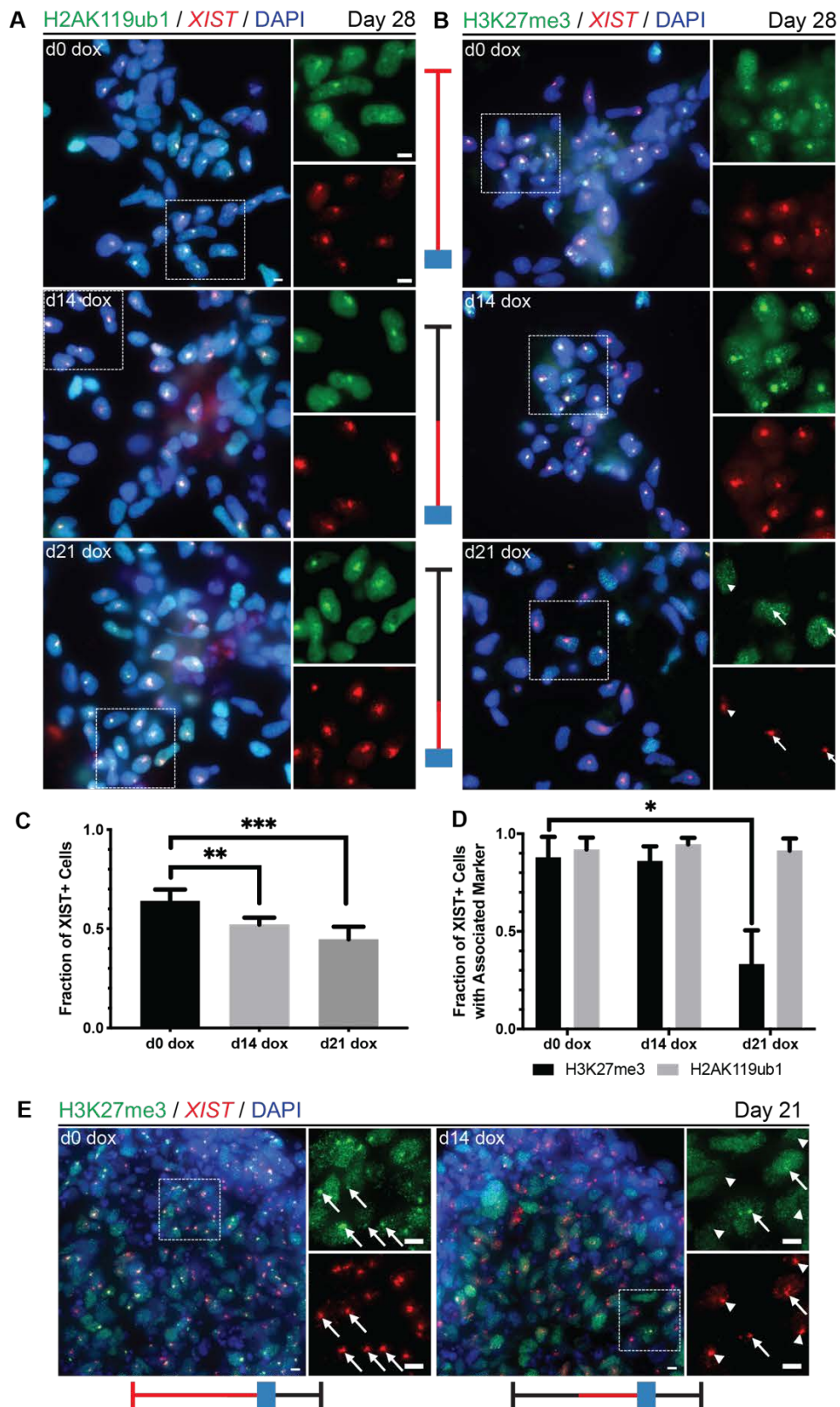
Figure II-2: Recruitment of heterochromatin hallmarks by *XIST* initiated in NSCs

treated with dox beginning at day 14 and day 21 clearly had H2AK119ub1 concentrated with the *XIST* signal (Figures II-2A and II-2D), indicating recruitment of this heterochromatin mark within 7 days. In cells induced at day 14, close to 90% of *XIST*+ cells had an associated H3K27me3 focus (Figures II-2B and II-2D), demonstrating that even in these differentiated cells *XIST* RNA can still recruit repressive modifications by two major polycomb complexes (PRC1 and PRC2).

Interestingly, when *XIST* RNA was induced at day 21, H3K27me3 was enriched in just 34% of *XIST*+ cells by day 28 (Figures II-2B and II-2D). Since the entire chromosome silencing process takes just a few days in pluripotent human iPSCs (Jiang et al., 2013) and mouse ES cells (Chaumeil et al., 2006; Wutz and Jaenisch, 2000), this indicated that by 21 days of differentiation many cells had either lost competence to efficiently trigger the H3K27me3 modification, or, alternatively, that the process may occur over a substantially longer timeframe in differentiated cells. To determine if H3K27me3 enrichment may require more than 7 days of *XIST* expression, we analyzed sectioned neurospheres at day 21 which had been treated with dox starting on day 14. In neurospheres expressing *XIST* for just a week, few cells had H3K27me3 enriched at the *XIST* RNA locus (Figure II-2E) in contrast to the robust enrichment of this hallmark in the same condition analyzed after two weeks at day 28 (Figure II-2B and II-2D). This observation suggested that the multi-step process of heterochromatin formation is more prolonged in differentiated cells. Most importantly, these findings provide the first indication that *XIST* can function to induce at least some heterochromatin modifications in differentiated neural cells.

XIST induces chromosome 21 transcriptional silencing in neural cells

Next, we used RNA sequencing (RNA-seq) to quantify the extent of silencing across chromosome 21 in neural cultures differentiated for 21 or 28 days, with *XIST* induced either at day 0 of differentiation or at later stages. As a benchmark for full chromosome 21 dosage compensation, we included comparison of a parental (trisomic) line to an isogenic disomic subclone. First, we examined the effectiveness of silencing for chromosome 21 genes in neural cells induced for *XIST* at day 0. We saw a significant decrease in the fraction of chromosome 21 reads in samples treated with dox (Figure II-3B). Dox treatment of the parental line, which does not contain the *XIST* transgene, did not cause a significant shift of chromosome 21 transcript levels, affirming that this effect is mediated by *XIST* RNA. Other chromosomes were not similarly affected by *XIST* (Figure II-3A). The effect seen on chromosome 21 gene expression is limited by the fraction of cells that express *XIST* (~60%, Figure 2.2C), which may account for a smaller decrease in chromosome 21 transcript levels in dox treated transgenic cells compared to isogenic disomic cells (Figure II-3B).

Importantly, addition of dox at later stages of differentiation also leads to significant repression of transcripts from chromosome 21 genes (Figure II-3B). This effect is not driven by a few highly expressed genes. Instead, genes across the length of chromosome 21 are repressed (Figure II-3D). Although the repression seen is less than for cells expressing *XIST* from day 0, this provides direct evidence that *XIST* can initiate substantial transcriptional silencing in differentiated cells. Further examination of individual genes reveals various silencing patterns. For example, while *USP25* and *BACH1* demonstrate moderate to absent repression, respectively, with later dox

Figure II-3: Transcriptional chromosome 21 repression at all time points of *XIST* initiation

A) Bar graph of bulk RNA-seq data of mean log2 fold change at day 28 of differentiation for all detected genes on each chromosome for each 3 vs. 3 comparison. Data are represented as mean ± SEM. Note: Y chromosome data represents only 9 detected genes. B) Day 21 and day 28 bulk RNA-seq data. Fraction of normalized chromosome 21 reads over all reads for each sample. n=3 for each condition. Dashed line represents a 33% drop in chr21 transcription from mean parental level. Samples colored in blue are trisomic; samples colored in red are disomic or functionally disomic (*XIST*+). C) Violin plots of scRNA-seq data at day 28 of differentiation showing median (horizontal line), interquartile range (rectangular box), 95% confidence interval, and the kernel probability density at each value. For each cell, the fraction of UMIs from chromosome 21 is divided by total UMIs to determine expression from chromosome 21. Number of cells in each sample is provided. D) Bulk RNA-seq fold change between indicated comparisons for all chromosome 21 genes with FPKM>1 and normalized average read count >10 plotted against ranked chromosomal position. Approximate chromosomal distance in megabases (Mb) relative to the *XIST* transgene locus is indicated on X-axis. Local average (LOESS) for each comparison is indicated by solid horizontal curves with 95% confidence intervals. Dotted horizontal line indicates 1.5-fold change. Significant differential expression (FDR<0.1) is indicated by larger dots. E) Dot plots of all samples for three selected chromosome 21 genes demonstrating various silencing kinetics. Dotted lines indicate each gene's position in (D). **p = ≤ 0.01, ***p ≤ 0.001; one-way ANOVA followed by Tukey's multiple comparisons test. Par, parental trisomic line; ParA, parental subclone A; Dis, disomic; C5A, Transgenic Clone 5a; nd, no dox; d0, day 0 dox initiation; d14, day 14 dox initiation; d21, day 21 dox initiation.

Figure II-3: Transcriptional chromosome 21 repression at all time points of *XIST* initiation
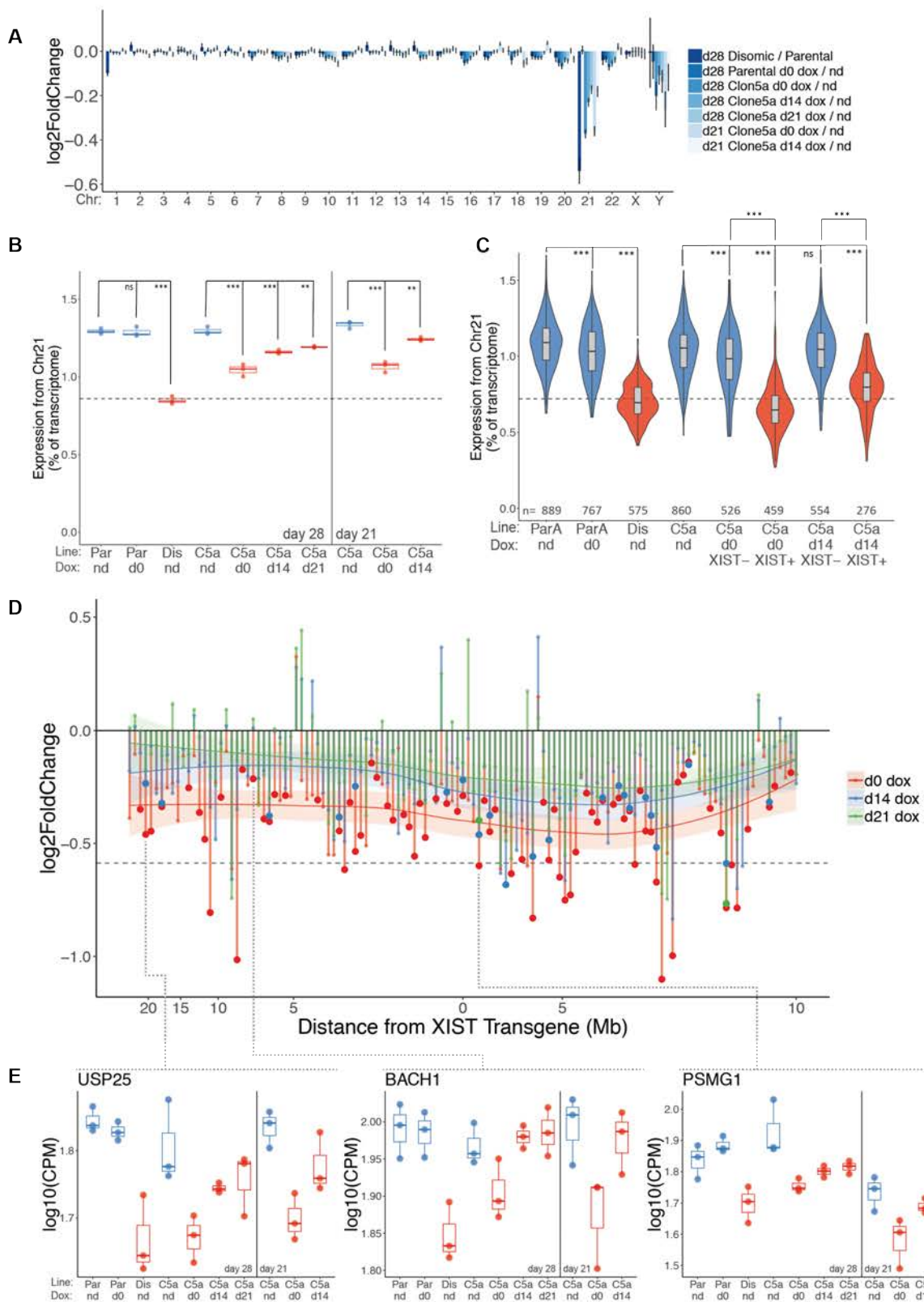
addition, *PSMG1* shows significant repression at all three dox addition timepoints (Figure II-3E). However, comparison of the degree of silencing is complicated by a lower fraction of cells expressing *XIST* at later dox addition timepoints (Figure II-2C), which will reduce overall silencing as measured by bulk RNA-seq. Therefore, to untangle the association between degree of silencing and proportion of cells expressing *XIST*, we performed single-cell RNA sequencing (scRNA-seq) on cells differentiated for 28 days to identify a cell population confirmed to express *XIST* RNA. This approach may miss some *XIST* expressing cells due to the relatively low number of unique RNA molecules (nUMI) identified per cell (median = 9,677). Using this technique, we find that by day 28, *XIST+* cells have decreased chromosome 21 expression by nearly one-third (31%) when *XIST* expression was initiated at the onset of differentiation, as compared to the same cells not treated with dox (Figure II-3C). This decrease in chromosome 21 expression would be expected for complete silencing of one of three copies of chromosome 21 and is nearly equivalent to the 32% decrease seen when comparing the disomic cell line to the trisomic. In contrast, the pool of single cells in which *XIST* RNA was not detected (*XIST-*) showed a much smaller (7%) reduction in chromosome 21 expression, reflecting a subset of false negative *XIST* expressing cells. As further illustrated below, using this approach to compare functionally trisomic to functionally disomic cells within the same population provides a powerful experimental approach to investigate how trisomy 21 impacts neural cells.

Next, we used scRNA-seq to examine the extent of transcriptional silencing in the *XIST+* population in cells with *XIST* induced at day 14, halfway into the 28-day differentiation time-course. In this case, *XIST+* cells at day 28 clearly showed

substantial repression of chromosome 21 mRNA levels, although this was incomplete compared to full silencing (55% of the reduction seen when dox is initiated at day 0). The less complete silencing may reflect a reduced developmental competence to respond to *XIST* with differentiation, or the shorter time-period that *XIST* was expressed (4 weeks versus 2 weeks). Slightly more silencing at day 28 compared to day 21 in cells treated with dox at day 14 (Figure II-3B) suggested that increased duration of *XIST* expression may allow for more chromosomal silencing. Nonetheless, induction of *XIST* both in iPSCs and 14 or 21 days into neural differentiation leads to a neural cell population with extensive transcriptional repression of chromosome 21 genes, providing the first demonstration that normal differentiated cells have substantial competence to respond to *XIST* to repress transcription.

XIST-mediated gene silencing in differentiated cells is a prolonged process

The reduced silencing seen at day 28 of differentiation in cells treated with dox starting on day 14 could reflect a chromosome-wide reduction in silencing or differences in the extent of silencing for individual genes. To investigate this, we compared the fold change of individual chromosome 21 genes (in *XIST*+ versus *XIST*- cells) for samples induced at day 0 and day 14. Many genes, such as *CSTB*, demonstrate an equivalent degree of silencing in both conditions (Figure II-4A-B), whereas several genes were substantially less repressed when *XIST* was induced later, including genes with high rates of detection/expression. This indicates there are late silencing genes, which we find are not correlated with gene distance from the *XIST* transgene locus (Figure II-4A).

Interestingly, *APP* stands out as having a particularly large difference in degree of silencing between the two conditions (Figure II-4A-B). To test whether increased

silencing of *APP* would be seen if the time-frame of *XIST* expression was extended from two to three weeks, we used RNA FISH to examine *APP* gene silencing at 28 days and 35 days of differentiation. As shown previously (Jiang et al., 2013; Xing et al., 1993), nuclear RNA FISH provides direct visualization of RNA transcription foci associated with each active *APP* allele, allowing for assessment of transcriptional silencing independent of any differences due to mRNA half-life. Silencing of *APP* transcription foci is essentially complete when *XIST* is initiated at the onset of differentiation and examined at either day 28 or day 35 of differentiation (Figure II-4C-D). In contrast, when *XIST* is initiated at day 14, the extent of *APP* silencing increases markedly at day 35 compared to day 28. Hence, when *XIST* expression is initiated later in differentiated neural cells the silencing process for some genes is still ongoing at day 28 and repression of this especially late-silencing gene is more complete 7 days later.

The longer timeframe required for chromosome silencing in more differentiated cells would not be anticipated from the rapid 3-4 day process that occurs when *XIST* expression is initiated in pluripotent cells. This shorter timeframe is well established in mouse ES cells (Chaumeil et al., 2006; Wutz and Jaenisch, 2000) and was seen for chromosome 21 silencing in pluripotent cells, including for the *APP* gene (Jiang et al., 2013; Valledor et al., in preparation). The prolonged kinetics of the process in differentiated cells could explain why gene silencing was not observed after *XIST* was expressed for a shorter period in differentiated mouse ES cells (Wutz and Jaenisch, 2000). We have preliminary evidence of chromosome silencing in post-differentiation
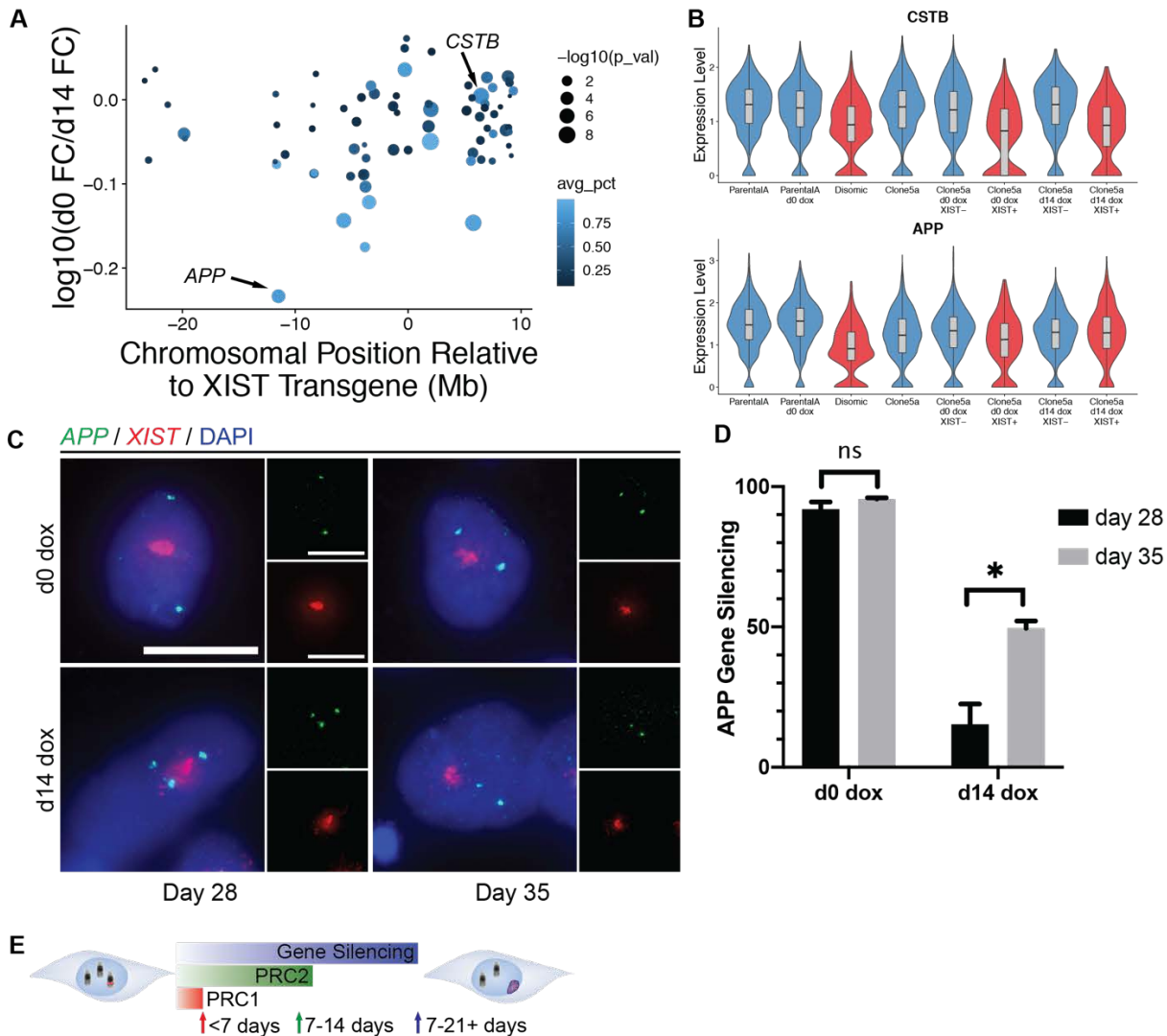
Figure II-4: *XIST*-mediated transcriptional silencing and heterochromatin recruitment are prolonged processes in differentiated cells

A) Difference in fold-change between d0 and d14 dox conditions plotted against chromosomal location relative to the *XIST* transgene locus. Each dot represents a gene; size of each dot is -log10(p-value). Color denotes the fraction of cells in which each gene is detected. Only genes demonstrating some degree of repression (>0.1 log10 FC) are plotted. B) Violin plots of expression level for *CSTB* and *APP* in each sample sequenced by sc-RNAseq. C) Representative images of RNA FISH for *APP* and *XIST* in cells differentiated for 28 and 35 days with dox initiated either at day 0 or day 14. Micrographs are maximal intensity projections of 3D z-stacks. Scale bars are 10µm. Insets are single channel images. D) Quantification of silencing of transcription foci in (C) for the *APP* gene as described in methods. Data are represented as mean ± SEM, n=2, with 293-473 cells scored per sample (median = 400) *p = ≤ 0.05; unpaired Student's t-test. E) Schematic indicating temporal order of steps in chromosome 21 silencing process as seen in differentiated cells.

cells using the same mouse transgenic cells induced to express *XIST* for 1-3 weeks, although analysis is complicated by monosomy-induced lethality (data not shown).

In sum, these findings reveal that initiation of silencing by *XIST* is not limited to the early developmental context of pluripotent stem cells. Differentiated cells require 2-3 weeks to complete various steps of this multifaceted process (Figure II-4E), but, most critically, chromosome-wide gene silencing still occurs when *XIST* is initiated in differentiated cells.

XIST produces dosage corrected trisomic neurons

The cell cultures analyzed above demonstrate that a mixed population of neurons and NSCs is capable of initiating *XIST*-mediated silencing at various stages of differentiation. Additionally, scRNA-seq analysis of chromosome 21 expression affirmed that this system can readily produce *XIST+* neurons with one silenced chromosome 21. We next attempted to initiate *XIST* transcription in a pure post-mitotic neuronal population by synchronously inducing differentiation with compound E, a γ-secretase inhibitor which rapidly induces all NSCs to differentiate into neurons (Figure II-5A) (Ogura et al., 2013). As expected, post-mitotic neurons generated in this way from cells treated with dox prior to differentiation had robust *XIST* expression (Figure 2.5B) associated with heterochromatin marks (Figure II-5C). However, if dox was added after differentiation into neurons, cells did not even show any transcription focus for *XIST* RNA (Figure II-5B). These findings suggested that neurons can continually maintain *XIST* RNA expression when induced prior to their terminal differentiation, but transgene activation is prevented in post-mitotic neurons.

To further investigate this phenomenon, we examined forebrain organoids grown for 50 days. These organoids contain clearly demarcated "ventricular-like" zones (VZ) containing NSCs which give rise to the surrounding differentiated neurons (Figure II-5D), thus providing a clear delineation of NSCs and post-mitotic neuron-containing regions. We induced organoids with dox at day 48 for just two days prior to examination, which minimizes the number of NSCs that will have differentiated into neurons after *XIST* induction. In this case, nearly all *XIST*+ cells were in the VZs rather than the surrounding neurons (Figure II-5E). In contrast, in organoids treated with dox beginning at day 22 of differentiation, many *XIST*+ cells are evident in both the VZ and the surrounding neurons (Figure II-5E), demonstrating robust *XIST* expression in neurons and NSCs in trisomy 21 cerebral organoids. Together, results demonstrate that post-mitotic neurons can continually express and localize *XIST* RNA and maintain comprehensive chromosome 21 silencing, even though the dox-inducible transgene needs to be activated prior to terminal differentiation, likely due to DNA methylation of the tetracycline response element with differentiation (Gödecke et al., 2017).

To our knowledge it has not been previously demonstrated whether *XIST* RNA requires S-phase to initiate recruitment of heterochromatin marks. To examine this, we induced *XIST* transcription concurrently with labelling of replicating DNA by BrdU incorporation, in both iPSCs and forebrain organoids. In both paradigms, there were cells that had not undergone DNA replication (BrdU-) but were *XIST*+, with a well-localized *XIST* RNA paint associated with a clear recruitment of the heterochromatin hallmark H2AK119ub1 (Figure II-5F), seen as early as eight hours in forebrain organoid cells. Hence, *XIST* RNA does not strictly require S-phase to initiate the chromosome

Figure II-5: Neurons support continued *XIST* expression to maintain silent chromatin provided the transgene is activated in the NSC stage

A) Representative IF images for SOX2 and TUBB3 of cells at day 28 of differentiation with and without compound E treatment at day 21 of differentiation. Scale bars are 50µm. B) RNA FISH for *XIST* in day 35 differentiated cells treated with compound E at day 21 and induced with dox at day 0 or 28. DAPI counterstain in blue. Scale bars are 5µm. C) Combined RNA FISH for *XIST* and IF for H3K27me3 and TUBB3 in day 35 differentiated cells treated with compound E at day 21. Insets are magnified single channel images of outlined area. Scale bars are 10µm. Arrows point to *XIST* RNA paints. D) IF of day 50 sectioned forebrain organoid for NeuN and SOX2. Dashed white line delineates NSC-containing SOX2+ VZ-like area and neuron containing NeuN+ area. Scale bars are 50µm. E) Combined RNA FISH for *XIST* and IF for NeuN in transgenic forebrain organoids. Micrographs are maximal intensity projections of 3D z-stacks. Scale bars are 50µm. F) Combined RNA FISH for *XIST* and IF for H2AK119ub1 and BrdU with concurrent addition of dox and BrdU in both iPSCs and day 50 organoids. Insets for the iPSCs are single channel images; insets for organoid are magnified single channel images of the outlined area. Arrows point to *XIST* RNA paints. Scale bars are 5µm.
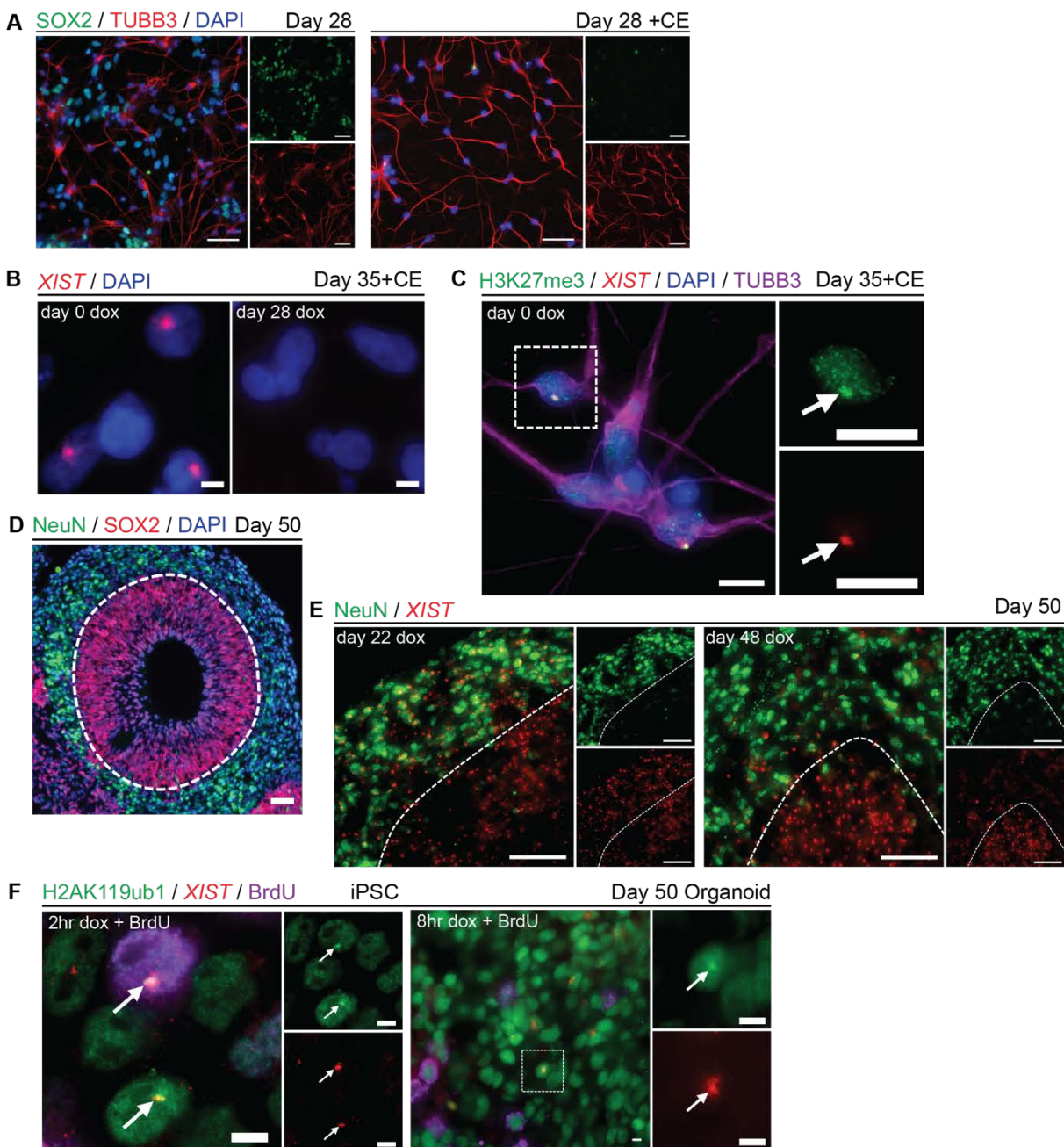
Figure II-5: Neurons support continued *XIST* expression to maintain silent chromatin provided the transgene is activated in the NSC stage

remodeling process, suggesting that the lack of cell-cycling in post-mitotic neurons does not preclude *XIST* RNA expression or function.

Together, these results demonstrate that this system can produce *XIST*-expressing dosage compensated neurons, despite the inability to initiate *XIST* expression with dox in neurons. Additionally, the S-phase of the cell cycle is not required for *XIST* expression and early recruitment of heterochromatin hallmarks.

XIST RNA enhances neuron formation indicating correction of a neurodevelopmental deficit

scRNA-seq was instrumental to quantify chromosome 21 gene silencing in differentiated neurons and NSCs, however this approach may also be advantageous to determine if trisomy 21 expression impacts neural differentiation, because it compares cells within the same sample sorted for *XIST* expression. This strategy minimizes sources of variability unrelated to trisomy and intrinsic to most other studies using patient-derived iPSCs (Liang and Zhang, 2013; Soldner and Jaenisch, 2012). Therefore, we examined the single-cell data to address whether *XIST*+ cells were distinct from *XIST*- cells beyond the difference in chromosome 21 expression.

As described above, after 28 days of differentiation cultures were histologically determined to be a mixture of NSCs and neurons. The scRNA-seq data confirmed the presence of two major cell-type clusters that we classified as NSC and neuron based on cell-specific markers such as SRY-box 2 (*SOX2*), Vimentin (*VIM*), Doublecortin (*DCX*), and Tau (*MAPT*) (Figure II-6A-B). Further affirming this classification, the NSC cluster contained about 40% of cells in G2/M and S-phase (based on cell-cycle specific gene

expression), whereas the neuron cluster contained only a small fraction (2%) of cells predicted to be cycling (Figure II-6C), as expected for post-mitotic neurons.

Next, we analyzed the proportion of *XIST*+ cells that were in the neuron cluster compared to *XIST*- cells in the same sample. Surprisingly, this revealed that *XIST*+ cells with dox initiated at day 0 were significantly more likely to be neurons compared to *XIST*- cells (Figure II-6D). This is despite the findings described above that neurons do not initiate *XIST* expression in this dox-inducible system, and evidence that the tetracycline transactivator (*TET3G*; required for *XIST* expression) transgene is more highly expressed in the NSC cluster, as shown by a dox-treated trisomic control line that contains just the *TET3G* transgene (data not shown). These results show that DS cells in which one chromosome 21 was silenced by *XIST* expression produced a higher proportion of neurons relative to NSCs. Given the potential importance for understanding DS neuropathology, this observation merited further investigation.

A difference in the rate with which trisomic NSCs transition to neurons could have its root at any point during the 28-day differentiation time course. The inducible *XIST* system makes it possible to investigate the onset of this defect by examining cells induced for *XIST* later during *in vitro* differentiation. We examined whether this phenotype could be reproduced by initiating *XIST* expression 14 days into differentiation. Remarkably, as shown in Figure II-6D, even when *XIST* expression was not initiated until midway through the time-course, *XIST*+ cells were still significantly more likely to form neurons, and to the same degree as cells induced for *XIST* at day 0. This indicates the developmental step which underlies reduced neuron formation occurs after 14 days of differentiation.

These results strongly suggest that trisomy 21 in DS is associated with some dysregulation in neurogenesis that delays and/or reduces neuron formation. In this study, we included one trisomic and one disomic cell line primarily as a benchmark for full chromosome 21 gene silencing, however we noted that no difference was seen in the proportion of neurons produced between these two isogenic lines. To rule out any possibility of this finding resulting from technical limitations of scRNA-seq or in analysis of one transgenic line, we tested the production of neurons in cultures of multiple transgenic lines using a different single-cell approach which combines RNA FISH for *XIST* RNA with immunofluorescence (IF) for SOX2 and TUBB3 (Figure II-6E). This was done in three independent transgenic clones each differentiated independently 1-2 times, with dox added at both day 0 and day 14. In accordance with sequencing results, *XIST*+ cells were again more likely to become neurons compared to *XIST*- cells (Figure II-6F). This was consistent for all three lines and, importantly, occurred to similar degrees at both dox addition timepoints.

Using this tightly controlled inducible system in multiple transgenic lines analyzed by two different approaches, we find strong evidence that trisomy 21 expression significantly impedes terminal differentiation of NSCs to neurons. This occurs after early formation of NSCs but prior to terminal differentiation of neurons. Together, the facts that we are comparing functionally disomic and trisomic cells within the same sample, that we are analyzing a difference in the proportion of neurons to NSCs rather than total neuron number, and that this proportion is altered even when *XIST* is initiated half-way into the differentiation protocol, all suggest that neuron formation is enhanced in *XIST*+ cells independent of an effect on cell density or proliferation. Implicit in this analysis is
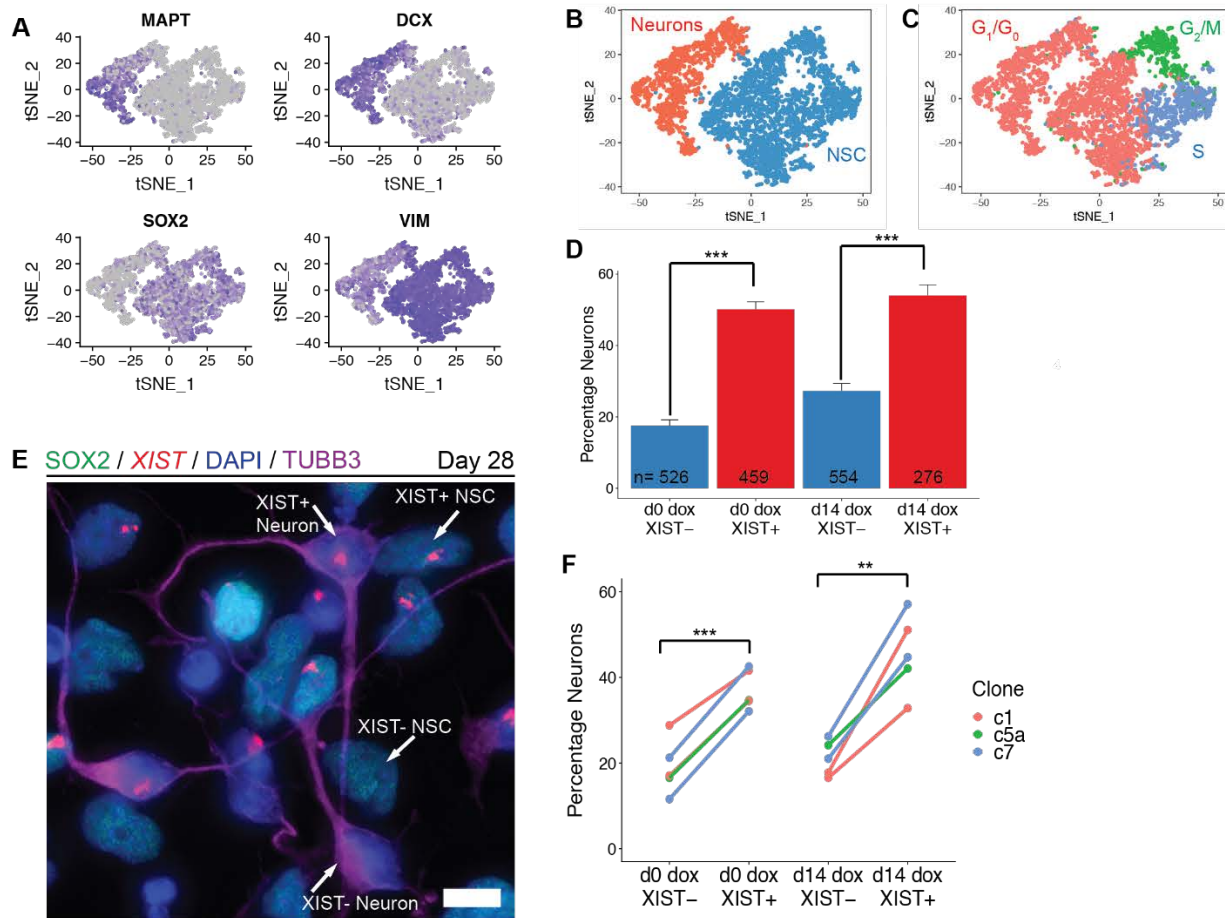
Figure II-6: *XIST* expressing cells are more likely to be neurons than cells that do not express *XIST*

A) t-distributed stochastic neighbor embedding (t-SNE) plot of day 28 scRNA-seq data for neuron- (*MAPT* and *DCX*) and NSC- (*SOX2* and *VIM*) specific genes. Gray dots represent low and purple represent high expression levels. Each dot represents a single cell. B) t-SNE plot colored for cell type classification. C) t-SNE plot colored for predicted phase of cell cycle. D) Fraction of cells in Clone5a d0 and d14 dox scRNA-seq samples identified as neurons separated based on *XIST* expression. Error bars are SE. E) Combined RNA FISH for *XIST* with IF for SOX2 and TUBB3 in day 28 cells. Example *XIST*+/-;NSC/Neuron cells are labeled. Micrograph is a maximal intensity projection of a 3D z-stack. Scale bars are 10μm. F) Quantification of (E) for 1-2 differentiations of three transgenic clones. Lines connect data points derived from the same sample. Between 419-1311 cells were analyzed for each sample (median = 868). **p = ≤ 0.01, ***p ≤ 0.001; Student's paired T-test.

that this specific neural phenotype can be rescued by expression of *XIST* RNA from one copy of chromosome 21.

Non-chromosome 21 differential expression identifies altered Notch pathway genes

Several studies have examined the transcriptomes of trisomic versus euploid (disomic) individuals in fetal or adult human brain samples (Lockstone et al., 2007a; Olmos-Serrano et al., 2016), yet transcriptome differences will be confounded by differences in cell-type representation, as well as due to variation between non-isogenic samples or sample processing. While not our primary goal, our scRNA-seq analysis could reveal in a specific cell population (neuron or NSCs) whether non-chromosome 21 genes are impacted, directly or indirectly, by chromosome 21 dosage. Due to limitations in sensitivity, the scRNA-seq data would not necessarily identify changes in weakly or variably expressed genes. Nonetheless, changes genuinely due to chromosome 21 dosage could be revealed with higher-confidence, because this approach compares *XIST*+ and *XIST*- cells within the same sample, with each cell serving as a biological replicate of the disomic and trisomic state.

Indeed, this analysis identifies a small number of genes which change expression in NSCs and/or neurons as a function of chromosome 21 silencing (Figure II-7A). Notably, when PANTHER Pathway analysis (Mi and Thomas, 2009; Mi et al., 2017) is conducted on the total of 9 unique non-chr21 genes that differentially expressed, "Notch signaling pathway" (accession: P00045) is the only significantly dysregulated pathway (FDR=0.0274). Additionally, the gene Tweety Family Member 1 (*TTYH1*), recently implicated in Notch signaling (Kim et al., 2018) but not included in the
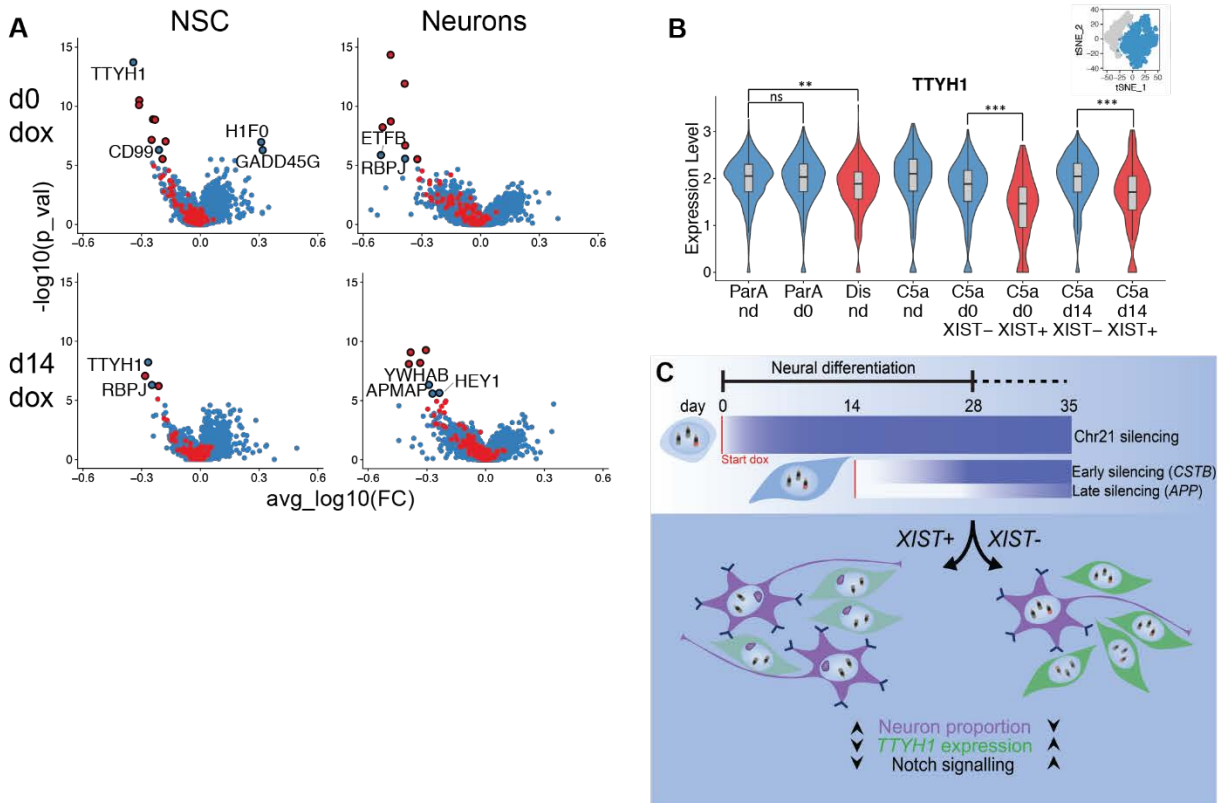
Figure II-7: Non-chromosome 21 differential expression identifies altered Notch pathway genes

A) Volcano plot of results from Wilcoxon rank-sum test between *XIST+* and *XIST-* cells in NSC and neuron clusters for d0 and d14 dox samples examined at day 28 in scRNA-seq dataset. Chromosome 21 genes are in red, and all other genes are in blue. Circled genes are significantly differentially expressed (p-adj<0.05). Non-chr21 DE genes are labeled. "Dox effect" genes found to be DE between ParA nd and parA d0 dox samples and transgenes were removed from the plots. B) Expression levels of *TTYH1* in NSCs of all samples sequenced. ParA, parental subclone A; Dis, disomic; C5A, Transgenic Clone 5a; nd, no dox; d0, day 0 dox initiation; d14, day 14 dox initiation. C) Schematic of experimental design and major results. Chromosome silencing is prolonged in differentiated cells and has variable kinetics between genes. *XIST+* cells in both d0 and d14 dox conditions have increased neuron proportions, decreased TTYH1 expression in NSCs, and increased Notch pathway gene expression compared to *XIST-* cells.

Notch pathway annotation, is also differentially expressed in NSCs expressing *XIST*. A similar more modest difference is seen between the trisomic and disomic cell lines (Figure II-7B), and no difference is seen between TET+ and TET- NSCs in the trisomic parental subclone treated with dox (data not shown). As summarized in Figure II-7C, the effects on neuron formation and non-chromosome 21 gene expression occur similarly whether one chromosome 21 is silenced from the beginning or far into the differentiation process, providing insight into the developmental timing of these effects. To our knowledge, this is the first data able to identify genes that likely reflect ongoing functional effects of trisomy 21 on expression of non-chromosome 21 genes in a given neural cell type.

Discussion

The results presented here have broad implications for basic developmental biology, DS neurobiology, and for potential translational applications of a unique non-coding RNA, *XIST*. Results with scRNA-seq further demonstrate the singular capacity of *XIST* to dosage compensate duplicated autosomal chromatin, allowing production of trisomy 21 neurons with a euploid transcriptomic profile. While this alone is valuable, this study addresses a critical question regarding the epigenetic plasticity of cells to respond to *XIST*, with encouraging results which heighten the experimental power and therapeutic prospects of *XIST*. These findings demonstrate for the first time in normal human cells that *XIST* can initiate chromosome silencing well beyond the "critical window" of pluripotency. Our findings demonstrate that the chromosome silencing process still occurs in differentiated cells, but is more prolonged, which could explain earlier findings suggesting a strict developmental window for initiation of chromosome

silencing. Additionally, single cell analyses of otherwise identical cells, with and without *XIST*-mediated dosage compensation, provide strong evidence that over-expression of chromosome 21 genes confers a developmental delay in the transition of NSC to neurons. Importantly, this change in the neuron versus NSC balance was consistently and equivalently corrected whether *XIST* expression was initiated at the onset or midway through the differentiation time-course. These findings demonstrate the value of this approach to investigate the developmental biology of DS. Moreover, these results overcome a perceived barrier to developing *XIST* as a potential therapeutic for DS and other trisomies, and further support the effectiveness of chromosome silencing to mitigate cell phenotypic effects of trisomy.

Our results provide new insights into basic developmental biology by revealing the unanticipated epigenetic plasticity of more differentiated cells to enact a multi-layered chromosome remodeling process that normally occurs within the inner cell mass. Previously it was thought that the limited reports of *XIST*-induced heterochromatin in somatic cells reflected a peculiarity of some cancer cells or an unusual regaining of competence specific to a subset of mouse hematopoietic cells. By thoroughly re-visiting this key point in normal human neural cells, our results bode well for the potential of *XIST* function in other cell-types. Using our 28-day time course to produce post-mitotic neurons, *XIST* expression begun at day 14 or 21 initiates chromosome-wide repression evident within just 1-2 weeks. Interestingly, some genes silence more slowly – and thus complete silencing requires over 3 weeks of *XIST* expression – as shown for the important *APP* gene, critical to the development of early-onset Alzheimer's disease in DS. Even when *XIST* expression was induced in cells of a

48-day old organoid, early heterochromatin hallmarks became apparent on the chromosome. Given that *XIST* triggers multi-layered repressive chromatin modifications, the highly-redundant process may still provide transcriptional repression even if certain chromatin modifying enzymes are absent or expressed at low levels in differentiated cells.

A complication we encountered using ectopic promoters to drive *XIST* expression is the common phenomenon of transgene silencing with differentiation (Gödecke et al., 2017; Huebsch et al., 2016; Laker et al., 1998; Oyer et al., 2009; Xia et al., 2007). In our system this could be occurring at the tetracycline transactivator locus on chr19 and/or the tetracycline response element/*XIST* locus on chr21, as discussed in detail in the appendix. This issue could have also influenced results of a previous study in which mice were fed dox to induce an *XIST* transgene on a disomic autosome, with cell/tissue lethality serving as the read-out for *XIST* function (Savarese et al., 2006). Our results clearly show that neurons maintain *XIST* expression and remarkably complete chromosome 21 silencing, yet the dox-inducible system was blocked in neurons unless it was already activated in NSCs prior to terminal differentiation. Additionally, there is precedent in the literature that dramatic epigenetic changes occur in post-mitotic neurons, such as activity-mediated neuronal plasticity achieved by epigenetic mechanisms (Ma et al., 2009), or the conversion of post-mitotic neurons from one distinct subtype to another by exogenous factors (Rouaux and Arlotta, 2013; Ye et al., 2015).

scRNA-seq not only affirmed chromosome 21 silencing in *XIST*-expressing neurons, but unexpectedly revealed that cells expressing *XIST* were more likely to

become neurons. The increased proportion of cells that transitioned from NSC to neurons was corroborated by a second approach and consistently seen for three *XIST* transgenic clones. We believe the ability to demonstrate a modest but reproducible developmental delay in neurogenesis *in vitro* rests on the inducible experimental approach that examines essentially identical cells in the same culture of the same subclone, with and without one chromosome 21 silenced, thereby circumventing other sources of variation even between isogenic iPSC clones (Koyanagi-Aoi et al., 2013; Liang and Zhang, 2013; Soldner and Jaenisch, 2012). For example, we can conclude that the increased rate of neuron formation in dosage-compensated cells is not due to culture density, which is difficult to control (Jiang et al., 2013). Importantly, the ability to induce dosage correction at later stages also makes it possible to examine the timing and reversibility of a developmental defect. Enhanced neuron differentiation occurred equivalently whether *XIST*-mediated silencing was initiated at day 0 or 14 days into neural differentiation, indicating a defect that is correctible at either time point. Additionally, given that complete transcriptional silencing takes over two weeks after *XIST* is induced, this suggests that either especially early-silenced chr21 gene(s) are responsible for this phenotype, or the defect arises after day 21 and potentially close to the final division of NSC to form post-mitotic neuron.

While studies using DS iPS cells have described variable results with neural differentiation (see introduction), some studies have reported hypocellularity in small samples of DS fetal cortex (Guidi et al., 2008; Larsen et al., 2008; Ross et al., 1984), which has been attributed to decreased neuron number. Using a very different approach involving experimental manipulation of chromosome 21 expression, results here provide

direct evidence that trisomy 21 over-expression causes a neurodevelopmental delay in the transition of NSCs to neurons. Recently, we showed that trisomy 21 silencing (beginning in pluripotency) prevents development of hematopoietic defects of DS, reducing and normalizing the well-known over-production of megakaryocytes and erythrocytes (Chiang et al., 2018). The contrasting effects seen where *XIST* enhances neuron production are consistent with the different clinical impact of trisomy 21 on the hematopoietic and neural systems.

Results further indicate that this experimental strategy can provide insights into target pathways dysregulated by trisomy 21, which might identify targets amenable to conventional drug therapies. Of particular interest are the significant number of Notch pathway related genes that stand out as impacted when comparing trisomic and functionally disomic cells. The role of Notch signaling in neural differentiation is well described, and its inhibition using γ-secretase inhibitors induces rapid differentiation of NSCs to neurons, as illustrated in this study and others (Kawaguchi et al., 2008; Ogura et al., 2013). However, the role of Notch in DS neurodevelopment is less clearly understood. Some transcriptome studies of DS adult brain samples have reported upregulation of Notch signaling genes, and the potential role of specific chromosome 21 genes in Notch signaling has been of interest (Fernandez-Martinez et al., 2009; Fischer et al., 2005; Lockstone et al., 2007a). *TTYH1* and related pathway genes*, HEY1* and *RBPJ,* are increased in the trisomic state and strongly downregulated after chromosome 21 silencing. Of particular interest was a recent report showing that *TTYH1* impacts the Notch pathway to promote maintenance of the NSC state (Kim et al., 2018), which could explain our finding of a higher fraction of NSCs in uncorrected trisomic cells.

Additionally, the role of Notch signaling in astrogliogenesis is well described (Louvi and Artavanis-Tsakonas, 2006), and aberrations in astrocyte number have been reported in DS brains and *in vitro* models (Chen et al., 2014; Colombo et al., 2005; Mito and Becker, 1993).

Finally, the fundamental finding here that differentiated cells retain substantial epigenetic plasticity is encouraging for the forward-looking prospect that *XIST* or derived sequences could be developed as a therapeutic strategy for aspects of DS and potentially a diversity of smaller duplication disorders only now being described (Theisen and Shaffer, 2010). Although our results implicate a specific cell transition during neural development, potentially due to mis-regulation of the Notch pathway, with over 200 genes on chromosome 21 it is likely that other steps in earlier neuroepithelial differentiation (Jiang et al., 2013) or later neuron function could also be impacted by a myriad of mechanisms. In addition, recent studies indicate that aneuploidy in general may cause proteomic cell stress due to low-level over-expression of many genes (Bonney et al., 2015; Sheltzer et al., 2012). Therefore, with enormous advances in genome editing and delivery technologies, we continue to advance the prospect that a single-gene could target the root cause for a complex chromosomal disorder to potentially mitigate effects of trisomy 21 in several physiologic systems. We have demonstrated that *XIST*-mediated silencing can correct known DS cell pathologies *in vitro* (Chiang et al., 2018), and now show that epigenetic correction may be possible long into development for the neural cell lineage and potentially other cell systems. Clearly, many challenges remain, including the need for a smaller *XIST* transgene amenable to current delivery methods and the fact that neurogenesis is largely

complete prenatally. However, several major aspects of neural development, such as myelination and synaptic pruning, continue long after birth (Silbereis et al., 2016), as does the development Alzheimer dementia in most DS individuals (Wiseman et al., 2015).

Great efforts from many investigators have led to breakthroughs in understanding dosage compensation of the X-chromosome, yet the translational relevance to common chromosomal dosage disorders of this basic epigenetic mechanism is only beginning to be explored.

Acknowledgements

Materials and Methods

iPS cell culture and neural differentiation

The isogenic XIST-transgenic and disomic subclones were derived and characterized as described in Jiang et al. (2013). The initial DS iPSC parental line (DS1-iPS4) was provided by G.Q. Daley (Park et al., 2008). Clone5a is a subclone of the previously characterized clone5 (Jiang et al., 2013) which was altered for this study to include a second copy of the tetracycline transactivator driven by the CAG promoter in the AAVS1 locus (Addgene plasmid #60431) in an attempt to minimize transgene silencing with differentiation. All clones except the original parental line contain the TET3G transgene in the AAVS1 locus. iPSCs were maintained on vitronectin-coated plates with Essential 8 medium (ThermoFisher) and tested periodically for mycoplasma. Cells were passaged every 3-4 days with 0.5mM EDTA.

Neural differentiations were performed as previously described (Cao et al., 2017; Chambers et al., 2009) with some modifications. Briefly, iPSCs were dissociated into single cells and plated at a density of 50,000 cells/well in a vitronectin-coated 24-well plate with 10µM of the ROCK inhibitor Y-27632 (Tocris Bioscience). The next day, media was changed to Neural differentiation media (NDM) consisting of 50% DMEM/F12, 50% Neurobasal, 0.5X Glutamax, 1X N-2 supplement, 1X penicillin/streptomycin (all from ThermoFisher), and supplemented with 2uM DMH1 and SB431542 (both from Tocris Bioscience). After 14 days, cells were broken into clumps after EDTA treatment and cultured in suspension for 7 days in NDM. On day 21 or 28 of differentiation, neurospheres were dissociated into single cells with StemPro Accutase (ThermoFisher) and plated onto coverslips (Electron Microscopy Sciences) coated with

Matrigel (Corning) at a density of 25,000-50,000 cells/coverslip and fed every 2-3 days

with Neuron media consisting of Neurobasal, 1X N-2, 0.5X B-27 without vitamin A, 1X

penicillin/streptomycin, 1X Glutamax (ThermoFisher), 0.3% Glucose, 10ng/ml GDNF

(Peprotech), 10ng/ml BDNF (Peprotech), 10ng/ml ascorbic acid (Sigma-Aldrich), and

1µM cyclic AMP (Sigma-Aldrich). Doxycycline diluted in distilled water was added to the

culture media starting at various time points at a concentration of 500ng/ml. In cultures

where NSCs were synchronously differentiated to neurons, compound E (EMD

Millipore) was added for 3 days at day 21 of differentiation at a concentration of 200nM.

Forebrain organoids were generated as previously described (Qian et al., 2016,

2018) with the following modifications: embryoid bodies were formed by dissociated of

iPSCs into single cells and re-aggregating in U-bottom 96-well plates (Lancaster and

Knoblich, 2014). On day 7, aggregates were transferred to ultra-low attachment 6-well

plates (Corning) for Matrigel embedding, and on day 14 the plates were moved to an

orbital shaker set at ~100rpm.

Cell fixation, RNA FISH, and immunofluorescence

For iPSC and monolayer neural culture, cell fixation with 4% paraformaldehyde

(PFA) was performed as previously described (Byron et al., 2013). Forebrain organoids

were fixed for 30min in PFA at room temperature, washed three times with PBS, and

cryopreserved in 30% sucrose/PBS at 4°C overnight. Fixed organoids were embedded

in O.C.T. compound (Sakura Finetek), frozen in an ispropanol/dry ice slurry, and

sectioned at 14µm on a cryotome. Sections were attached to Superfrost Plus slides

(Electron Microscopy Sciences) and stored at -20°C until staining. Prior to staining,

sections were rehydrated in PBS for 5min, and detergent extracted in 0.5% Triton X-100 (Roche) for 3min.

RNA FISH and IF were performed as previously described (Byron et al., 2013; Clemson et al., 1996). For RNA FISH and combined RNA FISH/IF in iPSCs and monolayer neural culture, detergent extraction was performed prior to fixation. For IF alone, fixation was performed prior to detergent extraction. The *XIST* probes used were G1A (Addgene plasmid #24690; Clemson et al., 1996) and a Stellaris FISH probe (Biosearch Technologies, SMF-2038-1), which was used according to the manufacturer's instructions. The APP probe is a BAC from BACPAC resources (RP11-910G8). DNA probes were labelled by nick translation with either biotin-16-dUTP or digoxigenin-11-dUTP (Roche). For simultaneous IF and RNA FISH, cells were immunostained normally with the addition of RNasin Plus (Promega) to the incubation buffer and fixed in 4% PFA prior to RNA FISH. The primary antibodies used in this study are provided in Table II-1. The conjugated secondary antibodies used in this study were Alexa Fluor 488, 594, and 647. BrdU staining was performed after RNA FISH and subsequent fixation by incubating coverslips or slides at 80°C in 70% formamide in 2X SSC for 5min (coverslips) or 30min (cryosections on slides) followed by dehydration in 70% and 100% cold ethanol for 5min each and standard IF.

RNA isolation, cDNA library preparation, and high-throughput sequencing

RNA was extracted using TRIzol reagent (ThermoFisher) according to manufacturer's instructions. RNA samples were cleared of contaminating genomic DNA by DNAse I (Roche) treatment for 1hr at 37°C. RNA cleanup and DNAse I removal was performed using RNeasy MinElute columns (Qiagen) according to manufacturer's

instructions. Clean RNA was assessed for quality on an Advanced Analytical Fragment Analyzer, and all samples had an RQN > 7.5. 100ng of RNA per sample was used to prepare mRNA strand-specific sequencing libraries using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® in conjunction with the NEBNext® Poly(A) mRNA Magnetic Isolation Module and NEBNext® Multiplex Oligos for Illumina® (New England Biolabs). Sequencing was performed by the UMass Medical School Deep Sequencing Core Facility on the Illumina HiSeq4000 platform to a depth of ~8 million reads/sample.

Reads were aligned to the hg19 human genome build (GRCh37) using hisat2 (v2.0.5). Reads were counted to genes using the featureCounts function of the subread package (v1.6.2). Within R, the DEseq2 package was used to normalize reads between samples and determine significantly differentially expressed genes. Significance in Figure 3D was determined by performing multiple comparison correction on all expressed chr21 genes (n=125) and setting an FDR of <0.1. The ggplot2 package was used to generate most graphs.

Single-cell RNA sequencing

On day 28 of differentiation, neurospheres were dissociated with StemPro Accutase (ThermoFisher) and passed through a 40μm strainer to remove remaining clumps. Cells were washed twice in PBS + 0.4% BSA, counted and assessed for viability (>80%). Cells were then processed using the 10x Genomics Chromium™ Single Cell 3' Library and Gel Bead Kit v2 per manufacturer's instructions. Sequencing was performed by the UMass Medical School Deep Sequencing Core Facility on the Illumina HiSeq4000 platform. Alignment, filtering, barcode counting, and UMI counting

was performed using the Cell Ranger pipeline (10x Genomics - v2.1.1) using the hg19

reference genome which was altered to include the TET3G transgene sequence.

Further normalization, filtering, and analysis was performed using the Seurat R package

(v2.3.4). Cell cycle scoring was performed as previously described (Tirosh et al., 2016).

Microscopy and cell scoring

Cells were visualized using a Zeiss AxioObserver 7, equipped with Chroma multi-

bandpass dichroic and emission filter sets (Brattleboro, VT), with a Flash 4.0 LT CMOS

camera (Hamamatsu). Images were minimally corrected for brightness and contrast to

best represent signals observed by eye using ZEN software (v2.3 Blue, Zeiss). Where

indicated, 3D z-stacks of several focal planes were computationally deconvolved and a

maximal image projection was created using ZEN software in order to visualize all

signals in one image. For scoring of heterochromatin marker association with XIST

signal, we examined at least 8 random fields in three independent differentiations per

sample. For APP gene silencing, we examined at least 6 random fields in two

independent differentiations per sample, and silencing was assessed using the following

formula, which corrects for variable hybridization efficiency between samples: Degree of

silencing = 100 * (1 - (fraction of XIST+ cells with 3 APP foci / fraction of XIST- cells with

3 APP foci)). For scoring of neuron/NSC cell type, TUBB3+ cells were counted as

neurons, SOX2+/TUBB3- cells were counted as NSC, and SOX2-/TUBB3- cells were

not counted. After a cell was determined to be a neuron or NSC, its XIST status was

assessed. For this experiment, 5 random low-power fields were examined for each

independent sample.

Table II-1: Primary antibodies used in chapter II

Antibody	Host	Source	Identifier
NeuN	mouse monoclonal	Millipore	MAB377
Trimethyl-Histone H3 Lys27 (H3K27me3)	rabbit polyclonal	Millipore	07-449
Oct3/4	goat polyclonal	Santa Cruz Biotechnology	sc-8629
Sox2	rabbit polyclonal	Millipore	AB5603
Tubulin β 3 (TUBB3)	mouse monoclonal	Biolegend	Tuj1 (MMS-435P)
BrdU	mouse monoclonal	Sigma-Aldrich	BU-33 (B8434)
Ubiquitl-Histone H2A Lys119 (H2AK119ub)	rabbit monoclonal	Cell Signaling Technology	D27C4 (#8240)
Sox1	goat polyclonal	R&D Systems	AF3369

CHAPTER III : Modeling DS neurodevelopment with cerebral organoids

Preface

I performed all of the experiments described in this chapter, with help from Meg Byron in the culturing and processing of over 1,000 organoids for the largest experiment. I performed the analysis of results from the early experiments myself, whereas most of the computational analysis on the largest experiment was performed by Dr. Oliver King, with input from me and Jeanne Lawrence.

Introduction

Cognitive disability is a universal feature of DS, and while the genetic basis of DS is clear, the direct molecular and cellular causes for this phenotype are not well defined. Studies in mouse models of DS have identified several brain pathologies and corresponding behavioral or cognitive phenotypes, although various studies of different or even the same mouse models do not always agree (Belichenko et al., 2009, 2007; Haydar and Reeves, 2012; Reeves et al., 1995). Additionally, discerning which models more faithfully reflect the various aspects of neurodevelopment and cognitive features of human DS is challenging. Several studies report evidence of genetic or pharmaceutical correction of specific phenotypes in specific mouse models of DS, such as for increased interneuron number and decreased cerebellar size (Chakrabarti et al., 2010; Das et al.,

2013). However, some of these findings do not correlate well with the human syndrome. For example, several studies have suggested that interneuron number may be decreased in DS patients and human cell models (Bhattacharyya et al., 2009; Huo et al., 2018; Ross et al., 1984; Wisniewski, 1986), in contrast to reports in trisomic mice that interneuron numbers are increased. Further complicating the current state of knowledge in this area, at the recent meeting of the Trisomy 21 Research Society, a panel discussion highlighted the important issue that neural phenotypes have not been consistent for the same DS mouse model bred and studied at different times or places.

In recent years, with the advent of whole genome sequencing approaches, studies have begun to examine differences in the transcriptomes of DS versus euploid samples. However, among several published studies there have been few consistent conclusions. While studies invariably find chr21 genes to be disproportionately upregulated in DS tissues and cells, the number and identity of these genes is inconsistent. A meta-analysis of 45 transcriptome studies found only 77 chr21 genes to be consistently upregulated in DS samples (Vilardell et al., 2011). If there is no feedback regulation of a specific gene, then trisomy for that gene would be expected to result in a relatively modest 1.5-fold increase in mRNA level, which could be further confounded by differences in cell types or pathological states. Most recently, several studies have claimed that trisomy 21 causes broad transcriptome-wide changes, with some studies reporting global upregulation of non-chr21 genes (Mowery et al., 2018) or the presence of domains of up- and down-regulation across the genome (Letourneau et al., 2014). However, the latter phenomenon has recently been called into question (Do et al., 2015), and very recently has been shown to be unrelated to trisomy and

detectable in both normal and trisomic samples (Ahlfors et al., 2019). Studies in adult DS brain tissue have found hundreds of differentially expressed genes even using extremely stringent statistical cutoffs (Lockstone et al., 2007b), which may reflect differences in cell type representation and/or cellular states. For example, some evidence from post-mortem DS brain samples as well as human cellular DS models have demonstrated increased astroglial numbers (Briggs et al., 2013; Chen et al., 2014; Lu et al., 2011; Mito and Becker, 1993; Zdaniuk et al., 2011). If true, this difference in cell-type representation alone could cause broad changes in the overall transcriptome of brain tissue, complicating identification of specific pathways directly perturbed by trisomy 21.

Hence, it remains a challenge to understand the neurodevelopmental basis for cognitive deficits in DS children and adults, deficits that can vary between patients in severity and are now generally accepted to progress throughout adulthood. It is now understood that triplication of the chr21 APP gene plays a critical role in the almost inevitable early-onset of AD in DS, but the neurodevelopmental changes are far less understood than the later-stage neurodegeneration. In summary, numerous molecular pathways have been touted as central to DS pathogenesis, however, little consensus has been reached as to the transcriptional changes and pathways impacted nor on the specific cellular pathologies that characterize differences in the DS human brain. Thus, new methods are needed to investigate and better characterize the cellular changes of DS neurodevelopment and allow identification of the molecular pathways involved, information essential for the development of effective therapeutic targets and strategies of any kind.

In the prior chapter, I used primarily monolayer neural differentiation to examine the functionality and effects of *XIST*-mediated chromosome silencing in a defined pathway of neurogenesis. This proved a valuable approach to examine a precise developmental window of neurogenesis. However, studies in this chapter pursued a complementary approach, using cerebral organoids, a new technology that I introduced into the Lawrence laboratory.

Recently, cerebral organoids have emerged as a new model system for studying early human neurodevelopment in a more developmentally appropriate three-dimensional environment (Di Lullo and Kriegstein, 2017). In addition to providing the potential to examine spatial relationships between developing neural cells and tissue layers, organoid systems also allow for the generation of a variety of cell types and to examine them over longer time frames without the need for passaging or other manipulation during differentiation. Not long after an early paper on human cerebral-organoid technology was published (Lancaster et al., 2013), I became intrigued by the opportunity to examine development of more complex neural tissues and over a longer time-frame. Given that this was a new and more complex approach to studying human neurodevelopment, the first major goal of the studies of this chapter was to establish this technology in the Lawrence lab. This ultimately involved reproducing and evaluating three different organoid protocols to assess their suitability for our experimental goals, to determine if the presence of trisomy 21 causes discernible neurodevelopmental differences of human organoids compared to those derived from isogenic euploid iPSCs. I quickly learned that the greater complexity provided by these structures presents substantial advantages as well as significant challenges requiring more

quantitative analysis techniques. Therefore, a theme that shaped the goals and experiments throughout this chapter was to develop experimental design strategies that avoid or minimize sources of variation between isogenic samples other than those due to presence of an extra chromosome 21. Some of the methodological points demonstrated by this work have value for the field of disease modeling with human iPSCs more generally, whereas other specific results have significant implications for the fundamental biology of trisomy 21.

Here, we test three organoid generation protocols out of a larger compendium of recently-developed organoid protocols as representing the range of undirected to directed organoid approaches. We use DS patient-derived iPSCs and utilize a directed forebrain spheroid method to perform in-depth transcriptional investigation of trisomic and disomic conditions. We examine both potential cell type representation differences as well as non-chr21 expression changes in three trisomic and three isogenic disomic cell lines.

Results

The results below detail the progression of experimental design improvements based on results of earlier observations. The last and largest experiment was formulated based on lessons learned from our initial cerebral organoid studies, which highlighted the need to address several sources of variation between samples that are often present but not due to trisomy 21. A summary of potential sources of confounding variation that are often present (but not due to trisomy 21) are listed in Table III-1, most of which are also relevant to human disease modeling with iPSCs generally. DS studies have reported numerous phenotypic and transcriptional differences attributed to trisomy

Table III-1: Potential sources of variability in iPSC disease modeling

Sources of variability	Strategies used in this study to lessen variability
Genetic differences between individuals	Isogenic cell lines
Between isogenic clones	Subclones from same reprogramming event
Within a clone - Genetic drift - Epigenetic drift - Freeze/thaw bottleneck	No freeze/thaw between differentiations in same experiment
Difference between individual organoids	Pooling large numbers of organoids
Differences between differentiations	Multiple differentiations using a semi-directed protocol

in human or mouse neural tissues and cells, however it is often difficult to know whether other potential differences between samples has been ruled out. In order to examine whether trisomy 21 *per se* impacts the development of cerebral organoids in any given assay, part of our goal here became to understand the extent to which differences may arise from other factors: e.g. variation between organoids, cell lines, experiments, cell representation, etc. This led us to focus on quantitative transcriptome analyses using an expanded experimental design of isogenic organoids, providing what we believe is the most comprehensive comparison of differences in trisomic versus disomic organoids available to date.

Evaluating three approaches for generating cerebral organoids with DS iPSCs

Several 3D cell culture models of cerebral development have recently been developed, each with its own set of advantages and drawbacks. Most significantly, protocols differ in the usage of exogenous patterning molecules. Lancaster et al. (2013; 2014) utilize a protocol with minimal patterning and make use of the "default fate" of differentiating pluripotent cells to become rostral neuroectoderm. This results in the generation of several cerebral cell types, including meninges, choroid plexus, and cortical zones including self-organizing neural stem cell niches and surrounding neurons. The potential advantages of minimal patterning in generating a model of neurodevelopmental disorders include diminishing the potentially overriding effects of non-physiologic levels of patterning and mitotic factors on a subtle defect in differentiation and/or proliferation. Additionally, modeling a wide range of cell types that could be involved in disease pathogenesis in a dense 3D environment could reveal

phenotypes absent in monoculture. On the other hand, the broader range of cell types produced may include those that are likely of peripheral relevance to cognitive disability.

Because of this, protocols based on advances in developmental neurobiology and 2D culture techniques have utilized patterning molecules to establish region-specific 3D models of human neurodevelopment. In particular, Paşca et al. (2015) utilize dual-SMAD inhibition, high concentrations of the mitogens FGF2 and EGF, as well as the neurotrophins BDNF and NT3 to generate spheroids that include only cortical-like cells, including both neurons and astroglia. A third protocol utilizes SMAD inhibitors as well as mild WNT signaling activation, which ameliorated apoptotic cell death and potentially further dorsalized the organoids (Qian et al., 2016, 2018).

Protocols also differ in their attempts to minimize necrosis in the center of organoids that is largely inevitable due to the lack of vascularization and poor oxygen penetration. While some have cultured organoids in very high (40%) oxygen environments (Kadoshima et al., 2013), most protocols involve aeration of the media with bioreactors and orbital shakers (Lancaster et al., 2013) or miniaturized bioreactors (Qian et al., 2016). In addition to aiding with necrosis and increasing overall organoid size, spinning in a bioreactor has also been shown to improve formation of large ventricular-like zones compared to stationary culture conditions (Lancaster et al., 2013). Interestingly, one protocol reports static culture of spheroids that grow up to 4mm in size, but does not attempt to reduce necrosis through aeration or increased oxygen concentration (Paşca et al., 2015).

Finally, some protocols rely on a provided extracellular matrix in the form of a mouse tumor-derived gelatinous protein mixture (Matrigel) to enhance production of

cortical ventricular-like zones (Lancaster et al., 2013; Qian et al., 2016). This comes at the cost of increased variability due to inconsistent embedding and lot-to-lot variability of growth factors present in this biologically derived material, as well as increased labor costs and decreased throughput associated with this added step. Protocols that omit Matrigel embedding still generate ventricular-like zones, though smaller and less complex (Mariani et al., 2012; Paşca et al., 2015).

In order to develop a model of DS neurodevelopment in the lab, I tested these protocols using isogenic iPSCs with trisomy 21 and their disomic controls. First, I generated cerebral organoids, as previously described (Lancaster and Knoblich, 2014; Lancaster et al., 2013). This protocol generated large ventricular-like zones with tightly packed neural stem cells surrounded by outwardly migrating postmitotic neurons (Figure III-1A). However, these structures constituted a minority of cells in each organoid, which were largely composed of self-organizing cells in a non-ventricular pattern, reminiscent of choroid plexus-like tissue as well as other cells lacking clear organization (Figure III-1A'). Because we aim to examine the effects of trisomy on cortical neurogenesis, we decided that this protocol was not sufficiently reproducible to achieve this goal.

Next, we created forebrain organoids with minor modifications (see methods) (Qian et al., 2018). After ~50 days, these organoids formed a large number of large, well-organized ventricular-like zones (Figure III-1B). The organoids were largely comprised of VZs with very few regions that showed organization of a different cerebral cell type. These organoids demonstrated a particularly striking contrast between NSC-containing VZs and surrounding neuron-containing regions. This characteristic was

Figure III-1: Evaluating three approaches for generating cerebral organoids with DS iPSCs

A-C) Immunofluorescence photomicrographs of representative cortical regions in three selected organoid generation protocols. A') Non-cortical region in organoid generated with Lancaster protocol, which resembles choroid plexus in organization. B') Cortical region of organoid generated with Qian protocol but lacking the distinct radial organization seen in (B). D) Visual summary of protocol generation protocols. All protocols utilize the same first step of single-cell dissociation and re-aggregation in 96-well plates. E) Example of 90-day organoids generated with Paşca protocol and demonstrating robust GFAP expression, suggestion formation of astrocytes. Wide variability between individual organoids can be seen, which makes quantification of cell representation difficult. Scale bars are 100μm in (A-C) and 1mm in (E).
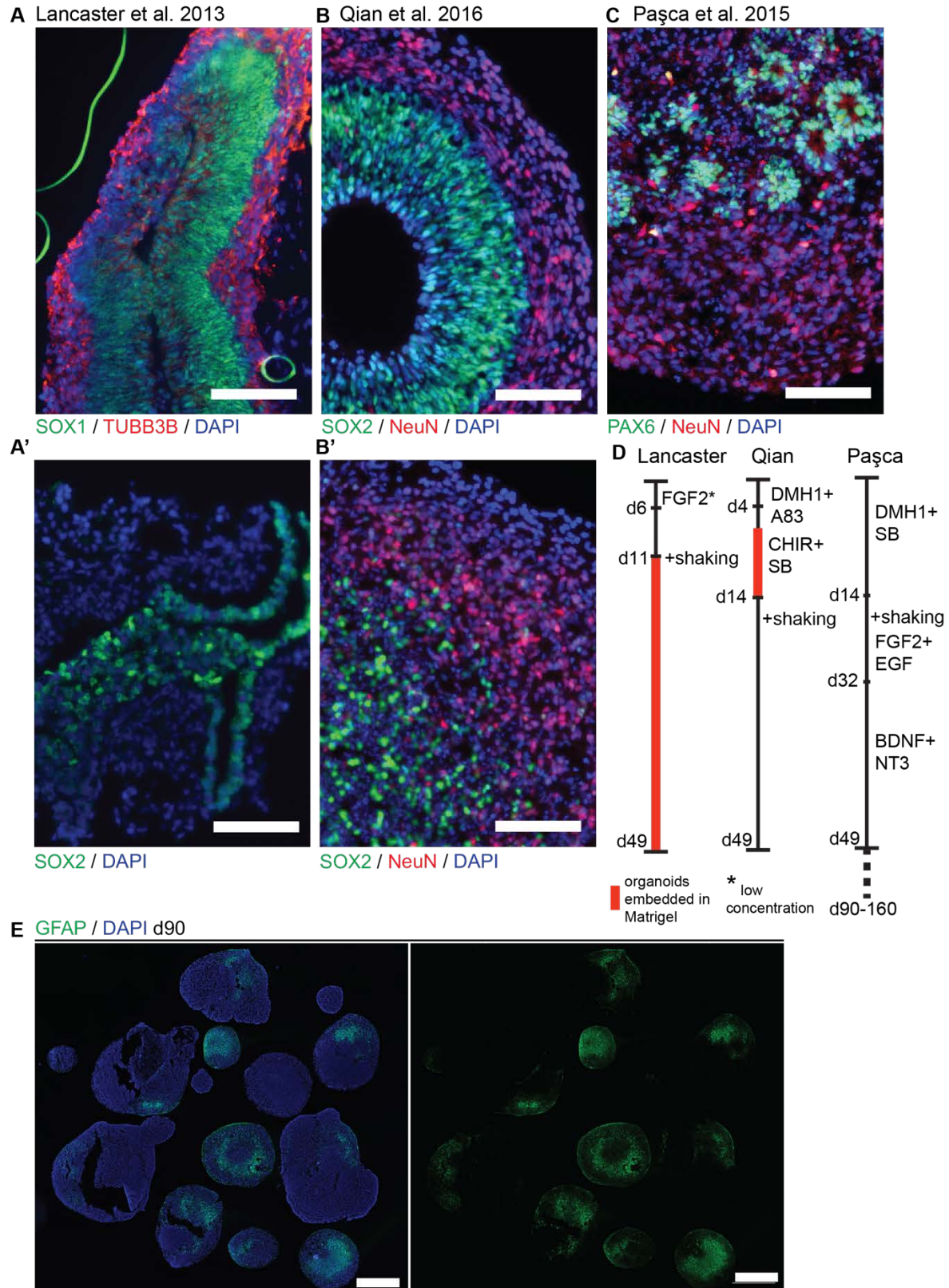
Figure III-1: Evaluating three approaches for generating cerebral organoids with DS iPSCs

taken advantage of in the previous chapter to validate the monolayer findings of impaired initiation of *XIST* transcription in postmitotic neurons, and this protocol could be used for other studies going forward, particularly for examination of early VZ formation and the cell dynamics within VZs. While some batches generated robust VZ-containing organoids, others did not produce the characteristic morphology, potentially due to inconsistencies in Matrigel embedding and subsequent disembedding (Figure III-1B'). This, along with the increased labor required to generate these organoids with Matrigel embedding, motivated us to find another protocol that could generate organoids with higher throughput.

To this end, we generated cortical spheroids, this time with some significant modifications in order to consistently generate organoids in our hands (Paşca et al., 2015). Most significantly, the exposure to SMAD inhibitors was increased from one to two weeks, and the subsequent mitogen and neurotrophin treatments were thus delayed by one week. Nevertheless, this protocol produced large spheroids containing smaller VZ-like zones along with some unorganized progenitor containing areas (Figure III-1C). A visual summary of the organoid differentiation protocols tested in this chapter is provided in Figure 3.1D. Additionally, prolonged culture generated significant numbers of GFAP-expressing astrocytes (Figure III-1E), production of which is limited in other protocols due to the lack of a progenitor expansion step. Together, these favorable characteristics encouraged us to utilize this organoid generation protocol going forward to examine the effects of trisomy on neurodevelopment in aged organoids.

After early attempts to analyze potential differences in cell type representation using histological methods, we quickly came to the conclusion that the still-large degree of variability from organoid to organoid makes accurate quantification a particularly difficult task (Figure III-1E). For this reason, we turned to bulk RNA sequencing to determine cell type representations of trisomic and disomic organoids.

Initial studies identify differences in organoid cell type representation

Our first pilot RNA sequencing experiment in organoids used bulk RNAseq of 10 organoids aged for 160 days, five from an isogenic trisomic (parental) line and five from the euploid control line. Use of isogenic lines avoids differences in genetic background, and the comparison of subclones of the same iPS line avoids differences in the iPS reprogramming process or the somatic cell of origin. The overall strategy was to generate bulk sequence data and use published gene sets for different cell-types to deconvolve the cell-type representation of the bulk sequencing. To evaluate the variation between individual organoids in this first experiment, we sequenced the 10 organoids individually.

We generated RNAseq data for the 10 organoids, each sequenced to a depth of ~30 million reads with 100bp paired-end reads, which provided strong quantification of mRNA levels for each gene, as evidenced by the reproducible difference in chr21 expression between trisomic and disomic organoids (Figure III-2A). Further analysis of the transcriptome differences between disomic and trisomic organoids indicated that, in addition to changes in upregulation of many chromosome 21 genes (red), there were widespread changes in expression levels of non-chromosome 21 genes between these samples (Figure III-2B). As described in the introduction, widespread differences in non-

Figure III-2: Initial studies identify differences in interneuron or glia cell representation between small pools of disomic and trisomic organoids

A) Fraction of chromosome 21 reads in each individual organoid sequenced. Trisomic organoids have close to the expected 1.5-fold increase in chromosome 21 expression compared to disomic organoids. B) Volcano plot comparing 5 trisomic vs. 5 disomic organoids. Chr21 genes are circled in red and expression level is signified by colors ranging from blue (low expression) to green (high expression). DLX family genes and GFAP are labelled. C) Estimated composition of each organoid into cell types using defined gene sets (further described in methods section). D-E) Pooled qPCR quantification of DLX1 (D) and GFAP (E) expression, normalized to GAPDH. Each dot represents a pool of ~8 organoids. Colors represent independent organoid differentiations.
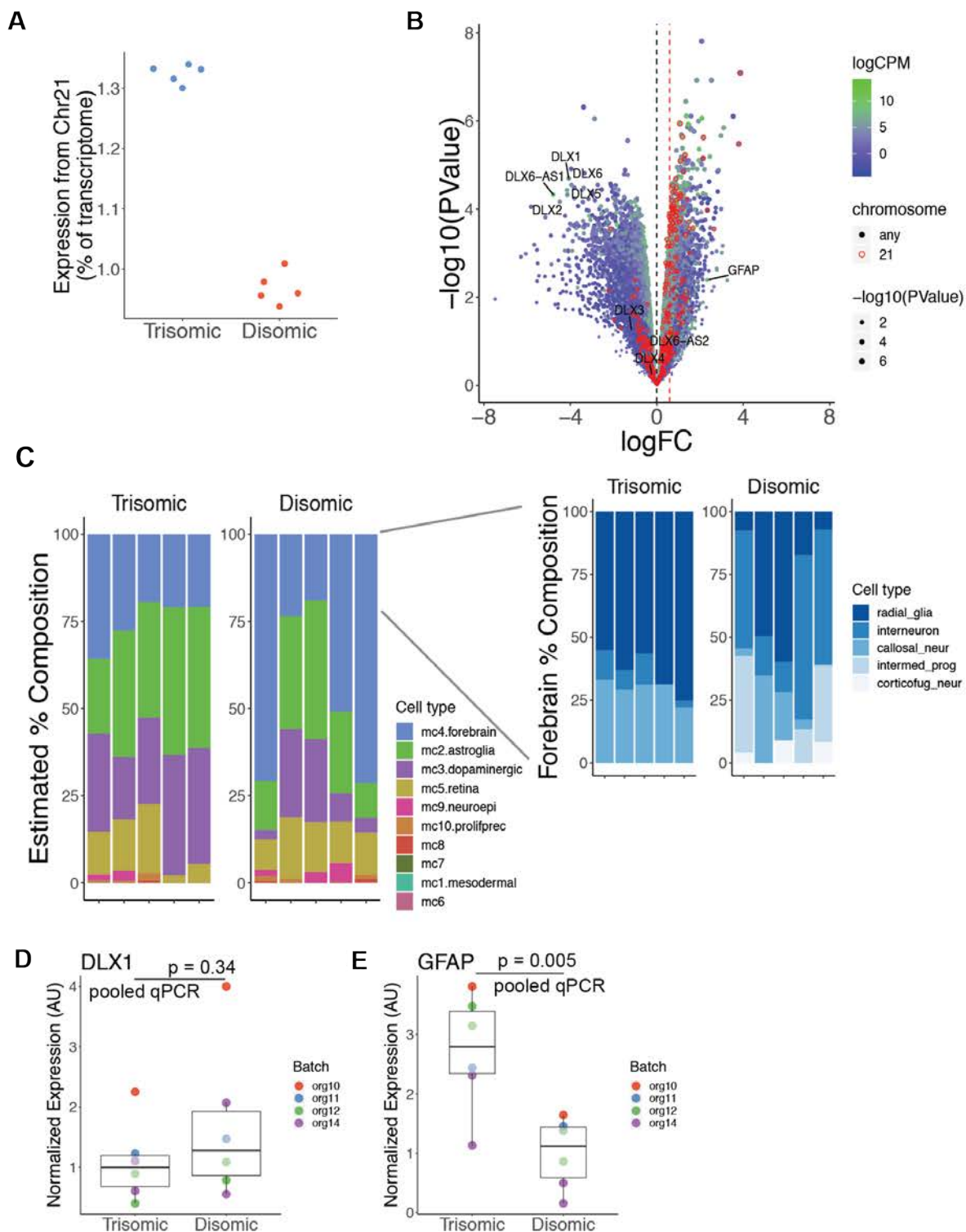
Figure III-2: Initial studies identify differences in interneuron or glia cell representation between small pools of disomic and trisomic organoids

chromosome 21 gene expression is currently a common finding in published studies of trisomy.

Most notably, analysis of differentially expressed non-chr21 genes between the two conditions showed many DLX family genes, each identified as downregulated on average in the trisomic organoids (Figure III-2B). These genes are well-known for their involvement in the specification and migration of ventral forebrain-derived interneurons (Anderson et al., 1997; Cobos et al., 2007; Paina et al., 2011; Stühmer et al., 2002), and there have been mixed reports in human samples and cell models of whether interneuron generation is decreased or increased due to trisomy (Bhattacharyya et al., 2009; Huo et al., 2018; Ross et al., 1984; Wisniewski, 1986; Xu et al., 2019).

To further investigate whether other interneuron-related genes followed the same pattern, and to determine whether other cell types also had altered representations in trisomic organoids, we utilized marker gene lists recently generated by single-cell RNA sequencing of human cerebral organoids (Quadrato et al., 2017). There could be a statistically significant difference on average between these two samples of five versus five organoids, but that does not itself address whether this reflects a consistent difference in neurodevelopment between these trisomic versus control cells. Deconvolution of cell type representations for each individual organoid demonstrated overrepresentation of forebrain-derived cells in the disomic condition, largely driven by an increase in interneuron generation (Figure III-3C). Importantly, this effect was driven by three disomic organoids which had large numbers of this cell type, whereas two disomic organoids had similar interneuron composition to the trisomic organoids. A second difference in cell-type representation was observed for radial glia cells, which

were overrepresented in trisomic organoids, with a corresponding increase in GFAP expression, a gene also expressed in astrocytes, in these samples (Figure III-2B). Thus, despite using a directed organoid generation protocol, significant variability between individual organoids in interneuron formation weakens any conclusions that can be drawn on this point.

In an attempt to minimize differences between individual organoids, and to investigate whether similar findings could be identified in younger organoids, we generated several batches of organoids grown for 90 days and examined them in pools of ~8 organoids by RT-qPCR for a prominent marker of interneurons, DLX1, as well as for GFAP. We found that DLX1 expression remained highly variable between experiments and inconsistently showed an increase in disomic organoids over trisomic, although we note that higher expression in organoids from the parental (trisomic) line was not seen in these experiments (Figure III-2D). On the other hand, GFAP expression was consistently upregulated in the trisomic organoids, by about 3-fold (Figure III-2E). This indicates that either the trisomic cells generate more radial glia and/or astrocytes, or that these cell types produce higher levels of GFAP mRNA in the trisomic condition.

Expanded experimental design to discriminate differences due to trisomy 21

The above findings provide some evidence of differences in organoid development that correlates with the trisomic condition, but questions remain as to whether these differences could reflect other sources of variability. Therefore, we greatly expanded the experimental design in order to increase the power to discriminate differences due to trisomy from differences due to variability between individual organoids, different experiments, or even between isogenic cell lines. In this experiment

Figure III-3: Expanded experimental design to discriminate differences due to trisomy 21

A) Micrographs of nearly all organoids generated in this experiment. Independent differentiations are signified by "org", isogenic trisomic lines by "par", and isogenic disomic lines by "dis". B) Schematic of samples generated. Each of the 48 dots represents 12 organoids and one sample for sequencing, while the 3D cylinders signify *in silico* collapsing for statistical comparison. C) Fraction of chromosome 21 reads for each pooled sample sequenced. D) Estimated composition of each sample into cell types using defined gene sets. E) Estimated cell-type composition of collapsed samples used for statistical comparison.
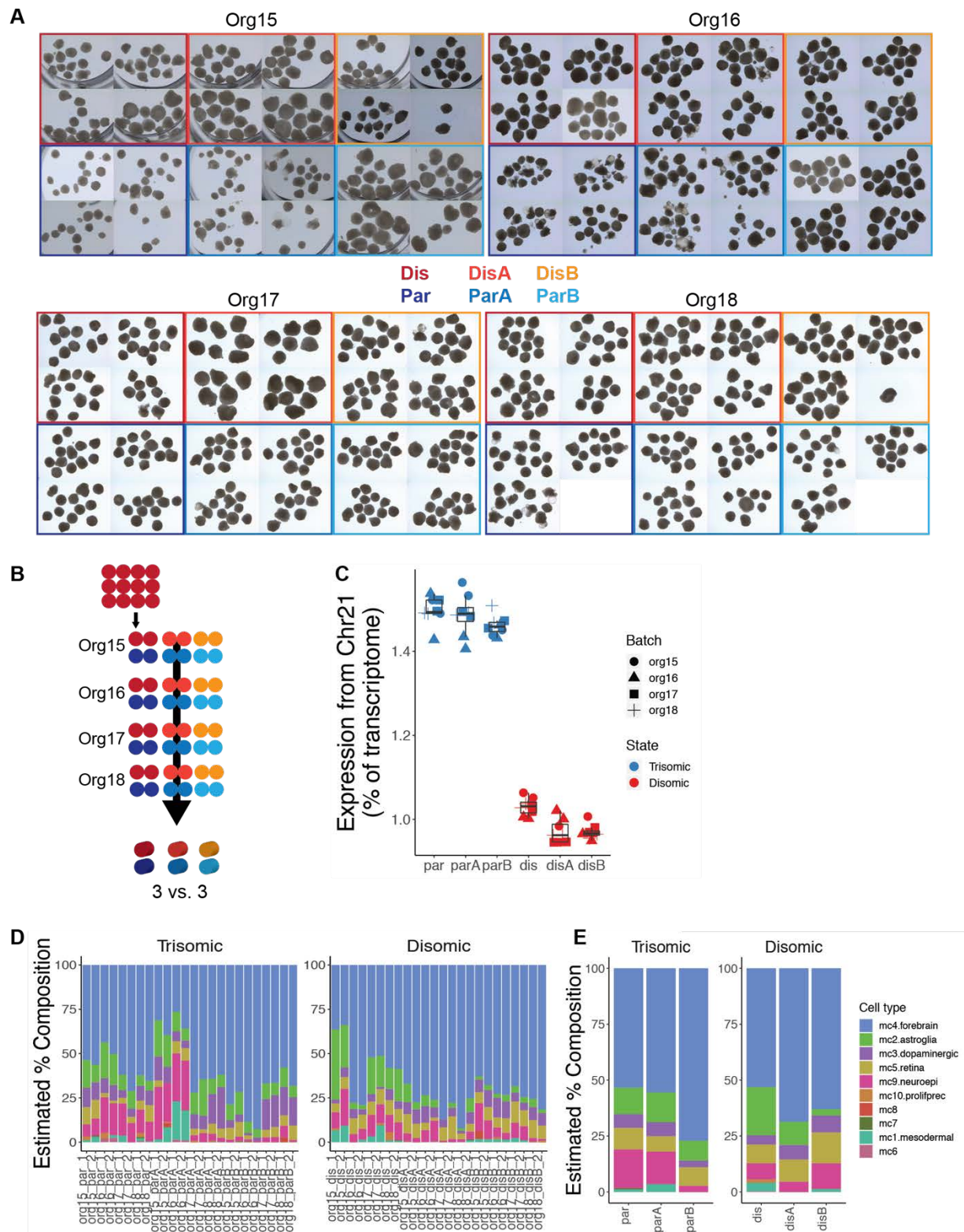
Figure III-3: Expanded experimental design to discriminate differences due to trisomy 21

we generated a total of over 1,000 organoids from three trisomic and three disomic isogenic iPSC lines (Figure III-3A), which were derived from the same iPS cell reprogramming event. To minimize effects of individual organoid differences, we examined pools of 12 organoids, generating four pools per cell line, and this scheme was repeated in four independent batches of organoids. Roughly half of these organoids were used for bulk RNA sequencing, with 2 pooled samples per cell line per batch, for a total of 48 samples (Figure III-3B). The remaining organoids were frozen for histology and media preserved for potential future analyses on parallel samples.

Initial sequencing analysis confirmed expected differences in chromosome 21 expression, with all trisomic lines having ~1.5-fold higher levels of chromosome 21 transcripts compared to disomic lines (Figure III-3C). We next set out to use this transcriptome data to determine the cell type composition of each sample using the same list of gene markers described above, based on published single-cell seq studies defining specific gene sets. We found that nearly every sample was mostly composed of forebrain-type cells, with significant contributions from astroglia, dopaminergic neurons, neuroepithelial cells. Surprisingly, this analysis revealed that some organoid samples contained a subset of mesodermally-derived cells (Figure III-3D), suggesting some degree of off-target differentiation.

Notably, there was no consistent or statistically significant difference in the proportions of the categorized cell-types between the disomic and trisomic states (Figure III-3E). Differences in cell-type representations between cell lines of the same state (disomic or trisomic) were detected, but these were not consistent between the disomic versus trisomic lines. Comparison of results for a given line between

independent differentiations suggested that some variation appears sporadic but may also reflect inherent epigenetic differences between even isogenic cell lines which may evolve in culture (Table III-1). We cannot rule out that the variability detected between cell lines may mask the presence of more subtle differences in the propensity of disomic and trisomic organoids to form different neural cell types.

A theme of recent transcriptome studies in DS cells and tissues is the finding of extensive transcriptome-wide differences between trisomic and euploid samples. This is also suggested in our initial experiment comparing individual organoids from trisomic and disomic iPSCs. To determine whether this expanded organoid experiment would affirm a similar finding, we examined differentially expressed genes (DEGs) between disomic and trisomic organoids. An important aspect of this statistical analysis was to avoid amplification of clone-specific differences by treating repeated measurements from different experiments on the same cell sample as four independent measurements, a form of pseudoreplication which results in inappropriately inflated p-values. Thus, our analysis collapsed replicate samples and samples from different differentiations of the same cell line and compared organoids generated from the 3 trisomic lines to 3 isogenic disomic lines (Figure III-3B).

This analysis detected strong upregulation of genes across chromosome 21, with 159 of 250 expressed chr21 genes meeting statistical criteria for differential expression, generally at or near the 1.5-fold level expected in trisomic cells (Figure III-4A). Interestingly, a notable exception was the RWDD2B gene, which was over 13-fold upregulated in trisomic samples. The most striking finding, however, was that despite the robust detection of differentially expressed chromosome 21 genes, no non-chr21

Figure III-4: Genome-wide transcriptome analysis of expanded organoid experiment

A) Volcano plot of collapsed 3 vs. 3 comparison of trisomic and disomic conditions. Chr21 genes are circled in red. No statistically significant (FDR<0.1) genes are detected. B) Volcano plot of the same comparison as in (A) but including the degree of forebrain representation as a covariate for each sample. The 3 non-chr21 genes that are identified as statistically significant (FDR<0.1) are labelled. C) Individual gene plots for two astrocyte marker genes, GFAP and AQP4 which do not meet statistical difference thresholds but trend towards higher levels in trisomic samples. D) Expression level in each sample of two of the non-chr21 genes identified in (B) as statistically significant plotted against estimated forebrain composition demonstrating strong correlation.
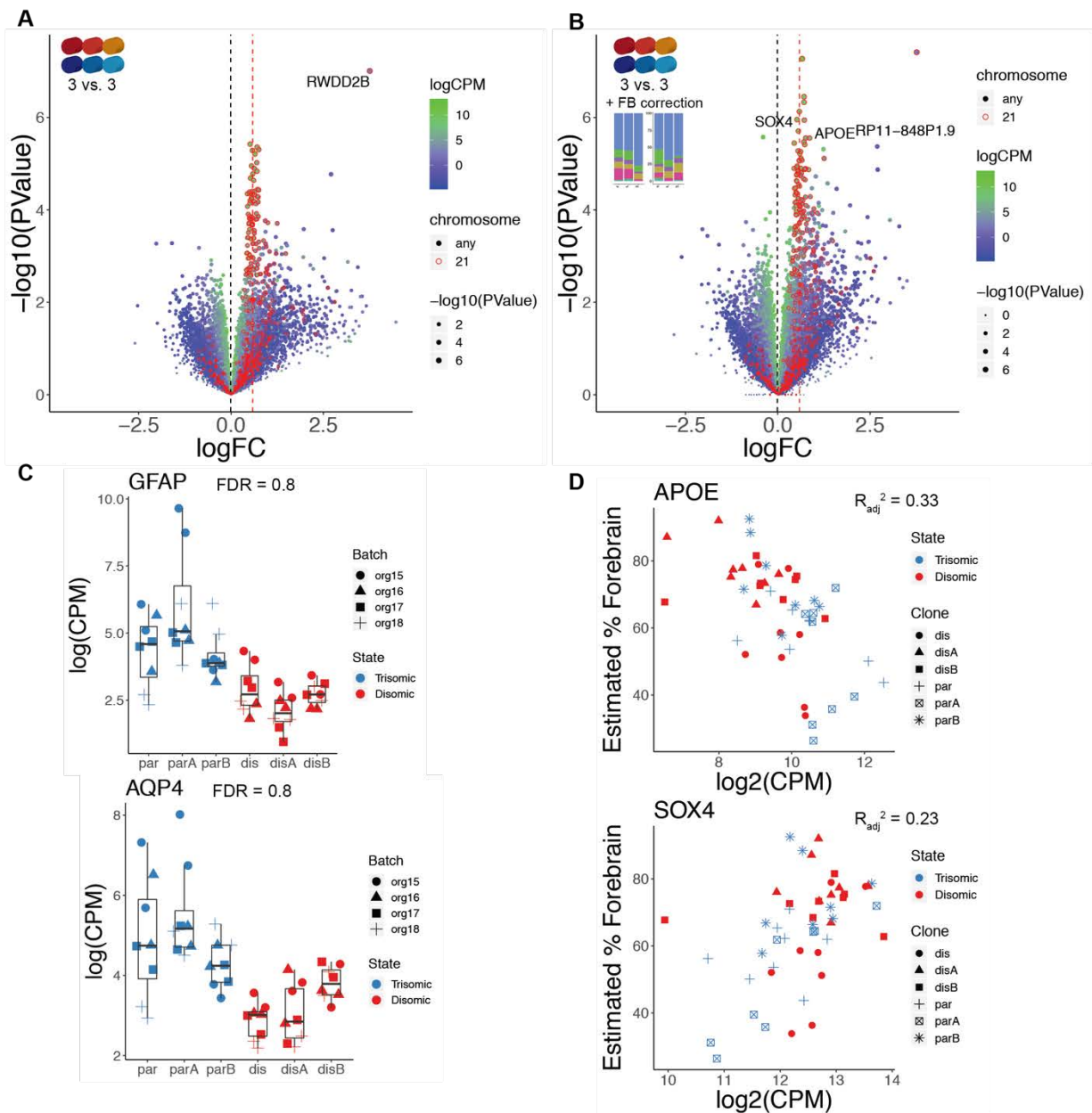
Figure III-4: Genome-wide transcriptome analysis of expanded organoid experiment

transcriptome changes were detected (FDR<0.1) in this greatly expanded experiment.

This suggests that the genome-wide differences found in the smaller-scale experiment

above (Fig III-2B) may reflect biological differences between the cell lines compared,

rather than due to trisomy. However, two astrocyte marker genes, GFAP and AQP4,

both showed a trend towards upregulation in trisomic samples, although these did not

meet this significance threshold, likely due to variable expression between samples

(Figure III-4C).

The paucity of non-chr21 DEGs detected in this expanded study fits with the lack

of consistent differences in cell type representation between the two groups shown

above, since consistent differences in cell-type representation would likely result in

many cell-type specific genes being differentially expressed between the two groups.

On the other hand, inconsistent variation in the cell type representations between all the

cell samples (not correlated with trisomy/disomy) might increase gene expression

variability that potentially could obscure any subtle changes in gene expression

between the two groups. In an attempt to correct for this possible effect, we normalized

the data in each sample for the dominant cell type, the proportion of forebrain cells.

Importantly, this correction increased the number of significantly DE chr21 genes to 175

(Figure III-4B), indicating an increase in power due to decreased variance by correcting

for differences in cell type representations between cell lines. Off of chr21, three hits

emerged, SOX4, APOE, and a lowly expressed pseudogene RP11-848P1.9. As

expected for the emergence of significant genes when correcting for cell type

representation, SOX4 and APOE are correlated, positively and negatively, respectively,

with the estimated proportion of forebrain cells (Figure III-4D). Of these, SOX4

expression was downregulated in trisomic samples, whereas APOE and RP11-848.9 were more highly expressed. The implications of these findings are considered in the discussion.

Discussion

The overall goal of this study was to use recently developed cerebral organoid technology to shed light on the molecular and cellular pathways altered in early DS neurodevelopment. Understanding how and when brain development and/or function is impacted in DS is critical to assess therapeutic prospects to mitigate cognitive or neurological deficits due to trisomy 21. Numerous studies report a variety of differences thought to be due to trisomy in mouse models or human DS tissues and cells. However, results are often inconsistent or even contradictory between studies. Experiments here focused on disease modeling with human iPSC and organoid technology and the overall findings have significant implications for understanding the extent to which trisomy 21 impacts early fetal neurodevelopment.

As this study progressed from smaller scale experiments to the much larger final experiment, we worked to minimize or account for potential sources of variation in human cell modeling of DS on several levels. From the start we used a totally isogenic system, generated organoids with a semi-directed (less variable) forebrain protocol, and used multiple large pools of organoids per sample in order to control for individual organoid variability. The larger numbers of pooled organoids were generated from each of six isogenic lines (three trisomic and three disomic) that are subclones representing the same iPSC reprogramming event, which may be important, as some have reported a differential capacity for neurogenesis of different iPSC lines derived from single

somatic cells (Koyanagi-Aoi et al., 2013). Finally, the entire large organoid production

scheme was repeated four times, allowing us to assess and account for variability

between experiments.

Due to these efforts we were able to improve detection of what is a relatively

subtle 1.5-fold expected increase in expression for individual chromosome 21 genes,

with over 70% of expressed chr21 genes meeting statistical criteria for differential

expression due to trisomy. This is substantially more chr21 DEGs than detected in our

smaller experiment or in most studies of DS tissues or cells, which, paradoxically, report

many more off-chr21 DEGs than found here (Letourneau et al., 2014; Mowery et al.,

2018; Olmos-Serrano et al., 2016; Vilardell et al., 2011; Weick et al., 2013). Despite

especially robust detection of chr21 DEGs, our largest experiment did not find

significant evidence for genome-wide transcriptional deregulation of non-chr21 genes.

In our smaller experiment (comparing five organoids each from a trisomic and a disomic

line), more genome-wide expression differences were detected between these samples,

but, importantly, the larger analysis leads us to reinterpret these results as likely

reflecting other sources of biological variability that we cannot conclude is

"dysregulation" due to trisomy 21.

Consistent with a lack of abundant non-chr21 DEGs, the more powerful

experimental design did not detect statistically significant differences in cell-type

representations between trisomic and disomic organoids. Instead, despite pooling large

numbers of organoids to eliminate individual organoid variability, we saw considerable

variability between cell lines of the same chr21 state. Additionally, while some lines,

such as DisB, consistently generated similar proportion of cell types from differentiation

to differentiation, others, like ParA, had considerable variability from one differentiation to another. These differences between cell lines of the same condition could obscure subtle changes in cell type representation caused by trisomy 21.

Organoid technology affords the opportunity to examine development of a greater complexity of cell-types, however that complexity also can introduce analytical challenges. Our analysis showing overall variation in cell-type proportions persists between samples, even in a more highly powered experiment than generally used, and this variation can weaken the power to identify differences genuinely due to trisomy 21. In an effort to minimize this noise, we normalized results for the proportion of forebrain cells in each sample. Following this correction, three statistically significant non-chr21 genes were found, two of which link to important cellular processes which could be aberrant in a range of DS neural cell types. Notably, SOX4 has been shown to affect oligodendrocyte development by induction of a Notch target gene (Braccioli et al., 2018) and missense mutations in SOX4 have recently been identified in patients with intellectual disability and facial dysmorphism (Zawerton et al., 2019). The role that SOX4 plays in DS neurodevelopment has not been examined but results here suggest a new hypothesis and opportunity to explore both the upstream cause (stemming from trisomy 21) of SOX4 dysregulation and its downstream consequences. Similarly, it seems striking that results also indicated dysregulation of the APOE gene which, through its isoforms, has been strongly linked to the risk of developing AD (Poirier et al., 1993). Interestingly, it has been suggested that the expression level of APOE, which we found to be elevated in trisomic samples, may play also play a role in AD pathogenesis (Riddell et al., 2008).

Previous studies in iPS-derived DS cells have described a range of findings with many reporting no difference in the neuronal differentiation capacity of DS cells (Briggs et al., 2013; Gonzales et al., 2018; Lu et al., 2013; Shi et al., 2012b; Weick et al., 2013). Other studies using unrelated disomic and trisomic iPSCs in a monolayer culture system have demonstrated an increase in the proportion of astroglia formed by trisomic cells (Chen et al., 2014). Another very recent study generated patterned ventral forebrain organoids using DS cells and found an increase in the propensity of trisomic cells to form interneurons, which was correctible by knockdown of a chr21 gene, OLIG2 (Xu et al., 2019). This finding is surprising given previous studies in iPSCs and primary human cells that describe the opposite finding (Bhattacharyya et al., 2009; Huo et al., 2018; Ross et al., 1984; Wisniewski, 1986). While our findings are not directly comparable to this study due to significant differences in the organoid generation protocol, one clear advantage of utilizing organoids patterned towards a specific forebrain subregion as in Xu et al. (2019), is a decrease in the variability between organoids and the number of different cell types formed, which may allow for enhanced detection of disease-specific differences.

Overall, these results raise caution about false-positive results, but also potential false negative results, arising due to variability from organoid to organoid, batch to batch, and cell line to cell line. Neurodevelopmental phenotypes may be quite subtle and difficult to model with iPSCs, as discussed elsewhere (Soldner and Jaenisch, 2012). In the current study we believe we have sufficiently minimized the variability between organoids by studying over 500 trisomic and 500 disomic organoids. However, our analysis reveals that in the end, when other sources of variability are addressed,

these organoids are derived from three trisomic and three trisomic isogenic cell lines, and differences between those cell lines exist and can over-shadow milder phenotypes. In fact, epigenetic drift in pluripotent cells was earlier demonstrated in our lab and others by demonstrating variability in X-chromatin modifications and *XIST* RNA status in female ES cell lines (Hall et al., 2008; Silva et al., 2008), which we showed evolve even between colonies within the same culture dish (Hall et al., 2008). Thus, even isogenic subclones can develop epigenetic differences over time that complicate disease modeling.

Ultimately, recently-developed cerebral organoid techniques offer the possibility to more accurately model the early stages of human neurodevelopment with the added potential to apply modern cellular biology techniques and manipulations to study previously inaccessible developmental programs in unprecedented detail. However, precise differentiation protocols must be tailored to the specific experimental question at hand and currently may result in highly variable differentiation conditions which necessitate examination of a large number of independent samples to identify interesting differences that emerge from the noise of variability.

Materials and Methods

iPSC culture

iPSCs were cultured and maintained as described in the previous chapter. Cell lines were verified for appropriate number of copies of chromosome 21 by FISH for a chr21 gene, *APP*, before each series of differentiations. RNA sequencing confirmed appropriate chr21 copy number.

Cerebral organoid differentiation

Lancaster protocol: organoids were generated as previously described (Lancaster and Knoblich, 2014; Lancaster et al., 2013). Briefly, iPSCs were dissociated into single cells and plated at a density of ~9,000 cells/well in 96-well round-bottom ultra-low attachment plates (Corning) in iPSC media containing 4ng/ml thermostable FGF-2 (Millipore) and 50μM Y-27632 (Tocris Bioscience). After 6 days, organoids were transferred to ultra-low-attachment 24-well plates in N2 and heparin-containing neural induction media. Organoids were embedded in Matrigel droplets on day 11 of differentiation and grown for 4 days before transferring to an orbital shaker set at ~100 RPM.

Paşca protocol: spheroids were generated as previously described (Paşca et al., 2015) with significant alterations. The first steps of the protocol were performed as above, using a re-aggregation strategy. Cells were re-aggregated in 96-well plates in iPSC media containing 20ng/ml thermostable FGF-2 and 50μM Y-27632. The next day, half the media was exchanged with neural differentiation media (NDM) containing 2uM DMH1 (Tocris Bioscience) and SB431542 (Tocris Bioscience) as described in the previous chapter. Organoids were fed with this media every day for 14 days. After 14 days, media was changed to neural media containing 20ng/ml FGF-2 and EGF (Peprotech) as described (Paşca et al., 2015) and moved to ultra-low attachment 24-well plates. From this point forward, organoids were grown on an orbital shaker set at ~100 RPM to improve aeration. At day 32, FGF-2 and EGF were replaced with 20ng/ml of BDNF (Peprotech) and NT-3 (Peprotech) for 18 days. At day 50, organoids were fed every other day with neural media without any supplements.

Qian protocol: forebrain organoids were generated as described in the previous chapter.

Cell fixation, RNA FISH, and immunofluorescence

These steps were performed as described in the previous chapter for organoid samples. Primary antibodies used in these experiments are listed in Table III-3.

RNA isolation, cDNA library preparation, and high-throughput sequencing

These steps were performed as described in the previous chapter with the following modifications. Whole organoids were washed once with 1X PBS and placed into 2ml microcentrifuge tubes containing one 5mm steel bead (Qiagen) and 1ml of Trizol reagent (ThermoFisher). These samples were homogenized using the TissueLyserII instrument (Qiagen) on the P1 setting. Beads were then removed using a magnet and samples were either stored at -80C or RNA extraction, DNAse treatment, and RNA cleanup was performed immediately as described in the previous chapter. All samples had an RQN > 7.5 and strand-specific sequencing libraries were prepared using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® in conjunction with the NEBNext® Poly(A) mRNA Magnetic Isolation Module and NEBNext® Multiplex Oligos for Illumina® (New England Biolabs).

Sequencing was performed by the UMass Medical School Deep Sequencing Core Facility on the Illumina HiSeq4000 platform to a depth of ~8 million reads/sample in the case of the large organoid experiment or on the NextSeq instrument to a depth of ~30 million reads/sample in the case of the pilot experiment.

RNA sequencing analysis

Read alignment and counting was performed as described in the previous chapter.

Cell type representation deconvolution was performed using the BSeq-SC algorithm (Baron et al., 2016). Pre-averaged pseudobulk estimates of single cell sequencing data from Quadrato et al. (2017) were used as the basis vectors for deconvolution. The top 20 marker genes ranked by p-value for each cell cluster were used to determine cell type representation estimates.

The quasi-likelihood test in the edgeR package for R was used to determine differential gene expression. Replicate samples and repeated differentiations of the same cell line were summed together to form a 3 vs. 3 comparison. Multiple comparison correction was performed separately for chr21 and non-chr21 genes to determine significant differential expression. For cell type representation correction, the estimated proportion of forebrain cells was included as a covariate in the statistical model.

Reverse transcription and qPCR

RNA was extracted and processed as described above and in chapter II. Reverse transcription was performed using SuperScript III reverse transcriptase (ThermoFisher) per manufacturer's instructions and using random hexamers for first strand synthesis. cDNA was then diluted, and qPCR reaction was set up using iTaq Universal SYBR Green Supermix (BioRad) per manufacturer's instructions with the primers listed in Table III-2. The qPCR reaction was performed on the BioRad C1000 Touch thermal cycler. GAPDH was used for normalization and quantification was performed using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

Table III-2: qPCR primers used in chapter III

Gene	Primer	Sequence
GFAP	F	5'-AAG-CTG-CTA-GAG-GGC-GAG-GAG-AAC-3'
	R	5'-TGA-CAC-AGA-CTT-GGT-GTC-CAG-GCT-3'
DLX1	F	5'-GGC-TGT-TTG-CCA-ATT-CAG-GGT-TCT-3'
	R	5'-TTC-GGC-TCC-AAA-CTC-TCC-ATA-CCA-3'
GAPDH	F	5'-TGC-ACC-ACC-AAC-TGC-TTA-GC-3'
	R	5'-GGC-ATG-GAC-TGT-GGT-CAT-GAG-3'

F = forward; R = reverse

Table III-3: Primary antibodies used in chapter III

Antibody	Host	Source	Identifier
NeuN	mouse monoclonal	Millipore	MAB377
Sox2	rabbit polyclonal	Millipore	AB5603
TUBB3 (Tuj1)	mouse monoclonal	Biolegend	MMS-435P
Sox1	goat polyclonal	R&D Systems	AF3369
GFAP	rabbit polyclonal	Chemicon	AB5804
PAX6	rabbit polyclonal	Biolegend	901301

CHAPTER IV : Discussion and conclusions

The work presented in this thesis aims to demonstrate the utility of dosage compensation and cerebral organoids as experimental model systems to study DS neurodevelopment and to advance the possibility of chromosome therapy as a therapeutic strategy. A discussion of the results using these experimental model systems will be presented here, and a forward-looking evaluation of the potential of *XIST* as a therapeutic will be considered in chapter V.

Dosage compensation to model disease

Work in our lab has translated the natural mechanism of dosage compensation orchestrated by the *XIST* gene to Down syndrome. We have demonstrated that an *XIST* transgene is capable of silencing an entire autosome in an inducible manner in DS iPSCs (Jiang et al., 2013), and that silencing of the extra chromosome can prevent known *in vitro* pathologies, such as in the hematopoietic system (Chiang et al., 2018). This system provides a novel way to study DS pathology by allowing for comparison of otherwise identical cells, with either two or three active copies of chromosome 21. This bypasses some of the limitations of *in vitro* modeling that relies on comparison of different cell lines, which, particularly in the case of iPSCs, can diverge over time in culture in ways that are unrelated to the pathology in question (Soldner and Jaenisch, 2012; Young-Pearse and Morrow, 2016). The work presented in this thesis further advances the utility of this model on several fronts.

Dosage compensation in differentiated cells

In chapter II I demonstrated that contrary to the current literature, *XIST* is capable of inducing chromosome silencing in differentiated cells. This was demonstrated for NSCs at several timepoints in differentiation. In addition to the therapeutic possibilities of *XIST* being able to silence in differentiated cells, this also has important implications for both basic epigenetics and disease modeling.

The initial finding that *XIST* was incapable of initiating silencing just 48 hours after differentiation in mouse ESCs (Wutz and Jaenisch, 2000) generally fits with the idea that pluripotent cells, unlike differentiated cells, exist in a malleable epigenetic state that is amenable to drastic chromosomal restructuring processes like X chromosome inactivation. Indeed, studies have confirmed that pluripotent cells, when compared to their lineage-committed daughters, have unusually high levels of open chromatin marks (Azuara et al., 2006; Mikkelsen et al., 2007). Regions of open chromatin in pluripotent cells are balanced by marks of heterochromatin at the same loci (Azuara et al., 2006), preventing precocious expression of lineage-specific genes. It is easy to imagine how this "razor's edge" of pluripotency is naturally permissible to chromosome-wide silencing, and how this ability may be lost in differentiated cells with narrowed developmental competence where large swaths of the genome have already been packed away in heterochromatic compartments.

Of course, this supposed unmalleable differentiated state has been shown to be malleable experimentally by the relatively recent finding that somatic cells can revert to the pluripotent state by the addition of a few key transcription factors (Takahashi and Yamanaka, 2006). Indeed, the chromatin state of iPSCs has been shown to be largely

identical to ESCs (Guenther et al., 2010), demonstrating a complete restructuring of nuclear architecture by the relatively minor perturbation of exogenous transcription factor expression. These findings force the reimagination of what a "stable" epigenetic state truly entails.

Our results demonstrating the natural ability of differentiated cells to enact a chromosome-wide silencing process provide further evidence that the differentiated state is more malleable than previously believed. While differentiated cells are capable of initiating the silencing process, the prolonged silencing process that we describe may reflect their altered epigenetic status compared to pluripotent cells. While others have demonstrated the ability of mouse hematopoietic progenitors to undergo chromosome silencing (Savarese et al., 2006), it is unknown whether all differentiated cell types are capable of undergoing this process. The current study focused on neurodevelopment and demonstrated the silencing capacity of neural stem cells. An important area of future work will involve empirical testing in other cell types to determine their silencing capacity. An intriguing possible outcome is that differentiated cells generally show significant capacity to initiate silencing, but potentially with varying competency or kinetics, which could enable high-resolution examination of the order of *XIST*-mediated chromosome silencing events. For example, the extended silencing timeline of NSCs in this study allowed us to easily discern the temporal order of polycomb-mediated heterochromatin modifications, which further supported recent findings suggesting that H2AK119ub1 is laid down prior to H3K27me3 (Almeida et al., 2017; Tavares et al., 2012). Further analysis of different cell types could help elucidate key factors required for the silencing process, and those involved in its modulation.

In addition to providing important insights into basic epigenetics, the ability of differentiated cells to initiate silencing also provides the unique ability to study the development of pathology in trisomy. Most genetic disease modeling studies use iPSCs or mouse models to compare control cells or animals to mutant cells harboring a genetic insult, but it is a challenge to determine when defects arise over the course of development. The studies presented here demonstrate the ability of our inducible system to harness a natural epigenetic mechanism to functionally correct the gene dosage imbalance of DS at different stages of differentiation. This powerful strategy will allow for a suite of studies in different cell types that may elucidate the specific cell-types and developmental origins of pathology in DS. Moreover, this approach can address at what points these pathologies are reversible.

For example, in this thesis we demonstrated the enhanced neurogenic capacity of neural stem cells in which *XIST* expression was not initiated until halfway through the differentiation process, implying a correctible defect in NSCs. This would provide a particularly powerful technique to study postmitotic neurons, whose possible ongoing dysfunction (Caviedes et al., 1990; Weick et al., 2013) could account for at least part of the cognitive disability in DS individuals. While we have shown that neurons are capable of maintaining *XIST* expression and chromosome silencing, if DS neurons were also shown to support the *initiation* of silencing, then this would afford a unique perspective into how trisomy 21 affects the ongoing function of a neuron. Comparison of trisomic and euploid cells with an identical differentiation history could eliminate several confounding variables unrelated to trisomic transcription and could more definitely address these key questions.

The ability to initiate chromosome-wide silencing in differentiated cells has wide-ranging implications beyond the dosage compensation field. It clearly illustrates the natural epigenetic plasticity of differentiated cells that has been increasingly appreciated in recent years and offers a previously unachievable opportunity to study the effects of transcriptional correction of trisomic cells at different stages of development.

Identification of affected pathways in DS neurodevelopment using dosage compensation

In addition to demonstrating the ability for differentiated cells to initiate dosage compensation, we also discovered a specific effect that reducing trisomic cells to functional disomy had on their neurogenic potential. We saw that cells with a silenced third copy of chromosome 21, either from the onset of differentiation or halfway through, formed a higher proportion of neurons than trisomic cells. This was initially revealed by single-cell RNA sequencing analysis and confirmed cytologically in multiple transgenic iPSC clones. Transcriptional analysis further identified a significant difference in Notch pathway genes between trisomic and disomic cell states. The well-known involvement of the Notch pathway in controlling the transition of NSCs to neurons suggests that the downregulation of this pathway in silenced cells may be responsible for their enhanced neurogenic potential or kinetics. Additionally, the finding that one of our most significant hits, *TTYH1*, has been reported to function upstream of Notch signaling (Kim et al., 2018), suggests a new potential area of investigation to look for regulators of *TTYH1* or its upstream mechanisms encoded on chr21.

The association of chr21 genes to specific cellular phenotypes has been a major challenge in the field, and future studies could utilize classical biological perturbation

tools for interrogating the involvement of single chr21 genes in generating this phenotype. One association that has been proposed, the dependence of an over-production of interneurons in a mouse model on the *Olig1/2* genes (Chakrabarti et al., 2010), has recently been suggested to play a similar role in a human *in vitro* cell model (Xu et al., 2019). However, substantial evidence from other mouse models and human studies suggest this association may not be a consistent effect across model systems (Bhattacharyya et al., 2009; Huo et al., 2018; Ross et al., 1984; Wisniewski, 1986). Perhaps one of the few reproducible associations with strong evidence in patients is the role of *APP* in AD pathology. Thus, novel strategies to elucidate genotype-phenotype associations are needed, and *XIST*-based tools being developed in our lab to inducibly silence small portions of chromosome 21, including the important *DYRK1A* locus, could play a role in narrowing key chr21 loci in DS pathology.

We believe our phenotypic findings in chapter II reflect the ability of inducible dosage compensation to identify chr21-dependent cellular effects because of the uniquely well-controlled nature of this system. As discussed in that chapter, we did not identify a difference in the neurogenic capacity between the trisomic and disomic cell lines examined. As detailed in chapter III, a wide assortment of variables affects iPSC disease modeling in general (Table III-1), most of which are mitigated or eliminated by comparison of trisomic cells to dosage corrected cells in the same culture.

For example, we found that the dosage corrected cells preferentially differentiated over trisomic cells while in the same exact environment with identical Notch ligand expression. The initial preferential differentiation of dosage compensated cells could be due to a cell autonomous effect of reduced intrinsic Notch signaling.

However, the Notch pathway acts through well-known mechanisms of lateral inhibition (Gaiano and Fishell, 2002) whereby a newly differentiated neuron expresses Notch ligands that enhance Notch signaling in adjacent cells, reducing their propensity to differentiate. Thus, newly-differentiated dosage compensated cells would upregulate Notch ligand expression, preventing differentiation of adjacent cells and potentially amplifying the difference seen between disomic and trisomic cells. This possibility has implications beyond DS for studying many developmental diseases. Examining disease and non-disease cells grown together has the potential to identify differences that could otherwise be masked by a variety of non-disease related factors.

Of course, the use of *XIST*-mediated chromosome silencing is not without its own set of limitations. As shown in chapter II, corrected *XIST*+ cells are more likely than *XIST*- cells to also express the tetracycline transactivator that is required for *XIST* expression in this system. While we did not see a difference in Notch gene expression between TET+ and TET- cells in a non-*XIST* transgenic line also treated with dox, other findings from our lab have shown that many genes are differentially expressed when such transactivator-containing cells (without an inducible *XIST* transgene) are treated with dox (data not shown). Thus, inclusion of such a line is important in any studies using an inducible system. Additionally, it is possible that *XIST* expression itself, independent of its chr21 silencing function, could somehow affect the neural differentiation potential of NSCs. We consider this to be a remote possibility based on the biology of *XIST* and its ubiquitous expression in female cells, but future studies could also include plasmid-based *XIST* expression as a control for this unlikely possibility.

Ultimately, we have demonstrated the ability of the recently-developed system of dosage compensation to correct neurodevelopmental defects in trisomy 21, potentially through its action on specific signaling pathways. Additionally, the demonstrated ability of this system to dissect precise developmental windows of opportunity has enormous potential to advance our understanding of DS pathogenies, which could ultimately lead to the development of conventional therapeutics targeting specific affected pathways. However, there has been suggestion in the literature that many aneuploid phenotypes could be caused by overall cellular stress caused by trisomic transcription and/or translation (Bonney et al., 2015; Oromendia et al., 2012; Sheltzer et al., 2012), which would imply that there may not be simple gene-phenotype associations for at least some aspects of DS. Addressing these phenotypes would thus necessitate a chromosome silencing approach, as discussed further in chapter V.

Cerebral organoids to study DS neurodevelopment

Challenges to organoid modeling of subtle neurodevelopmental phenotypes

In chapter III, we evaluated several novel organoid protocols with the goal of modeling trisomy 21 neurodevelopment in a more biologically relevant three-dimensional environment. Our smaller early experiments identified promising alterations in cell type representation between trisomic and disomic organoids. However, due to high organoid-to-organoid variability we questioned if these differences were genuinely due to trisomy, and thus pursued expanded studies utilizing large numbers of organoids and three isogenic lines per condition, which could not confirm these conclusions. Thus, we did not identify consistent cell type representation differences due to trisomy in this

system. This raises the question as to whether any strong cellular phenotypes should be expected at this stage of DS neurodevelopment.

As discussed in detail in chapter III, there are a number of sources of variation that we worked to control for in this study (Table III-1). While we believe this is a stronger study than most, variability between samples could still have influenced our results. While the experimental design has likely minimized false positive findings, the significant degree of variability in cell type representation between subclones of the same condition could mask any subtle differences in cell type representation that are due to trisomy. Additionally, while some lines had consistent cell type composition from differentiation to differentiation, others had a drastically different cell make-up from one experiment to the next. This could imply either epigenetic or environmental factors that significantly affect the differentiation of organoids and could make elucidation of cell type differences due to trisomy quite difficult. As in any *in vitro* cell culture modeling, it is also possible that the experimental conditions and exogenous factors present in the media, like mitogens and neurotrophins, could override real biological differences between chr21 states and lead to roughly similar cellular composition. Finally, it is possible that at the modeled stage of neurodevelopment, there is negligible or no effect of trisomy 21 on cerebral organoid development. The same factors described above could also explain the associated finding of very few non-chr21 gene transcription changes detected in the cerebral organoids.

Future studies utilizing isogenic iPSC clones and cerebral organoids to model DS neurodevelopment should take into account these numerous possible sources of variability. Additionally, future studies could further improve upon this initial investigation

by increasing the number of isogenic clones used, which could increase the power to detect true differences related to chr21 state. Minimizing passage number from the initial derivation of isogenic lines could also decrease the chance for lines to undergo significant epigenetic drift over time in culture. Finally, utilizing an even more directed organoid generation protocol could decrease the number of different cell types formed and thus further minimize organoid to organoid and batch to batch variability. Several such brain-region-specific protocols have been described in the literature (Bagley et al., 2017; Birey et al., 2017; Monzel et al., 2017; Qian et al., 2016; Xu et al., 2019), and very recent advances in organoid generation protocols (Velasco et al., 2019) promise to further improve the reproducibility of results between organoids, batches, and lines.

Prospects and hurdles towards combining dosage compensation and cerebral organoids

Of course, many of the sources of variability described above could be completely eliminated by comparing cells within the same organoid, those with three transcriptionally active copies of chr21 and those that, due to *XIST*, have two. In chapter II, we demonstrated that this approach could be used in a monolayer culture system to reveal both a difference in the propensity for dosage corrected cells to terminally differentiate, and a potential mechanism for this finding. In principle, a similar approach could be used to study functionally disomic and trisomic cells in long-term organoid cultures. This would have the advantage over short-term monolayer cultures of being able to study a wider range of cell types, such as later-born astrocytes, inhibitory neurons, and upper-layer projection neurons. Additionally, studying more mature neurons has the potential to address if there are ongoing functional deficits in

synaptically active neurons, which could provide a more therapeutically accessible target for pharmaceutical intervention.

While this was our initial goal and represents an exciting area of potential future studies, we struggled to maintain long-term *XIST* transgene expression in aged organoids, as described in further detail in the appendix. However, some of the insights that we've come to as we attempted to mitigate this common problem in transgene studies could be applied to generate a system capable of long-term *XIST* expression. Since natural or endogenous promoters may be less likely to be silenced, this could include using brain-specific promoters to drive either transactivator or *XIST* expression, especially if temporal inducibility is not a requirement for the particular experimental question. Additionally, in an inducible system, constitutive expression of a transactivator-demethylase construct may potentially prevent methylation of the tetracycline response element in fully differentiated neurons (as further discussed in the appendix).

Still, several potential caveats of this approach must be taken into consideration. One difficulty is that even if a considerable number of cells remain *XIST+* in aged organoids, it would be important to compare this number to the starting proportion of *XIST*-expressing cells. If there is significant discrepancy in these proportions, then there is likely a large population of cells that expressed *XIST* at one point in the past or are derived from *XIST*-expressing cells. This could complicate interpretation of any differences found between these populations, because it is possible that seemingly *XIST-* cells still retain some transcriptional silencing due to past *XIST* expression, or that their developmental trajectory was altered by their dosage compensated progenitors.

Ultimately, if a robust *XIST* expression system could be put in place with minimal change in the proportion of *XIST* expressing cells over time in culture, this could prove to be a powerful resource for revealing the true effects of trisomy 21 on DS neurodevelopment.

Concluding remarks

Together, the results presented in this thesis demonstrate the utility of a powerful new approach to studying DS neurodevelopment. I have demonstrated the ability of *XIST* to initiate chromosome-wide transcriptional silencing in differentiated cells, which opens the door to a wide variety of studies examining the dynamics of *XIST*-mediated transcriptional silencing in many cells types, allows for precise determination of critical developmental steps in DS pathogenesis, and overcomes a perceived obstacle towards the application of *XIST* transgenes as a therapy for DS. I identify a specific alteration in neurogenesis that is potentially driven by alterations in a defined pathway, Notch, that may be responsible for key aspects of altered DS neurodevelopment. Finally, I explored the ability of cerebral organoids to model DS neurodevelopment in a natural three-dimensional environment which could allow for studying more mature *in vitro* cell types that are altered in DS. Overall, I hope these studies provide some insight into the cellular events that lead to cognitive disability in DS, and that these insights may someday be translated into therapies that improve the lives of patients and families with DS.

CHAPTER V : *XIST*: from natural trisomy rescue to potential therapy

The mechanisms of female X-inactivation for dosage compensation have been studied for decades, and yet the therapeutic possibilities of this unique biological phenomenon have hardly been considered. Chromosomal disorders, which are detected in nearly 0.6% of newborns (Shaffer and Lupski, 2000), have largely been regarded as beyond the reach of modern advances in gene therapy due to their complex underlying genetic basis. Largely unappreciated case studies in patients with unbalanced X;autosome (X;A) translocations have demonstrated the remarkable ability of *XIST* to rescue otherwise lethal trisomies. Additionally, recent breakthroughs have confirmed the ability of *XIST* transgenes to dosage correct chromosome 21 transcription in Down syndrome (DS) iPSCs. Of course, many challenges remain regarding translating this natural mechanism of dosage compensation to disorders of gene dosage imbalance. Here, I review the case study literature on trisomy rescue via X;A translocation, discuss the state of the art and limitations of current gene therapy technologies, and evaluate the biological questions that still need addressing to bring this transformative strategy for chromosomal disorders closer to reality.

XIST rescues lethal trisomies in patients

Due to the increased dosage of hundreds to thousands of genes, trisomies are nearly always incompatible with life. The few exceptions include small chromosomes with relatively fewer genes, such as chr21, trisomy for which causes DS, and the X

chromosome, which possesses the unique ability to undergo dosage compensation via the gene *XIST*. In fact, many women with trisomy X, who have two silenced X chromosomes instead of the usual one, lead perfectly normal lives and show no signs of harboring an extra chromosome in each of their cells (Tartaglia et al., 2010). These individuals demonstrate two findings critical for the translational potential of *XIST*: the complete compatibility of two *XIST*-expressing chromosomes with normal health, and, as evidenced by all XX women, that it is not the physical presence of millions of basepairs of extra DNA that is pathogenic in trisomy, as has been proposed by others (Plona et al., 2016), but rather their transcriptional output. Trisomy X patients demonstrate the ability of *XIST* to silence multiple chromosomes and render trisomy for a chromosome containing thousands of genes non-pathogenic. Additionally, these patients demonstrate that the presence of multiple *XIST* RNA coated Barr bodies in every nucleus of a person is non-toxic, and indeed necessary to rescue an otherwise lethal chromosomal abnormality. However, it is rare patients with X;A translocations that establish the power of this remarkable RNA to function beyond the X chromosome.

One such case involves a boy with Klinefelter syndrome and a few mild malformations despite the presence of nearly an entire extra copy of chromosome 14, which contains nearly 2,000 genes (Allderdice et al., 1978). This condition, generally incompatible with life, was possible due to an X;14 translocation containing *XIST*. Further analysis demonstrated that the translocated chromosome late replicating, indicating it was transcriptionally silenced (Allderdice et al., 1978), leading to effectively disomic chromosome 14 dosage. Another case involved a woman with two normal copies of chromosome 9 in addition to a derivative chromosome containing the long

XIST-containing arm of the X chromosome translocated to the long arm of a third copy

of chromosome 9 (Leisti et al., 1975). This patient did exhibit morphological

abnormalities and learning disabilities, but considering her karyotype, the effects were

remarkably mild due to the silencing of the trisomic genetic material. Examination of

cultured cells from these patients demonstrated that *XIST* RNA from the translocated

chromosome spread across the autosomal chromatin, albeit incompletely, along with

hallmarks of inactivation (Hall et al., 2002b). Finally, a third case of a girl with mild

developmental delay and dysmorphic features was shown to be caused by trisomy 15

with an X;15 translocation (Stankiewicz et al., 2006). Even partial trisomy for 15q has

been associated with severe cognitive disability and dysmorphic features (Kristoffersson

and Bergwall, 1984; Pedersen, 1976), further implying that at least a large portion of

15q is silenced in this patient leading to her mild phenotype. Together, these cases

demonstrate that *XIST* is capable of effective autosomal silencing that can rescue

otherwise lethal trisomies and lead to relatively mild phenotypes in patients.

Recent work in our lab has further applied these natural phenomena to the most

common autosomal trisomy, DS (Jiang et al., 2013). We inserted an inducible transgene

for *XIST* into one copy of chromosome 21 in DS iPSCs as a proof of concept that these

natural phenomena could be reproduced in the lab using modern stem cell and gene

editing technologies. Importantly, this work demonstrated that an *XIST* transgene

inserted into an autosome leads to robust transcriptional silencing chromosome-wide, in

addition to deposition of several heterochromatin marks associated with X-inactivation

as well as DNA methylation. While this work opened the door to the possibility of a

"chromosome therapy" for DS and other trisomies, several biological questions and technological barriers still need to be addressed.

Biological considerations for chromosome therapy

The general concept of chromosome therapy has been demonstrated in living patients with trisomies that would be otherwise lethal were it not for the transcriptional silencing action of *XIST*. Additionally, our lab has provided a proof-of-concept that this powerful technique can be harnessed *in vitro* to target a trisomic chromosome and lead to its transcriptional inhibition. However, patients with a rescued trisomy have been dosage compensated by *XIST* since conception, and thus there are several outstanding questions relating to the function of *XIST* in differentiated cells and the reversibility of cellular phenotypes later in development that must be addressed in order to evaluate the viability of chromosome therapy.

Somatic cell silencing

In order for chromosome therapy to be feasible *in utero* or postnatally, *XIST* must be able to initiate silencing in differentiated cells. As discussed in detail in the introduction and in chapter I, inducing *XIST*-mediated silencing in differentiated cells, potentially with the exception of hematopoietic cells (Savarese et al., 2006), has long been considered unfeasible because these cells are thought to lack the epigenetic plasticity to respond to *XIST*. I have shown in iPSC-derived neural stem cells that inducing silencing in differentiated cells is no longer a strict barrier and is not a unique property of hematopoietic progenitors. I have shown that silencing indeed takes place in differentiated neural stem cells, albeit requiring a longer timeframe than in pluripotent

cells. It remains possible that certain cell types will be found to be incapable of initiating silencing via *XIST*. This requires empirical testing in a variety of cell types that are particularly affected in trisomy. I have shown evidence that S-phase is not required for *XIST* expression, localization, and recruitment of early marks of heterochromatin, however the ability of postmitotic cells to initiate silencing should be tested in such cells capable of initiating *XIST* transcription. The confirmed competence of differentiated cells to enact the chromosome silencing process is a vital step forwards towards bringing chromosome therapy closer to reality.

Correction of cellular function

Perhaps the biggest unknown in chromosome therapy is whether transcriptional silencing of the trisomic chromosome in a cell will lead to improvement in cellular function, and ultimately improved health and well-being of patients. This thesis demonstrates that early neurodevelopmental defects, such as impaired neurogenesis, can be corrected with chromosome silencing. Additionally, we have shown that known hematopoietic defects can be prevented when silencing is initiated in iPSCs (Chiang et al., 2018). Importantly, in this thesis we have shown that initiation of silencing in differentiated cells can also improve neurogenesis, suggesting that initiating silencing in pluripotent cells is not a requirement for phenotypic benefits.

Currently, diagnosis of DS and other trisomies is only possible at around 8 weeks of gestation. At this point, neurogenesis has already begun and will continue until the third trimester (Bystron et al., 2006; Malik et al., 2013). This provides a potential window of opportunity for correcting some of the earliest defects in neurogenesis via *in utero* delivery. Clearly, animal models of trisomy silencing are required to gain an

understanding into whether correction at different developmental stages could mitigate trisomic phenotypes, such as delayed neurogenesis. Even if the earliest effects of trisomy are already in place at the time of intervention, later cellular events that have been found to be dysregulated in DS brains, such as defective myelination (Olmos-Serrano et al., 2016), could still be mitigated since the myelination process continues well past adolescence.

Additionally, trisomy 21 may not just impact cell differentiation and development, but also cellular function. Chromosome 21 encodes a number of ion channels that are expressed in the brain (Cramer et al., 2010; Lipsky and Goldman, 2003), and may affect ongoing function of trisomic neurons, which evidence indicates is altered *in vitro* (Caviedes et al., 1990; Weick et al., 2013). If *XIST* expression and chromosome silencing can be initiated in postmitotic neurons, it is possible that reversal of these ongoing defects could improve neuronal function and potentially cognition.

Perhaps most significantly, the overproduction of amyloid precursor protein (APP) is thought to be the driving factor in the extremely high prevalence of early-onset AD in DS patients (Zigman et al., 1996). Reducing the transcription of this gene to functionally disomic levels in young DS patients prior to detectable AD pathology (Mann and Esiri, 1989) could prevent the development of this devastating neurodegenerative process. While many individuals with DS and their families have adjusted to the challenges of living with DS, there is considerable concern about the looming prospect of cognitive decline and prolonged course of AD-related dementia in relatively young individuals.

While the focus of this thesis has been on the cognitive deficits in DS, chromosome silencing, like all gene therapies, is of course potentially applicable to any tissue and cell type. Other cell systems, such as the hematopoetic system, could also be amenable to *ex vivo* therapies, which have a much longer history of success in clinical trials and offer potentially higher efficiencies (Grossman et al., 1994; Hacein-Bey-Abina et al., 2002; Naldini, 2011). Additionally, while *XIST*-mediated chromosome therapy may be the only prospect for reducing cells to functional euploidy *in vivo*, *ex vivo* approaches could also involve other strategies for chromosomal correction, such as ring chromosome induction (Bershteyn et al., 2014) or selectable chromosome knock-out (Li et al., 2012).

There is considerable pre-clinical testing that needs to be undertaken in order for the prospect of chromosome therapy to gain further momentum. We have demonstrated that there is no longer evidence for the previously perceived hard barrier preventing differentiated cells from initiating chromosome silencing. Additionally, we have shown that chromosome silencing is capable of correcting some *in vitro* phenotypes, even when induced in differentiated cells. Further work in mouse models of trisomy and advanced human cellular models, such as cerebral organoids, is necessary to determine what the developmental limits are for dosage compensation to correct trisomic transcription and cellular phenotypes.

Technological hurdles towards chromosome therapy

The concept for chromosome therapy reduces the problem of hundreds of trisomic genes down to the challenge of insertion of one gene. In this respect, revolutionary progress in gene therapy for less genetically complex single gene

disorders becomes relevant to DS and other trisomies. However, chromosome therapy with *XIST* would require other technical hurdles to be overcome. In its current form, chromosome therapy would require efficient delivery of a large *XIST* DNA transgene to a high proportion of cells followed by allele-specific insertion into one trisomic chromosome and subsequent expression of the transgene leading to transcriptional silencing of the chromosome.

Efficient transgene delivery *in vivo*

The necessary number of cells required for phenotypic benefit varies from disease to disease and from tissue to tissue. While it is hard to predict the precise number of corrected cells needed to improve DS phenotypes, it is safe to assume that delivery will need to be much more efficient than for disorders of secreted enzyme deficiency like β-thalassemia (Ricciardi et al., 2018), for example. Additionally, unlike for loss of function disorders for which plasmid-based expression or random genomic integration is suitable, the targeted insertion of an *XIST* transgene requires efficient delivery in the same cells of both gene editing factors and the transgene itself.

Luckily, great strides in gene delivery techniques have been made in recent years, largely focused on viral vectors. While randomly integrating vectors, such as lentiviruses, would not be amenable to chromosome therapy requiring targeted insertion, others, such as adeno-associated viruses (AAV) do not readily integrate and instead form stable episomes carrying transgenes capable of targeted insertion (Hirsch et al., 2010). Several serotypes of AAV have been shown to effectively cross the blood brain barrier (BBB) leading to robust long-term expression in neurons and astrocytes (Gray et al., 2010; Miyake et al., 2011) and novel capsid selection methods have led to

the creation of an increasing number of neurotrophic AAV variants (Deverman et al., 2016). Most significantly, the promising results of clinical trials (Lowes et al., 2019) and recent FDA approval of intravenously delivered AAV-based therapy for spinal muscular atrophy reinforces the incredible progress that has been made in developing viral vectors with efficient delivery to cells even across the BBB. One important limitation of many viral vectors, and AAV in particular, is their limited cargo capacity of ~5kb. This poses a particular problem for *XIST*, which has a cDNA of ~17kb, as will be discussed in the next section. Recombinant viruses with larger cargo capacity have been identified and used in preclinical trials, but their safety profiles are not as well described (Lachmann, 2004; Sweeney et al., 2017).

In addition to viral delivery systems, non-viral approaches have also seen enormous progress in recent years. While non-viral systems lack the natural tropism of viral vectors and therefore generally have lower delivery efficiency, their main advantage is lower toxicity and immunogenicity. Lipid nanoparticles (LNPs) are currently the leading non-viral delivery method, with clinical trials currently underway for small RNA-containing LNPs, and progress is being made towards encapsulating and delivering mRNAs and DNA (Cullis and Hope, 2017). Additionally, viral-like particles containing gene editing tools and repair templates have also been developed (Mangeot et al., 2019), along with hybrid models utilizing LNP-delivered gene editing tools and virally-delivered repair templates (Yin et al., 2016). Taken together, as this fast-moving field progresses with ever-more efficient technologies, chromosome therapy will be able to take advantage of delivery tools with broad applicability to any gene therapy requiring highly efficient delivery.

Smaller functional *XIST* transgenes

As alluded to above, the *XIST* gene poses a particularly difficult problem for delivery technologies due to its large size. Additionally, because it does not encode a protein, elucidation of the function of each of its domains is particularly difficult. Despite this, progress has been made toward identifying regions of the *XIST* genes necessary for certain attributes of this lncRNA, such as recruitment of heterochromatin modifiers and gene silencing. Most significantly, the A-repeat region of *XIST* has long been known to be necessary for its silencing function (Wutz et al., 2002) and has also been shown to be sufficient for silencing of adjacent reporter genes (Minks et al., 2013). Recent work suggests that the A-repeat alone is also capable of silencing endogenous genes several Mb away from its site of insertion (Valledor et al., in preparation). Intriguingly, the A-repeat has been shown to modestly repress gene expression when recruited to a genomic location via catalytically inactive dCas9 enzymes (Shechner et al., 2015), suggesting that integration into the chromosome may not be required for its silencing function. Together, there is considerable evidence to suggest that insertion of a full-length *XIST* transgene may not be necessary to lead to a relevant degree of transcriptional repression, which could considerably lower the barriers to chromosome therapy.

Directed allele-specific transgene insertion

Once the transgene can be delivered to a large proportion of cells it must then be inserted into one copy of the trisomic chromosome in order to initiate transcriptional silencing. The first step in this process is accurate generation of double strand breaks (DSB) using a targeted nuclease. This has been perhaps the most prolific area of

research particularly in the past 5 years since the discovery of the easily programmable CRISPR-Cas9 system (Dai et al., 2016). While there are still some limitations regarding where in the genome a nuclease can be targeted, the rapid proliferation of newly discovered and engineered nucleases with varying protospacer adjacent motif (PAM) sequences promises to render nearly the entire genome available for editing. The specificity of these nucleases has been demonstrated to be capable of allele-specific genome editing in disease loci (Smith et al., 2015), yet a remaining concern is the degree of off-target DNA cleavage, which could potentially lead to off-target integration and, more likely, off-target mutagenesis (Fu et al., 2013). This has led to the development of progressively more precise nuclease variants with minimal off-target effects (Kleinstiver et al., 2016) as well as a move towards transient expression of nuclease components in an attempt to minimize off-target DSBs (Yin et al., 2016).

Once the DSB is created, the transgene must be efficiently inserted into the genome. In dividing cells, homology directed repair (HDR) is capable of inserting a donor DNA template at the cut site, albeit at a relatively low frequency (Hirsch et al., 2010). However, the efficiency of HDR drops to extremely low levels in non-dividing cells, such as neurons, necessitating a different insertion strategy. Several such strategies have been developed (Nami et al., 2018; Yamamoto et al., 2015), one of which is termed homology-independent targeted integration (HITI). HITI utilizes non-homologous end joining machinery which is active in non-dividing cells and has been shown to be extremely efficient *in vitro* with close to 50% of cells containing the targeted gene modification (Suzuki et al., 2016). *In vivo* editing using HITI is significantly more

efficient than HDR and ongoing efforts in numerous labs are resulting in substantial incremental improvements.

Stable transgene expression

As described in chapter I of this thesis as well as in the appendix, transgene silencing is a common occurrence even in *in vitro* models of chromosome silencing. *XIST* possesses the unique feature of laying down several redundant layers of heterochromatin marks. Thus, once the repressive chromatin structure is triggered by *XIST*, the RNA is no longer strictly required to maintain the silent chromosome state (Brown and Willard, 1994). However, expression of the *XIST* transgene will be absolutely required to initiate chromosome silencing in cells. Additionally, as described in chapter I, the silencing process can be significantly prolonged in differentiated cells, requiring *XIST* expression for at least several weeks in neural cells to lead to complete chromosome silencing.

Transgene silencing has also affected early gene therapy trials (Bestor, 2000), with one patient dying of his underlying disease likely due to the progressive silencing of the therapeutic gene (European Society of Gene Therapy (ESGT), 2006). Eukaryotic cells have evolved considerable defenses to prevent ectopic gene expression, particularly from viral sources, which makes their use a particular challenge. Several strategies can be employed to optimize transgene expression such as careful selection of ubiquitous or cell-specific promoters, inclusion of an efficient polyadenylation signal to the transgene of interest, as well as the inclusion of introns that can increase transgene expression (Powell et al., 2015).

Chromosome position effects also have a major impact on transgene silencing but are difficult to predict. Selection of purported "safe-harbor" locations for insertion of the transgene would be impractical for a chromosome therapy strategy due to the limited number of such sites which are not present on chr21 or other chromosomes compatible with trisomy. While certain strategies can be employed to maintain a euchromatic state at the site of insertion, such as utilizing a ubiquitous chromatin opening element (UCOE) (Kunkiel et al., 2017), ultimately selection of a target site may require empirical testing without the guarantee that a universally permissive site exists (Papapetrou and Schambach, 2016). The pervasive issue of transgene silencing is poorly understood and remains a technological hurdle for effective gene replacement therapies but can likely be overcome through a process of trial and error including testing in suitable human cell models.

The biological tools are largely in place to accurately insert a transgene into one copy of a trisomic chromosome *in vivo*, at least in theory. However, considerable incremental progress on multiple technical fronts is still required in order to increase the efficiency of each step described above, and to provide a meaningful chance at clinical improvement in trisomies.

Conclusions

Thanks to recent advances in delivery systems, editing technologies, and improved safety profiles, the gene therapy revolution is finally making a significant impact on the lives of patients and families with rare monogenetic disorders. The rescue of natural trisomies by X;A translocations and the recent proof-of-concept that this strategy can be harnessed in the lab provide hope that *XIST*-mediated chromosome

therapy may one day offer a viable therapeutic approach to individuals and families with

common trisomies like DS. Figure V-1 summarizes the biological considerations, some

of which have been addressed in this thesis and previous work in the lab, as well as the

technological barriers towards the development of chromosome therapy. Significant

progress must still be made for this dream to become reality, and several important

biological questions about potential efficacy remain unanswered. This new therapeutic

strategy to trisomy also brings with it many important ethical considerations, including

the need to safeguard the interests of vulnerable populations such as children with

intellectual disabilities, and the need to consider the potential germline effects of

targeted *XIST* insertion. Ultimately, we hope that the decades of research into the

unique natural phenomenon of dosage compensation will finally be realized into

promising strategies for improving the health and well-being of individuals with DS and

other chromosomal duplication disorders.
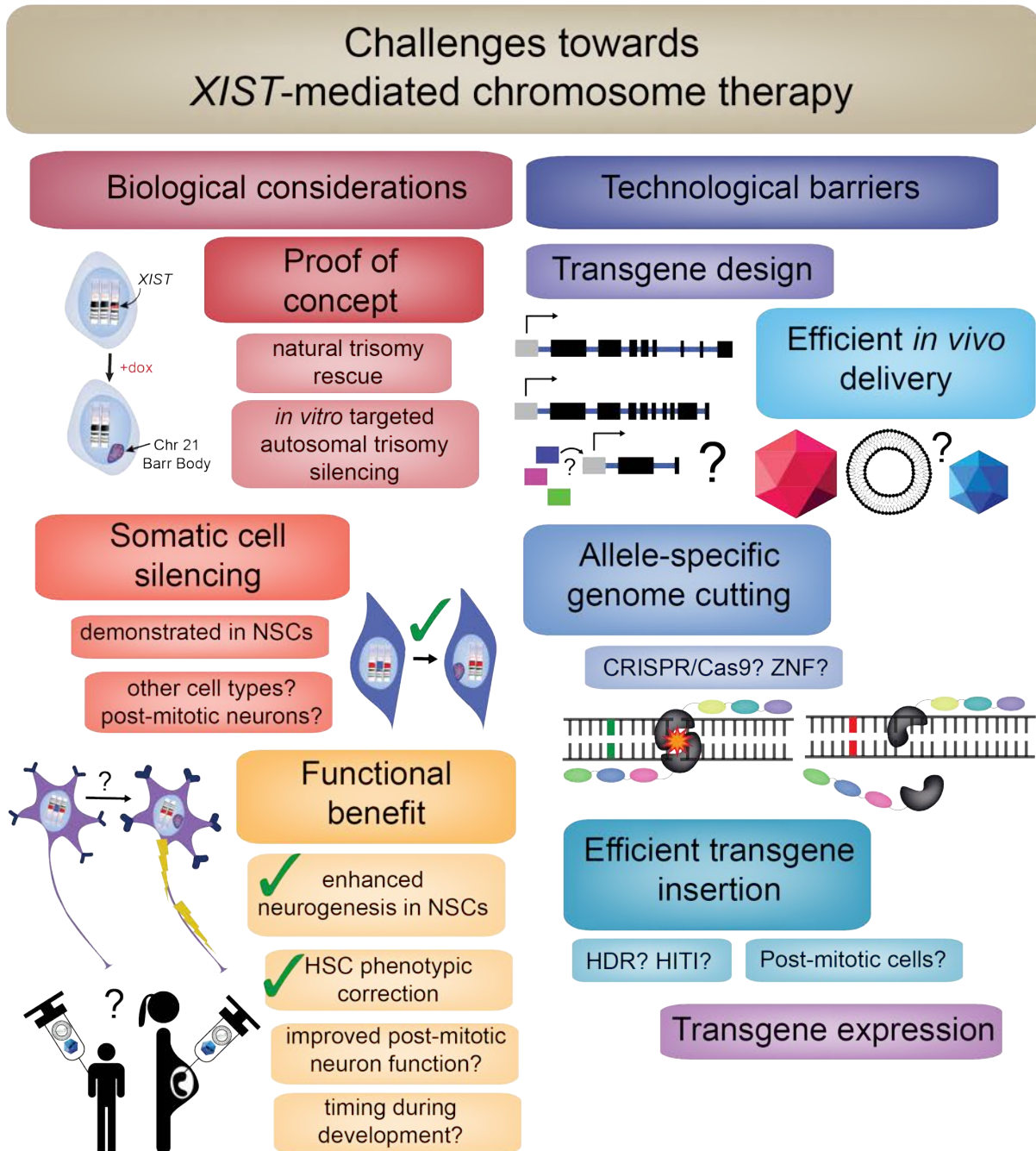
Figure V-1: Summary of biological considerations and technical hurdles towards chromosome therapy

APPENDIX : Transgene silencing: *XIST* gets a taste of its own medicine

Preface

All of the experiments and analysis described in this appendix were performed by me, with help from Meg Byron growing and processing organoids treated with antibiotics. The lentiviral plasmid was created by Melvys Valledor. Zdenka Matijašević performed experiments treating iPSCs with antibiotics that informed some of the studies described here.

Introduction

Transgene silencing is a broad problem affecting numerous biological systems and transgene vectors, and several pathways of silencing have been identified (Matzke et al., 2000). In general, transgenes are thought to be silenced by genome defense systems that have evolved over millions of years to prevent parasitic genomic elements, such as viruses, from invading and overtaking host machinery (Waterhouse et al., 2001).

In this thesis I have described the usage of an *XIST* transgene as a powerful experimental system with which to study DS pathogenesis. Additionally, I have outlined the potential therapeutic applications of this unique gene for treating trisomies. However, as alluded to in the introduction, in the course of this thesis research I and others in the lab have experienced significant *XIST* transgene silencing using our *in vitro* system. As described in chapter V, transgene silencing can have drastic

consequences when it involves a therapeutic gene replacement. While the stakes were not nearly so high in this case, transgene silencing poses a problem for effectively studying *XIST*-mediated silencing as an experimental tool and must be addressed before therapeutic approaches can be considered.

Results

XIST silencing in iPSCs

We first noticed a transgene silencing problem in iPSCs. While several early passages of the transgenic *XIST*-inducible cell lines (Jiang et al., 2013) showed stable expression of *XIST* in a very large fraction of iPSCs (Jun Jiang, personal communication), we noticed that over time, many cells lost the expression of the *XIST* transgene (Figure A-1A). The loss of *XIST* expression was patchy, in that some entire colonies would robustly express the transgene, while others had no *XIST*+ cells, and still others had a cluster of *XIST*+ cells clearly separated from cells with a silenced transgene. This suggested that the iPSCs were epigenetically silencing either the reverse tetracycline-controlled transactivator (rtTA) required for inducible *XIST* expression, or the tetracycline response element (TRE) upstream of the *XIST* transgene. Attempts to subclone the cells in order to obtain a stable line with a high fraction of *XIST* expressing cells were unsuccessful. Thus, many of the neural differentiation experiments conducted in this thesis used a heterogenous starting population of iPSCs with roughly 70-80% of cells expressing *XIST* at the onset of differentiation.

XIST silencing in aged organoids

In addition to a reduced starting population of *XIST*-expressing cells, I also ran into a much more drastic example of transgene silencing in long-term differentiation cultures. I found that in organoids grown for 90 days and treated with dox from the onset of differentiation, there was an extremely low fraction of *XIST*-expressing cells (Figure A-1B). We quickly made the realization that the "ubiquitous" promoter that was used to drive rtTA expression in our transgenic lines, EF-1α, has been reported to be completely silenced in human iPSC-derived neurons (Guillaume et al., 2006). This suggested to us that replacement of rtTA driven by another promoter could rescue the transgene silencing phenotype that we see in aged organoids. To test this hypothesis, I generated lentiviruses carrying rtTA driven by the ubiquitin C promoter and containing a fluorescent marker (Figure A-1C – pLV-rtTA). 7 days after transduction of day 83 organoids that had been treated with dox since the onset of differentiation (d0 dox), we saw restricted patches of red fluorescent cells, indicating patchy transduction of the organoids (data not shown). After fixing, sectioning, and performing RNA FISH on these organoids, we saw areas with high numbers of *XIST*+ cells (Figure A-1B). Such a high degree of *XIST* expression was unprecedented in d90 organoids and was not seen in organoids without lentiviral transduction. This encouraged us to generate a stable cell line containing rtTA driven by CAG, a promoter that has been shown to resist silencing in human neurons (Muotri et al., 2005), inserted into one allele of the AAVS1 site using CRISPR/Cas9 technology (Figure A-1C – CAG-rtTA). This generated a cell line that was resistant to geneticin (G418) due to the neomycin resistance gene present in this

Figure A-1: Rescuing transgene expression with reintroduction of rtTA

A) Representative image of RNA FISH in iPSC colony with a patch of *XIST* expressing cells. B) RNA FISH for *XIST* in 90d organoids treated with dox from the onset of differentiation. Bottom panel demonstrates increase in *XIST* expression with pLV-rtTA transduction. C) Schematic of rtTA expression cassettes used in this study. Top two panels are cassettes inserted at AAVS1 locus on chr19. Bottom panel is randomly integrating lentiviral plasmid. Note the lack of dedicated promoter for NeoR in CAG-rtTA. D) RNA FISH for *XIST* in organoids at days 14, 31, and 49 of differentiated treated with dox from day 0 and either treated with no antibiotic, puromycin, or G418. E) Representative phase contrast images of organoids at day 49. F) IF for SOX2 in d49 organoids treated with puro or no antibiotics. Scale bars for (A, B, D, and F) are 100μm. Scale bars for (E) are 1mm.
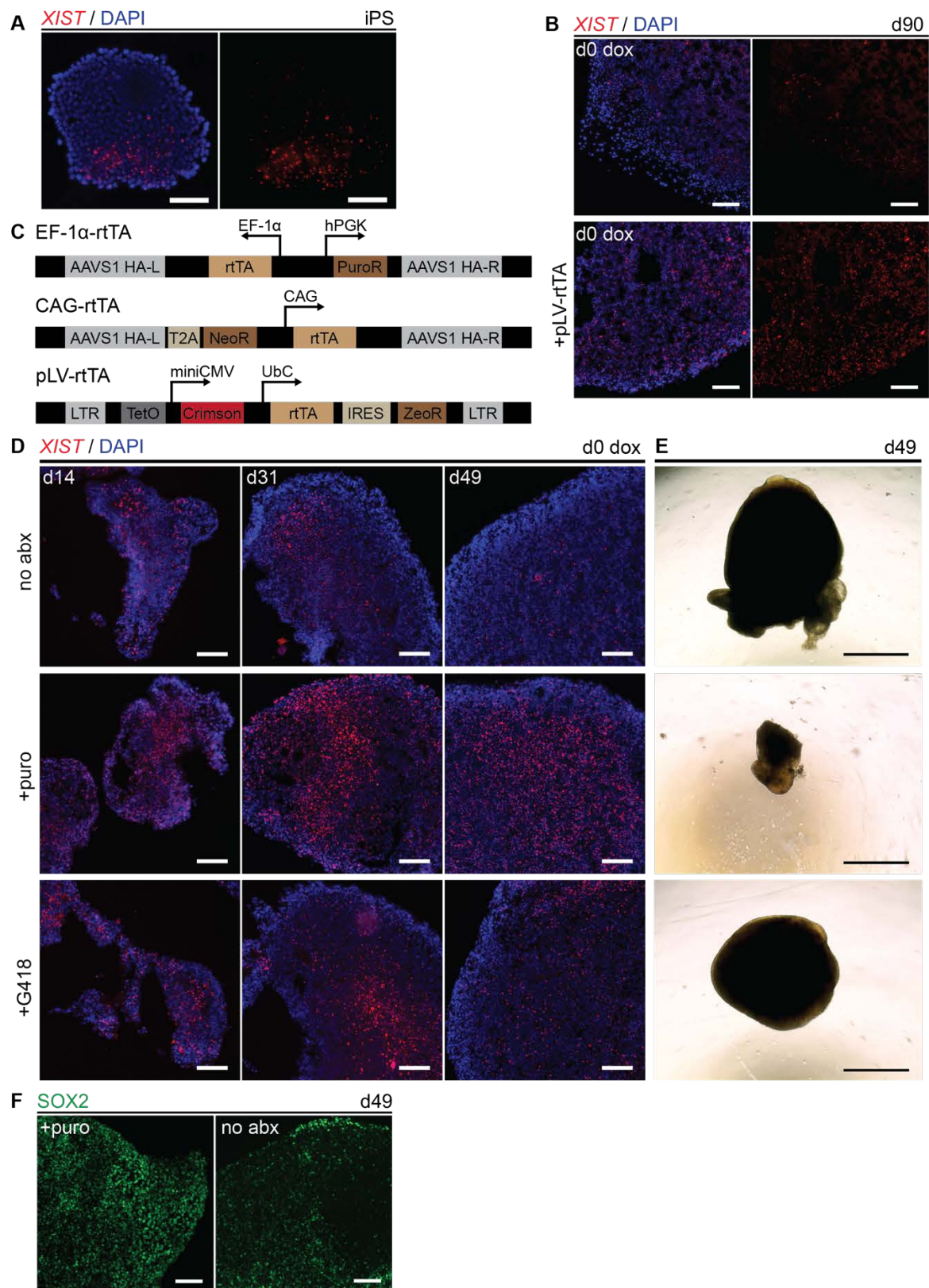
Figure A-1: Rescuing transgene expression with reintroduction of rtTA

construct. I termed this cell line clone 5a (C5A), and it was used extensively in chapter II of this thesis.

Knowing that rtTA was the limiting factor in aged organoids that were treated with dox from the onset of differentiation, we reasoned that selecting for rtTA expression using the antibiotics present on the rtTA cassettes could increase *XIST* expression. To test this, we differentiated cerebral organoids, as described in chapter III, from the C5A line described above. Dox treatment was started at day 0, along with either no antibiotic selection, puromycin (puro), or G418. As shown in Figure A-1D, the level of *XIST* expression was similar at early stages of differentiation. At later stages, both the untreated and G418 treated samples lost significant *XIST* expression, while the puro treated organoids retained *XIST* expression in nearly all cells. However, the puro treated organoids were also significantly smaller at day 49 (Figure A-1E) when compared to untreated organoids and were composed almost entirely of SOX2+ neural progenitors (Figure A-1F), suggesting that puromycin is toxic to terminally differentiated neurons which silence the EF-1α promoter. These same effects of puro and G418 were confirmed in iPSCs, whereby puromycin selection leads to a pure population of *XIST* expressing cells, while G418 treatment has no effect.

The different action of these two drugs may reflect differences in how the promoters are arranged in the two rtTA-expression cassettes (Figure A-1C). In the puro-containing cassette, selection for puromycin resistance also selects for expression of rtTA likely due to the back-to-back arrangement of the promoters. In non-expressing cells, these promoters may be silenced by CpG methylation at that one locus. On the other hand, the CAG-rtTA cassette utilizes the endogenous AAVS1 site promoter to

drive neomycin resistance and the spatially separated CAG promoter to drive rtTA

expression. In this case, selection with G418 does not concomitantly select for rtTA

expression.

Overall, these experiments suggest that in cells treated with dox from the onset

of differentiation, silencing of the rtTA locus leads to loss of *XIST* expression with

differentiation even in a cell line containing CAG-rtTA. A similar process occurs in

iPSCs, where the rtTA locus is stochastically silenced in a population of cells leading

them to lose the ability to initiate *XIST* expression.

Attempting to initiate *XIST* expression in postmitotic neurons

As discussed in chapter II, in both in a pure monolayer culture of postmitotic

neurons and in organoids where the NSCs and neurons can be spatially distinguished,

addition of dox does not lead to the initiation of *XIST* expression in neurons. Based on

the results from the previous section, we reasoned that the EF-1α promoter is likely

silenced in these cells leading to a loss of rtTA expression, which may be responsible

for the inability of these cells to initiate *XIST* expression.

To test this hypothesis, I differentiated iPSCs into a pure population of neurons

by adding the γ-secretase inhibitor compound E (CE) at day 21 of differentiation, which

causes synchronized neuronal differentiation with no remaining neural stem cells by day

28 (Figure II-5A). On day 28, I added dox in order to initiate *XIST* expression and also

transduced the cells with the previously described pLV-rtTA in order to exogenously

express rtTA. Unlike neurons that had been treated with dox from the onset of

differentiation (d0 dox), there were essentially no *XIST*+ cells in this condition (Figure A-

2A-B). This is despite the robust increase in rtTA expression levels in all conditions, as
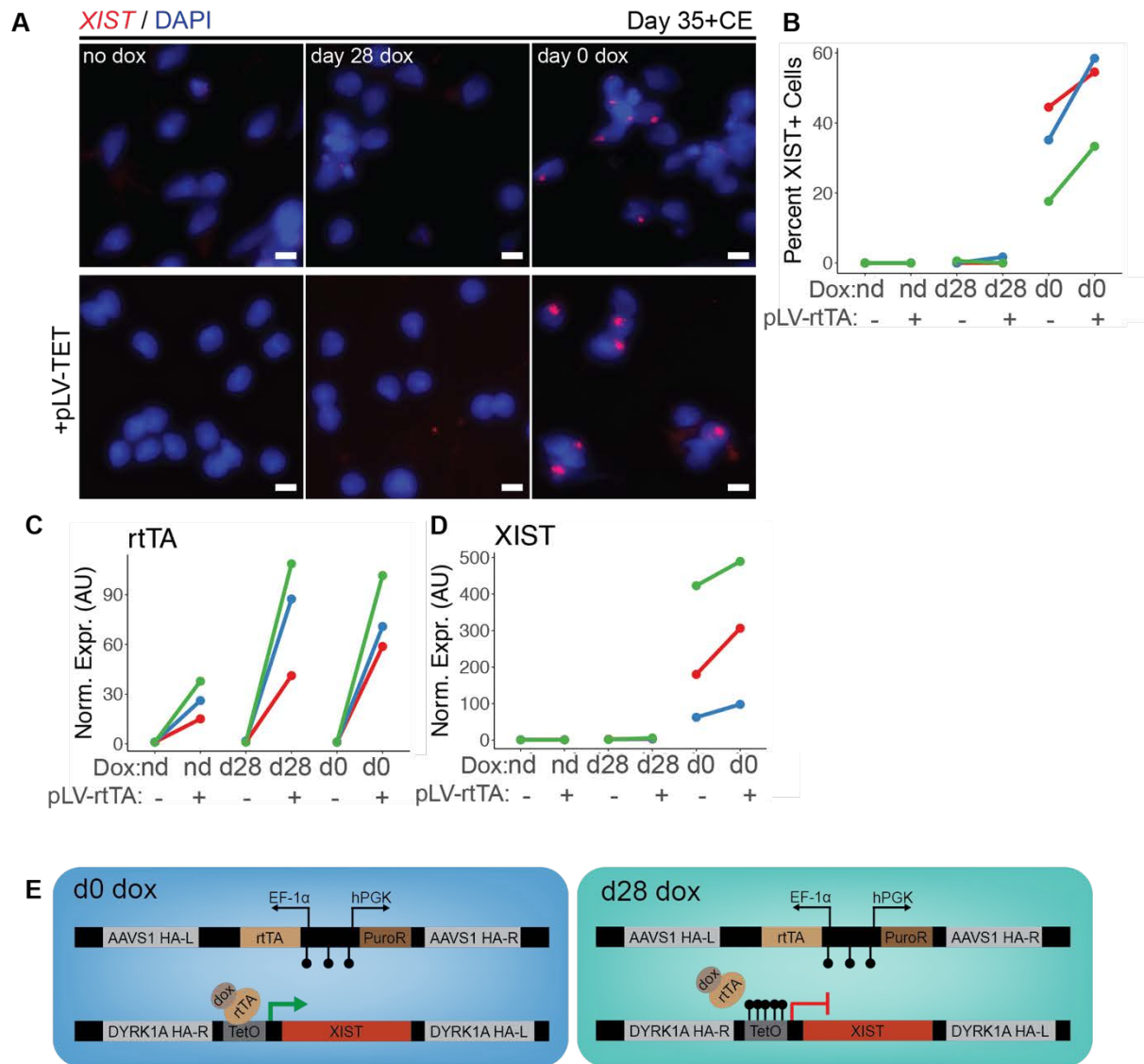
Figure A-2: Introducing exogenous rtTA in postmitotic neurons to initiate *XIST* expression

A) RNA FISH for *XIST* in d35 monolayer neurons treated with compound E on day 21 for 7 days. pLV-rtTA was transduced in cells where indicated. Dox was added on the day indicated. B) Quantification of the fraction of *XIST*-expressing cells in each condition of (A). 123-305 cells were counted for each condition (median=176). C-D) qPCR for rtTA (C) and *XIST* (D) normalized to GAPDH for cells in each condition of (A). AU, arbitrary units. E) Schematic of hypothesized methylation status of rtTA and *XIST* loci in transgenic cells. EF-1α promoter is likely progressively silenced over time in neurons, but not completely at this stage. TRE upstream of *XIST* is suspected to be robustly silenced in dox naïve neurons.

measured by qPCR (Figure A-2C), which led to an expected increase in both the fraction of *XIST*+ cells (Figure A-2B) and *XIST* expression (Figure A-2D) in the d0 dox condition.

Together, these results suggest that in d0 dox cells, rtTA is the limiting factor for *XIST* expression, while in dox-naïve neurons something other than rtTA is limiting. It is most likely that the TRE upstream of the *XIST* transgene is silenced by DNA methylation (Figure A-2E), as has been reported in the literature for tetracycline inducible systems (Gödecke et al., 2017).

Discussion

The experiments described above outline my attempts to counteract the finding of a decreased proportion of *XIST* expression cells in long-term iPSC culture and in differentiated cells, especially neurons. There are several important lessons to be taken away from these findings that can be applied to any studies using the tetracycline inducible system as well as for general design of expression cassettes.

Foremost, in this study the major cause of progressive loss of inducible transgene expression with differentiation was loss of rtTA expression. This happened both stochastically in iPSCs and due to silencing of a developmentally regulated promoter with differentiation. Transgene expression could be rescued with re-introduction of rtTA. It seems that rtTA may protect the TRE from methylation, which is why this rescue was possible only in cells that had previously been exposed to dox and may mean that rescue is limited by the starting proportion of *XIST* expressing cells. In dox naïve cells, the TRE was not protected from methylation, likely leading to the inability for *XIST* to be initiated even with adequate rtTA expression. The fact that

NSCs, but not neurons, are capable of initiating *XIST* expression suggests that there could be developmentally regulated methylation of the TRE in the final transition from NSCs to neurons.

Additionally, back-to-back transgene cassettes in this case led to joint expression profiles, which can be advantageous in that it allows for selection of continued transgene expression. It could also lead to silencing of a ubiquitous promoter when paired with a developmentally regulated promoter. The fact that C5A did not demonstrate an increase in *XIST* expressing cells could have several root causes. Perhaps most likely is that the spatial separation, and therefore uncoupled expression, of promoters driving antibiotic selection and rtTA expression means that selection for G418 only guarantees that the rtTA cassette is inserted in the AAVS1 locus, not that it is expressed. A low starting level of CAG driven rtTA expression would prevent robust expression of *XIST* in differentiated cells.

Because transgene expression is such a widespread phenomenon in biology, several common strategies for maintained expression have improved biological research. One recent paper examined a similar phenomenon to that described in this appendix, where tetracycline inducible expression was silenced with differentiation (Gödecke et al., 2017). In order to reverse the DNA methylation detected at the TRE locus, the researchers fused rtTA to the demethylase Ten-eleven translocation methylcytosine dioxygenase 1 (TET1). This led to demethylation of the TRE locus and allowed for transgene expression, albeit with relatively low efficiency.

Excitingly, we have preliminary evidence that the rtTA-TET1 fusion may reverse transgene silencing in our system, allowing for the induction of *XIST* expression in

differentiated neurons. This prospect would allow the study of *XIST*-mediated silencing in a postmitotic cell type for the first time. From a disease modeling perspective, if neurons are capable of initiating silencing this would greatly increase the experimental utility of dosage compensation for studying DS neurobiology. The ability to transition a neuron from a trisomic to disomic state would provide a unique opportunity to the examine whether trisomic neurons have ongoing functional deficits that could be reversed with dosage compensation. This could also have exciting translational ramifications for the potential of *XIST* as a therapeutic strategy for DS.

Materials and Methods

iPSC maintenance and differentiation

iPSCs were grown and maintained as described in chapter II. Organoids were generated using the Paşca (2015) protocol with the modifications described in chapter III. Monolayer neurons were generated with the protocol described in chapter II and were treated with $200\mu M$ CE at day 21 for 7 days. Dox was used at a concentration of 500ng/ml. Puromycin was used at a concentration of $3\mu g/ml$. G418 was used at a concentration of $40\mu g/ml$.

Generation of iPSC line with CAG-driven rtTA

First, transgenic iPSC clones (Jiang et al., 2013) were screened for heterozygous insertion of EF-1α-driven rtTA using genomic PCR. These clones were then transfected with a CAG-driven rtTA plasmid with AAVS1 homology arms (CAG-rtTA) which was a gift from Paul Gadue (Addgene plasmid #60431) and a sgRNA/Cas9 plasmid directed at AAVS1 which was a gift from Masato Kanemaki (Addgene plasmid #72833) using

PBAE nanoparticles (Eltoukhy et al., 2012; Zugates et al., 2007) at a 30:1 dilution. After

3 hours, media was changed to E8 with 10μM of the ROCK inhibitor Y-27632. A total of

5μg DNA were transfected. After 48 hours, selection with 40μg/ml G418 was started

and continued for 2 weeks. Insertion of the transgene was confirmed with genomic

PCR.

Lentiviral production and transduction

293FT cells (ThermoFisher) were used to generate lentiviral particles. 293FT

cells were passaged 1:2 into a T75 flask the day before transfection. On the day of

transfection, 9μg of transfer plasmid, 3μg of envelope plasmid, and 5.5μg of packaging

plasmid were transfected using Lipofectamine 2000 (ThermoFisher) per the

manufacturer's instructions into one T75 flask containing 293FT cells at ~80%

confluence. The pLV-rtTA plasmid is also known as pSLIK3 and was created by Melvys

Valledor (Valledor et al., 2018). The envelope (PMD2.G, addgene plasmid #12259) and

packaging (PsPax2, addgene plasmid #12260) plasmids were a gift from Didier Trono.

The day after transfection, the media was changed to either neuron media (for

monolayer culture, as described in chapter II) or organoid media. Two days after

transfection, the conditioned media was filtered through a 0.45μm low binding filter

(ThermoFisher) and added directly to the neurons (d28 of differentiation) or organoids

(d83 of differentiation). The next day, the media was replaced with fresh unconditioned

media. About 3 days after transduction and dox treatment, cells began to fluoresce in

the red channel with close to 100% efficiency in monolayer neurons and at a much

lower efficiency with patchy distribution in organoids.

Cell fixation, immunofluorescence, and RNA FISH

These steps were performed as described in chapter II for both iPSCs,

monolayer neurons, and organoids. The Stellaris *XIST* probe was used for RNA FISH.

RNA extraction, reverse transcription, and qPCR

These steps were performed as described in chapter III. 50-100ng of RNA was

reverse transcribed and cDNA was used at a 1:10 dilution for qPCR. The primers used

for qPCR are listed in Table A.1. GAPDH was used for normalization and quantification

was performed using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) with normalization

performed separately for each clone.

Table A-1: qPCR primers used in appendix

Gene	Primer	Sequence
rtTA	F	5'-AAA-TCA-GCT-CGC-GTT-CCT-GT-3'
	R	5'-TGT-TCC-AAT-ACG-CAG-CC-3'
XIST	F	5'-GCA-GGT-CCA-AGA-AAT-TTG-AAC-AC-3'
	R	5'-AGA-GTG-CCA-GGC-ATG-TTG-ATC-3'
GAPDH	F	5'-TGC-ACC-ACC-AAC-TGC-TTA-GC-3'
	R	5'-GGC-ATG-GAC-TGT-GGT-CAT-GAG-3'

F = forward; R = reverse

References

Abeliovich, A., and Doege, C.A. (2009). Reprogramming therapeutics: iPS cell prospects for neurodegenerative disease. Neuron *61*, 337–339.

Ábrahám, H., Vincze, A., Veszprémi, B., Kravják, A., Gömöri, É., Kovács, G.G., and Seress, L. (2012). Impaired myelination of the human hippocampal formation in Down syndrome. Int. J. Dev. Neurosci. *30*, 147–158.

Agrelo, R., Souabni, A., Novatchkova, M., Haslinger, C., Leeb, M., Komnenovic, V., Kishimoto, H., Gresh, L., Kohwi-Shigematsu, T., Kenner, L., et al. (2009). SATB1 defines the developmental context for gene silencing by Xist in lymphoma and embryonic cells. Dev. Cell *16*, 507–516.

Ahlfors, H., Anyanwu, N., Pakanavicius, E., Dinischiotu, N., Lana-Elola, E., Watson-Scales, S., Tosh, J., Wiseman, F., Briscoe, J., Page, K., et al. (2019). Gene expression dysregulation domains are not a specific feature of Down syndrome. Nature Communications *10*, 2489.

Ahmad, I., Zaqouras, P., and Artavanis-Tsakonas, S. (1995). Involvement of Notch-1 in mammalian retinal neurogenesis: association of Notch-1 activity with both immature and terminally differentiated cells. Mech. Dev. *53*, 73–85.

Allderdice, P.W., Miller, O.J., Miller, D.A., and Klinger, H.P. (1978). Spreading of inactivation in an (X;14) translocation. Am. J. Med. Genet. *2*, 233–240.

Almeida, M., Pintacuda, G., Masui, O., Koseki, Y., Gdula, M., Cerase, A., Brown, D., Mould, A., Innocent, C., Nakayama, M., et al. (2017). PCGF3/5-PRC1 initiates Polycomb recruitment in X chromosome inactivation. Science *356*, 1081–1084.

Anderson, S.A., Eisenstat, D.D., Shi, L., and Rubenstein, J.L. (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science *278*, 474–476.

Anthony, T.E., Klein, C., Fishell, G., and Heintz, N. (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron *41*, 881–890.

Arshad, A., Vose, L.R., Vinukonda, G., Hu, F., Yoshikawa, K., Csiszar, A., Brumberg, J.C., and Ballabh, P. (2016). Extended Production of Cortical Interneurons into the Third Trimester of Human Gestation. Cereb. Cortex *26*, 2242–2256.

Aziz, N.M., Guedj, F., Pennings, J.L.A., Olmos-Serrano, J.L., Siegel, A., Haydar, T.F., and Bianchi, D.W. (2018). Lifespan analysis of brain development, gene expression and behavioral phenotypes in the Ts1Cje, Ts65Dn and Dp(16)1/Yey mouse models of Down syndrome. Dis Model Mech *11*.

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jørgensen, H.F., John, R.M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M., et al. (2006). Chromatin signatures of pluripotent cell lines. Nature Cell Biology *8*, 532.

Baek, K.-H., Zaslavsky, A., Lynch, R.C., Britt, C., Okada, Y., Siarey, R.J., Lensch, M.W., Park, I.-H., Yoon, S.S., Minami, T., et al. (2009). Down's syndrome suppression of tumour growth and the role of the calcineurin inhibitor DSCR1. Nature *459*, 1126–1130.

Bagley, J.A., Reumann, D., Bian, S., Lévi-Strauss, J., and Knoblich, J.A. (2017). Fused cerebral organoids model interactions between brain regions. Nat. Methods *14*, 743–751.

Bahn, S., Mimmack, M., Ryan, M., Caldwell, M.A., Jauniaux, E., Starkey, M., Svendsen, C.N., and Emson, P. (2002). Neuronal target genes of the neuron-restrictive silencer factor in neurospheres derived from fetuses with Down's syndrome: a gene expression study. Lancet *359*, 310–315.

Barnea-Goraly, N., Menon, V., Eckert, M., Tamm, L., Bammer, R., Karchemskiy, A., Dant, C.C., and Reiss, A.L. (2005). White matter development during childhood and adolescence: a cross-sectional diffusion tensor imaging study. Cereb. Cortex *15*, 1848–1854.

Baron, M., Veres, A., Wolock, S.L., Faust, A.L., Gaujoux, R., Vetere, A., Ryu, J.H., Wagner, B.K., Shen-Orr, S.S., Klein, A.M., et al. (2016). A Single-Cell Transcriptomic Map of the Human and Mouse Pancreas Reveals Inter- and Intra-cell Population Structure. Cell Syst *3*, 346-360.e4.

Barr, M.L., and Bertram, E.G. (1949). A morphological distinction between neurones of the male and female, and the behaviour of the nucleolar satellite during accelerated nucleoprotein synthesis. Nature *163*, 676.

Baxter, L.L., Moran, T.H., Richtsmeier, J.T., Troncoso, J., and Reeves, R.H. (2000). Discovery and genetic localization of Down syndrome cerebellar phenotypes using the Ts65Dn mouse. Hum. Mol. Genet. *9*, 195–202.

Becker, L.E., Armstrong, D.L., and Chan, F. (1986). Dendritic atrophy in children with Down's syndrome. Ann. Neurol. *20*, 520–526.

Belichenko, N.P., Belichenko, P.V., Kleschevnikov, A.M., Salehi, A., Reeves, R.H., and Mobley, W.C. (2009). The "Down syndrome critical region" is sufficient in the mouse model to confer behavioral, neurophysiological, and synaptic phenotypes characteristic of Down syndrome. J. Neurosci. *29*, 5938–5948.

Belichenko, P.V., Masliah, E., Kleschevnikov, A.M., Villar, A.J., Epstein, C.J., Salehi, A., and Mobley, W.C. (2004). Synaptic structural abnormalities in the Ts65Dn mouse model of Down Syndrome. J. Comp. Neurol. *480*, 281–298.

Belichenko, P.V., Kleschevnikov, A.M., Salehi, A., Epstein, C.J., and Mobley, W.C. (2007). Synaptic and cognitive abnormalities in mouse models of Down syndrome: exploring genotype-phenotype relationships. J. Comp. Neurol. *504*, 329–345.

van den Berg, I.M., Laven, J.S.E., Stevens, M., Jonkers, I., Galjaard, R.-J., Gribnau, J., and Hikke van Doorninck, J. (2009). X Chromosome Inactivation Is Initiated in Human Preimplantation Embryos. The American Journal of Human Genetics *84*, 771–779.

Bershteyn, M., Hayashi, Y., Desachy, G., Hsiao, E.C., Sami, S., Tsang, K.M., Weiss, L.A., Kriegstein, A.R., Yamanaka, S., and Wynshaw-Boris, A. (2014). Cell-autonomous correction of ring chromosomes in human induced pluripotent stem cells. Nature *507*, 99–103.

Bestor, T.H. (2000). Gene silencing as a threat to the success of gene therapy. J. Clin. Invest. *105*, 409–411.

Bhattacharyya, A., McMillan, E., Chen, S.I., Wallace, K., and Svendsen, C.N. (2009). A critical period in cortical interneuron neurogenesis in down syndrome revealed by human neural progenitor cells. Dev. Neurosci. *31*, 497–510.

Bianchi, D.W., Platt, L.D., Goldberg, J.D., Abuhamad, A.Z., Sehnert, A.J., Rava, R.P., and MatErnal BLood IS Source to Accurately diagnose fetal aneuploidy (MELISSA) Study Group (2012). Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. Obstet Gynecol *119*, 890–901.

Birey, F., Andersen, J., Makinson, C.D., Islam, S., Wei, W., Huber, N., Fan, H.C., Metzler, K.R.C., Panagiotakos, G., Thom, N., et al. (2017). Assembly of functionally integrated human forebrain spheroids. Nature *545*, 54–59.

Blewitt, M.E., Gendrel, A.-V., Pang, Z., Sparrow, D.B., Whitelaw, N., Craig, J.M., Apedaile, A., Hilton, D.J., Dunwoodie, S.L., Brockdorff, N., et al. (2008). SmcHD1, containing a structural-maintenance-of-chromosomes hinge domain, has a critical role in X inactivation. Nat. Genet. *40*, 663–669.

Boldrini, M., Fulmore, C.A., Tartt, A.N., Simeon, L.R., Pavlova, I., Poposka, V., Rosoklija, G.B., Stankov, A., Arango, V., Dwork, A.J., et al. (2018). Human Hippocampal Neurogenesis Persists throughout Aging. Cell Stem Cell *22*, 589-599.e5.

Bonney, M.E., Moriya, H., and Amon, A. (2015). Aneuploid proliferation defects in yeast are not driven by copy number changes of a few dosage-sensitive genes. Genes Dev. *29*, 898–903.

Bousard, A., Raposo, A.C., Zylicz, J.J., Picard, C., Pires, V.B., Qi, Y., Syx, L., Chang, H.Y., Heard, E., and Rocha, S.T. da (2018). Exploring the role of Polycomb recruitment in Xist-mediated silencing of the X chromosome in ES cells. BioRxiv 495739 [Preprint].

Braccioli, L., Vervoort, S.J., Puma, G., Nijboer, C.H., and Coffer, P.J. (2018). SOX4 inhibits oligodendrocyte differentiation of embryonic neural stem cells in vitro by inducing Hes5 expression. Stem Cell Research *33*, 110–119.

Briggs, J.A., Sun, J., Shepherd, J., Ovchinnikov, D.A., Chung, T.-L., Nayler, S.P., Kao, L.-P., Morrow, C.A., Thakar, N.Y., Soo, S.-Y., et al. (2013). Integration-free induced pluripotent stem cells model genetic and neural developmental features of down syndrome etiology. Stem Cells *31*, 467–478.

Brockdorff, N. (2017). Polycomb complexes in X chromosome inactivation. Philos. Trans. R. Soc. Lond., B, Biol. Sci. *372*.

Brown, C.J., and Willard, H.F. (1994). The human X-inactivation centre is not required for maintenance of X-chromosome inactivation. Nature *368*, 154–156.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991a). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature *349*, 38–44.

Brown, C.J., Lafreniere, R.G., Powers, V.E., Sebastio, G., Ballabio, A., Pettigrew, A.L., Ledbetter, D.H., Levy, E., Craig, I.W., and Willard, H.F. (1991b). Localization of the X inactivation centre on the human X chromosome in Xq13. Nature *349*, 82–84.

Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafrenière, R.G., Xing, Y., Lawrence, J., and Willard, H.F. (1992). The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell *71*, 527–542.

Buchsbaum, I.Y., and Cappello, S. (2019). Neuronal migration in the CNS during development and disease: insights from in vivo and in vitro models. Development *146*.

Burda, J.E., and Sofroniew, M.V. (2014). Reactive gliosis and the multicellular response to CNS damage and disease. Neuron *81*, 229–248.

Byron, M., Hall, L.L., and Lawrence, J.B. (2013). A multifaceted FISH approach to study endogenous RNAs and DNAs in native nuclear and cell structures. Curr Protoc Hum Genet *Chapter 4*, Unit 4.15.

Bystron, I., Rakic, P., Molnár, Z., and Blakemore, C. (2006). The first neurons of the human cerebral cortex. Nature Neuroscience *9*, 880.

Cabrejo, L., Guyant-Maréchal, L., Laquerrière, A., Vercelletto, M., De la Fournière, F., Thomas-Antérion, C., Verny, C., Letournel, F., Pasquier, F., Vital, A., et al. (2006). Phenotype associated with APP duplication in five families. Brain *129*, 2966–2976.

Calegari, F., and Huttner, W.B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. J. Cell. Sci. *116*, 4947–4955.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043.

Cao, S.-Y., Hu, Y., Chen, C., Yuan, F., Xu, M., Li, Q., Fang, K.-H., Chen, Y., and Liu, Y. (2017). Enhanced derivation of human pluripotent stem cell-derived cortical glutamatergic neurons by a small molecule. Sci Rep *7*, 3282.

Carlesimo, G.A., Marotta, L., and Vicari, S. (1997). Long-term memory in mental retardation: evidence for a specific impairment in subjects with Down's syndrome. Neuropsychologia *35*, 71–79.

Cattanach, B.M. (1975). Control of chromosome inactivation. Annu. Rev. Genet. *9*, 1–18.

Caviedes, P., Ault, B., and Rapoport, S.I. (1990). The role of altered sodium currents in action potential abnormalities of cultured dorsal root ganglion neurons from trisomy 21 (down syndrome) human fetuses. Brain Research *510*, 229–236.

Chakrabarti, L., Galdzicki, Z., and Haydar, T.F. (2007). Defects in embryonic neurogenesis and initial synapse formation in the forebrain of the Ts65Dn mouse model of Down syndrome. J. Neurosci. *27*, 11483–11495.

Chakrabarti, L., Best, T.K., Cramer, N.P., Carney, R.S.E., Isaac, J.T.R., Galdzicki, Z., and Haydar, T.F. (2010). Olig1 and Olig2 triplication causes developmental brain defects in Down syndrome. Nat. Neurosci. *13*, 927–934.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. *27*, 275–280.

Chapman, R.S., and Hesketh, L.J. (2000). Behavioral phenotype of individuals with Down syndrome. Ment Retard Dev Disabil Res Rev *6*, 84–95.

Charlesworth, B. (1996). The evolution of chromosomal sex determination and dosage compensation. Curr. Biol. *6*, 149–162.

Charlton, J.L., Ihsen, E., and Lavelle, B.M. (2000). Control of manual skills in children with Down syndrome. Perceptual-Motor Behavior in Down Syndrome 25–48.

Chattopadhyaya, B., and Cristo, G.D. (2012). GABAergic circuit dysfunctions in neurodevelopmental disorders. Front Psychiatry *3*, 51.

Chaumeil, J., Le Baccon, P., Wutz, A., and Heard, E. (2006). A novel role for Xist RNA in the formation of a repressive nuclear compartment into which genes are recruited when silenced. Genes Dev. *20*, 2223–2237.

Chen, C., Jiang, P., Xue, H., Peterson, S.E., Tran, H.T., McCann, A.E., Parast, M.M., Li, S., Pleasure, D.E., Laurent, L.C., et al. (2014). Role of astroglia in Down's syndrome revealed by patient-derived human-induced pluripotent stem cells. Nat Commun *5*, 4430.

Chiang, J.-C., Jiang, J., Newburger, P.E., and Lawrence, J.B. (2018). Trisomy silencing by XIST normalizes Down syndrome cell pathogenesis demonstrated for hematopoietic defects in vitro. Nat Commun *9*, 5180.

Choi, B.H., and Lapham, L.W. (1978). Radial glia in the human fetal cerebrum: a combined Golgi, immunofluorescent and electron microscopic study. Brain Res. *148*, 295–311.

Choi, S.H., Kim, Y.H., Hebisch, M., Sliwinski, C., Lee, S., D'Avanzo, C., Chen, H., Hooli, B., Asselin, C., Muffat, J., et al. (2014). A three-dimensional human neural cell culture model of Alzheimer's disease. Nature *515*, 274–278.

Chow, J.C., Hall, L.L., Baldry, S.E.L., Thorogood, N.P., Lawrence, J.B., and Brown, C.J. (2007). Inducible XIST-dependent X-chromosome inactivation in human somatic cells is reversible. Proc. Natl. Acad. Sci. U.S.A. *104*, 10104–10109.

Chu, C., Zhang, Q.C., da Rocha, S.T., Flynn, R.A., Bharadwaj, M., Calabrese, J.M., Magnuson, T., Heard, E., and Chang, H.Y. (2015). Systematic discovery of Xist RNA binding proteins. Cell *161*, 404–416.

Clemson, C.M., McNeil, J.A., Willard, H.F., and Lawrence, J.B. (1996). XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. J. Cell Biol. *132*, 259–275.

Clemson, C.M., Chow, J.C., Brown, C.J., and Lawrence, J.B. (1998). Stabilization and localization of Xist RNA are controlled by separate mechanisms and are not sufficient for X inactivation. J. Cell Biol. *142*, 13–23.

Cobos, I., Borello, U., and Rubenstein, J.L.R. (2007). Dlx transcription factors promote migration through repression of axon and dendrite growth. Neuron *54*, 873–888.

Colombo, J.A., Reisin, H.D., Jones, M., and Bentham, C. (2005). Development of interlaminar astroglial processes in the cerebral cortex of control and Down's syndrome human cases. Exp. Neurol. *193*, 207–217.

Contestabile, A., Fila, T., Ceccarelli, C., Bonasoni, P., Bonapace, L., Santini, D., Bartesaghi, R., and 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*, 665–678.

Corder, E.H., Saunders, A.M., Risch, N.J., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Rimmler, J.B., Locke, P.A., Conneally, P.M., and Schmader, K.E. (1994).

Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat. Genet. *7*, 180–184.

Costa, R.M., Honjo, T., and Silva, A.J. (2003). Learning and memory deficits in Notch mutant mice. Curr. Biol. *13*, 1348–1354.

Cramer, N.P., Best, T.K., Stoffel, M., Siarey, R.J., and Galdzicki, Z. (2010). GABAB– GIRK2-Mediated Signaling in Down Syndrome. In Advances in Pharmacology, T.P. Blackburn, ed. (Academic Press), pp. 397–426.

Creamer, K.M., and Lawrence, J.B. (2017). XIST RNA: a window into the broader role of RNA in nuclear chromosome architecture. Philos. Trans. R. Soc. Lond., B, Biol. Sci. *372*.

Csankovszki, G., Nagy, A., and Jaenisch, R. (2001). Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J. Cell Biol. *153*, 773–784.

Cullis, P.R., and Hope, M.J. (2017). Lipid Nanoparticle Systems for Enabling Gene Therapies. Molecular Therapy *25*, 1467–1475.

Dai, W.-J., Zhu, L.-Y., Yan, Z.-Y., Xu, Y., Wang, Q.-L., and Lu, X.-J. (2016). CRISPR-Cas9 for in vivo Gene Therapy: Promise and Hurdles. Molecular Therapy - Nucleic Acids *5*, e349.

Das, I., Park, J.-M., Shin, J.H., Jeon, S.K., Lorenzi, H., Linden, D.J., Worley, P.F., and Reeves, R.H. (2013). Hedgehog agonist therapy corrects structural and cognitive deficits in a Down syndrome mouse model. Sci Transl Med *5*, 201ra120.

deAzevedo, L.C., Fallet, C., Moura-Neto, V., Daumas-Duport, C., Hedin-Pereira, C., and Lent, R. (2003). Cortical radial glial cells in human fetuses: depth-correlated transformation into astrocytes. J. Neurobiol. *55*, 288–298.

Deb, S., Hare, M., and Prior, L. (2007). Symptoms of dementia among adults with Down's syndrome: a qualitative study. J Intellect Disabil Res *51*, 726–739.

Deverman, B.E., Pravdo, P.L., Simpson, B.P., Kumar, S.R., Chan, K.Y., Banerjee, A., Wu, W.-L., Yang, B., Huber, N., Pasca, S.P., et al. (2016). Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. Nat. Biotechnol. *34*, 204–209.

Di Lullo, E., and Kriegstein, A.R. (2017). The use of brain organoids to investigate neural development and disease. Nat. Rev. Neurosci. *18*, 573–584.

Dierssen, M., Benavides-Piccione, R., Martínez-Cué, C., Estivill, X., Flórez, J., Elston, G.N., and DeFelipe, J. (2003). Alterations of neocortical pyramidal cell phenotype in the Ts65Dn mouse model of Down syndrome: effects of environmental enrichment. Cereb. Cortex *13*, 758–764.

DiGuiseppi, C., Hepburn, S., Davis, J.M., Fidler, D.J., Hartway, S., Lee, N.R., Miller, L., Ruttenber, M., and Robinson, C. (2010). Screening for Autism Spectrum Disorders in Children With Down Syndrome. J Dev Behav Pediatr *31*, 181–191.

Do, L.H., Mobley, W.C., and Singhal, N. (2015). Questioned validity of Gene Expression Dysregulated Domains in Down's Syndrome. F1000Res *4*, 269.

Doody, R.S., Thomas, R.G., Farlow, M., Iwatsubo, T., Vellas, B., Joffe, S., Kieburtz, K., Raman, R., Sun, X., Aisen, P.S., et al. (2014). Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. N. Engl. J. Med. *370*, 311–321.

Doran, E., Keator, D., Head, E., Phelan, M.J., Kim, R., Totoiu, M., Barrio, J.R., Small, G.W., Potkin, S.G., and Lott, I.T. (2017). Down Syndrome, Partial Trisomy 21, and Absence of Alzheimer's Disease: The Role of APP. J. Alzheimers Dis. *56*, 459–470.

Down, J.L.H. (1867). Observations on an Ethnic Classification of Idiots. Journal of Mental Science *13*, 121–123.

Du, Z.-W., Chen, H., Liu, H., Lu, J., Qian, K., Huang, C.-L., Zhong, X., Fan, F., and Zhang, S.-C. (2015). Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. Nat Commun *6*, 6626.

Dykens, E.M., and Kasari, C. (1997). Maladaptive behavior in children with Prader-Willi syndrome, Down syndrome, and nonspecific mental retardation. Am J Ment Retard *102*, 228–237.

Eiraku, M., Watanabe, K., Matsuo-Takasaki, M., Kawada, M., Yonemura, S., Matsumura, M., Wataya, T., Nishiyama, A., Muguruma, K., and Sasai, Y. (2008). Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem Cell *3*, 519–532.

Eltoukhy, A.A., Siegwart, D.J., Alabi, C.A., Rajan, J.S., Langer, R., and Anderson, D.G. (2012). Effect of molecular weight of amine end-modified poly(β-amino ester)s on gene delivery efficiency and toxicity. Biomaterials *33*, 3594–3603.

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R.A.M., Bulfone, A., Kowalczyk, T., and Hevner, R.F. (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J. Neurosci. *25*, 247–251.

Esposito, G., Imitola, J., Lu, J., De Filippis, D., Scuderi, C., Ganesh, V.S., Folkerth, R., Hecht, J., Shin, S., Iuvone, T., et al. (2008). Genomic and functional profiling of human Down syndrome neural progenitors implicates S100B and aquaporin 4 in cell injury. Hum. Mol. Genet. *17*, 440–457.

European Society of Gene Therapy (ESGT) (2006). One of three successfully treated CGD patients in a Swiss-German gene therapy trial died due to his underlying disease:

A position statement from the European Society of Gene Therapy (ESGT). J Gene Med *8*, 1435.

Fang, J., Chen, T., Chadwick, B., Li, E., and Zhang, Y. (2004). Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X inactivation. J. Biol. Chem. *279*, 52812–52815.

Fernandez, F., Morishita, W., Zuniga, E., Nguyen, J., Blank, M., Malenka, R.C., and Garner, C.C. (2007). Pharmacotherapy for cognitive impairment in a mouse model of Down syndrome. Nat. Neurosci. *10*, 411–413.

Fernandez-Martinez, J., Vela, E.M., Tora-Ponsioen, M., Ocaña, O.H., Nieto, M.A., and Galceran, J. (2009). Attenuation of Notch signalling by the Down-syndrome-associated kinase DYRK1A. J. Cell. Sci. *122*, 1574–1583.

Ferrer, I., and Gullotta, F. (1990). Down's syndrome and Alzheimer's disease: dendritic spine counts in the hippocampus. Acta Neuropathol. *79*, 680–685.

Fiddes, I.T., Lodewijk, G.A., Mooring, M., Bosworth, C.M., Ewing, A.D., Mantalas, G.L., Novak, A.M., van den Bout, A., Bishara, A., Rosenkrantz, J.L., et al. (2018). Human-Specific NOTCH2NL Genes Affect Notch Signaling and Cortical Neurogenesis. Cell *173*, 1356-1369.e22.

Fischer, D.F., van Dijk, R., Sluijs, J.A., Nair, S.M., Racchi, M., Levelt, C.N., van Leeuwen, F.W., and Hol, E.M. (2005). Activation of the Notch pathway in Down syndrome: cross-talk of Notch and APP. FASEB J. *19*, 1451–1458.

Fish, J.L., Dehay, C., Kennedy, H., and Huttner, W.B. (2008). Making bigger brains-the evolution of neural-progenitor-cell division. J. Cell. Sci. *121*, 2783–2793.

Franke, A., and Baker, B.S. (1999). The rox1 and rox2 RNAs are essential components of the compensasome, which mediates dosage compensation in Drosophila. Mol. Cell *4*, 117–122.

Fraser, A.S., Sobey, S., and Spicer, C.C. (1953). Mottled, a sex-modified lethal in the house mouse. Journ. of Genetics *51*, 217–221.

Freeman, S.B., Taft, L.F., Dooley, K.J., Allran, K., Sherman, S.L., Hassold, T.J., Khoury, M.J., and Saker, D.M. (1998). Population-based study of congenital heart defects in Down syndrome. Am. J. Med. Genet. *80*, 213–217.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., and Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature Biotechnology *31*, 822–826.

Gaiano, N., and Fishell, G. (2002). The role of notch in promoting glial and neural stem cell fates. Annu. Rev. Neurosci. *25*, 471–490.

Gödecke, N., Zha, L., Spencer, S., Behme, S., Riemer, P., Rehli, M., Hauser, H., and Wirth, D. (2017). Controlled re-activation of epigenetically silenced Tet promoter-driven transgene expression by targeted demethylation. Nucleic Acids Res. *45*, e147.

Godfrey, M., Hepburn, S., Fidler, D.J., Tapera, T., Zhang, F., Rosenberg, C.R., and Raitano Lee, N. (2019). Autism spectrum disorder (ASD) symptom profiles of children with comorbid Down syndrome (DS) and ASD: A comparison with children with DS-only and ASD-only. Research in Developmental Disabilities *89*, 83–93.

Godridge, H., Reynolds, G.P., Czudek, C., Calcutt, N.A., and Benton, M. (1987). Alzheimer-like neurotransmitter deficits in adult Down's syndrome brain tissue. J. Neurol. Neurosurg. Psychiatry *50*, 775–778.

Goedert, M., Spillantini, M.G., Cairns, N.J., and Crowther, R.A. (1992). Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms. Neuron *8*, 159–168.

Gonzales, P.K., Roberts, C.M., Fonte, V., Jacobsen, C., Stein, G.H., and Link, C.D. (2018). Transcriptome analysis of genetically matched human induced pluripotent stem cells disomic or trisomic for chromosome 21. PLoS ONE *13*, e0194581.

Goodliffe, J.W., Olmos-Serrano, J.L., Aziz, N.M., Pennings, J.L.A., Guedj, F., Bianchi, D.W., and Haydar, T.F. (2016). Absence of Prenatal Forebrain Defects in the Dp(16)1Yey/+ Mouse Model of Down Syndrome. J Neurosci *36*, 2926–2944.

Gould, S.J., Howard, S., and Papadaki, L. (1990). The development of ependyma in the human fetal brain: an immunohistological and electron microscopic study. Developmental Brain Research *55*, 255–267.

Granholm, A.C., Sanders, L.A., and Crnic, L.S. (2000). Loss of cholinergic phenotype in basal forebrain coincides with cognitive decline in a mouse model of Down's syndrome. Exp. Neurol. *161*, 647–663.

Gray, S.J., Blake, B.L., Criswell, H.E., Nicolson, S.C., Samulski, R.J., and McCown, T.J. (2010). Directed Evolution of a Novel Adeno-associated Virus (AAV) Vector That Crosses the Seizure-compromised Blood–Brain Barrier (BBB). Molecular Therapy *18*, 570–578.

Gribble, S.M., Wiseman, F.K., Clayton, S., Prigmore, E., Langley, E., Yang, F., Maguire, S., Fu, B., Rajan, D., Sheppard, O., et al. (2013). Massively parallel sequencing reveals the complex structure of an irradiated human chromosome on a mouse background in the Tc1 model of Down syndrome. PLoS ONE *8*, e60482.

Griffin, W.S., Stanley, L.C., Ling, C., White, L., MacLeod, V., Perrot, L.J., White, C.L., and Araoz, C. (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. PNAS *86*, 7611–7615.

Grossman, M., Raper, S.E., Kozarsky, K., Stein, E.A., Engelhardt, J.F., Muller, D., Lupien, P.J., and Wilson, J.M. (1994). Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. Nature Genetics *6*, 335.

Guenther, M.G., Frampton, G.M., Soldner, F., Hockemeyer, D., Mitalipova, M., Jaenisch, R., and Young, R.A. (2010). Chromatin Structure and Gene Expression Programs of Human Embryonic and Induced Pluripotent Stem Cells. Cell Stem Cell *7*, 249–257.

Guidi, S., Bonasoni, P., Ceccarelli, C., Santini, D., Gualtieri, F., Ciani, E., and Bartesaghi, R. (2008). Neurogenesis impairment and increased cell death reduce total neuron number in the hippocampal region of fetuses with Down syndrome. Brain Pathol. *18*, 180–197.

Guidi, S., Ciani, E., Bonasoni, P., Santini, D., and Bartesaghi, R. (2011). Widespread proliferation impairment and hypocellularity in the cerebellum of fetuses with down syndrome. Brain Pathol. *21*, 361–373.

Guihard-Costa, A.-M., Khung, S., Delbecque, K., Ménez, F., and Delezoide, A.-L. (2006). Biometry of face and brain in fetuses with trisomy 21. Pediatr. Res. *59*, 33–38.

Guillaume, D.J., Johnson, M.A., Li, X.-J., and Zhang, S.-C. (2006). Human embryonic stem cell-derived neural precursors develop into neurons and integrate into the host brain. J. Neurosci. Res. *84*, 1165–1176.

Gupta, M., Dhanasekaran, A.R., and Gardiner, K.J. (2016). Mouse models of Down syndrome: gene content and consequences. Mamm. Genome *27*, 538–555.

Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., Bouneaud, C., Hue, C., De Villartay, J.-P., Thrasher, A.J., Wulffraat, N., Sorensen, R., Dupuis-Girod, S., et al. (2002). Sustained Correction of X-Linked Severe Combined Immunodeficiency by ex Vivo Gene Therapy. New England Journal of Medicine *346*, 1185–1193.

Hall, L.L., Byron, M., Sakai, K., Carrel, L., Willard, H.F., and Lawrence, J.B. (2002a). An ectopic human XIST gene can induce chromosome inactivation in postdifferentiation human HT-1080 cells. Proc. Natl. Acad. Sci. U.S.A. *99*, 8677–8682.

Hall, L.L., Clemson, C.M., Byron, M., Wydner, K., and Lawrence, J.B. (2002b). Unbalanced X;autosome translocations provide evidence for sequence specificity in the association of XIST RNA with chromatin. Hum. Mol. Genet. *11*, 3157–3165.

Hall, L.L., Byron, M., Butler, J., Becker, K.A., Nelson, A., Amit, M., Itskovitz-Eldor, J., Stein, J., Stein, G., Ware, C., et al. (2008). X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected. J. Cell. Physiol. *216*, 445–452.

Hansen, D.V., Lui, J.H., Parker, P.R.L., and Kriegstein, A.R. (2010). Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature *464*, 554–561.

Hansen, D.V., Lui, J.H., Flandin, P., Yoshikawa, K., Rubenstein, J.L., Alvarez-Buylla, A., and Kriegstein, A.R. (2013). Non-epithelial stem cells and cortical interneuron production in the human ganglionic eminences. Nat. Neurosci. *16*, 1576–1587.

Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science *297*, 353–356.

Hardy, K., Hardy, P.J., Jacobs, P.A., Lewallen, K., and Hassold, T.J. (2016). Temporal changes in chromosome abnormalities in human spontaneous abortions: Results of 40 years of analysis. Am. J. Med. Genet. A *170*, 2671–2680.

Hasle, H., Friedman, J.M., Olsen, J.H., and Rasmussen, S.A. (2016). Low risk of solid tumors in persons with Down syndrome. Genet. Med. *18*, 1151–1157.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F., and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development *131*, 5539–5550.

Haydar, T.F., and Reeves, R.H. (2012). Trisomy 21 and early brain development. Trends Neurosci. *35*, 81–91.

Heard, E., Rougeulle, C., Arnaud, D., Avner, P., Allis, C.D., and Spector, D.L. (2001). Methylation of histone H3 at Lys-9 is an early mark on the X chromosome during X inactivation. Cell *107*, 727–738.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science *298*, 840–843.

Hirsch, M.L., Green, L., Porteus, M.H., and Samulski, R.J. (2010). Self-complementary AAV mediates gene targeting and enhances endonuclease delivery for double-strand break repair. Gene Therapy *17*, 1175–1180.

Holland, A.J., Hon, J., Huppert, F.A., Stevens, F., and Watson, P. (1998). Population-based study of the prevalence and presentation of dementia in adults with Down's syndrome. Br J Psychiatry *172*, 493–498.

Holland, A.J., Hon, J., Huppert, F.A., and Stevens, F. (2000). Incidence and course of dementia in people with Down's syndrome: findings from a population-based study. J Intellect Disabil Res *44 (Pt 2)*, 138–146.

Holtzman, D.M., Santucci, D., Kilbridge, J., Chua-Couzens, J., Fontana, D.J., Daniels, S.E., Johnson, R.M., Chen, K., Sun, Y., Carlson, E., et al. (1996). Developmental abnormalities and age-related neurodegeneration in a mouse model of Down syndrome. Proc. Natl. Acad. Sci. U.S.A. *93*, 13333–13338.

Hu, B.-Y., Weick, J.P., Yu, J., Ma, L.-X., Zhang, X.-Q., Thomson, J.A., and Zhang, S.-C. (2010). Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. Proc. Natl. Acad. Sci. U.S.A. *107*, 4335–4340.

Huebsch, N., Loskill, P., Deveshwar, N., Spencer, C.I., Judge, L.M., Mandegar, M.A., Fox, C.B., Mohamed, T.M.A., Ma, Z., Mathur, A., et al. (2016). Miniaturized iPS-Cell-Derived Cardiac Muscles for Physiologically Relevant Drug Response Analyses. Sci Rep *6*, 24726.

Huo, H.-Q., Qu, Z.-Y., Yuan, F., Ma, L., Yao, L., Xu, M., Hu, Y., Ji, J., Bhattacharyya, A., Zhang, S.-C., et al. (2018). Modeling Down Syndrome with Patient iPSCs Reveals Cellular and Migration Deficits of GABAergic Neurons. Stem Cell Reports *10*, 1251–1266.

Huttenlocher, P.R. (1979). Synaptic density in human frontal cortex - developmental changes and effects of aging. Brain Res. *163*, 195–205.

Huttner, W.B., and Brand, M. (1997). Asymmetric division and polarity of neuroepithelial cells. Curr. Opin. Neurobiol. *7*, 29–39.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., and Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J. Neurosci. *30*, 3489–3498.

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. *9*, 3136–3148.

Ishihara, K., Amano, K., Takaki, E., Shimohata, A., Sago, H., Epstein, C.J., and Yamakawa, K. (2010). Enlarged brain ventricles and impaired neurogenesis in the Ts1Cje and Ts2Cje mouse models of Down syndrome. Cereb. Cortex *20*, 1131–1143.

Jakovcevski, I., and Zecevic, N. (2005). Sequence of oligodendrocyte development in the human fetal telencephalon. Glia *49*, 480–491.

Jarrold, C., and Baddeley, A.D. (1997). Short-term Memory for Verbal and Visuospatial Information in Down's Syndrome. Cogn Neuropsychiatry *2*, 101–122.

Jiang, J., Jing, Y., Cost, G.J., Chiang, J.-C., Kolpa, H.J., Cotton, A.M., Carone, D.M., Carone, B.R., Shivak, D.A., Guschin, D.Y., et al. (2013). Translating dosage compensation to trisomy 21. Nature *500*, 296–300.

Jones, P.A., Issa, J.-P.J., and Baylin, S. (2016). Targeting the cancer epigenome for therapy. Nat. Rev. Genet. *17*, 630–641.

Jørgensen, O.S., Brooksbank, B.W., and Balázs, R. (1990). Neuronal plasticity and astrocytic reaction in Down syndrome and Alzheimer disease. J. Neurol. Sci. *98*, 63–79.

Kadoshima, T., Sakaguchi, H., Nakano, T., Soen, M., Ando, S., Eiraku, M., and Sasai, Y. (2013). Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. Proc. Natl. Acad. Sci. U.S.A. *110*, 20284–20289.

Kalantry, S., and Magnuson, T. (2006). The Polycomb group protein EED is dispensable for the initiation of random X-chromosome inactivation. PLoS Genet. *2*, e66.

Kang, H.J., Kawasawa, Y.I., Cheng, F., Zhu, Y., Xu, X., Li, M., Sousa, A.M.M., Pletikos, M., Meyer, K.A., Sedmak, G., et al. (2011). Spatio-temporal transcriptome of the human brain. Nature *478*, 483–489.

Kasuga, K., Shimohata, T., Nishimura, A., Shiga, A., Mizuguchi, T., Tokunaga, J., Ohno, T., Miyashita, A., Kuwano, R., Matsumoto, N., et al. (2009). Identification of independent APP locus duplication in Japanese patients with early-onset Alzheimer disease. J. Neurol. Neurosurg. Psychiatry *80*, 1050–1052.

Kawaguchi, A., Ikawa, T., Kasukawa, T., Ueda, H.R., Kurimoto, K., Saitou, M., and Matsuzaki, F. (2008). Single-cell gene profiling defines differential progenitor subclasses in mammalian neurogenesis. Development *135*, 3113–3124.

Kelava, I., and Lancaster, M.A. (2016). Stem Cell Models of Human Brain Development. Cell Stem Cell *18*, 736–748.

Khokha, M.K., Yeh, J., Grammer, T.C., and Harland, R.M. (2005). Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures. Dev. Cell *8*, 401–411.

Kim, J., Han, D., Byun, S.-H., Kwon, M., Cho, J.Y., Pleasure, S.J., and Yoon, K. (2018). Ttyh1 regulates embryonic neural stem cell properties by enhancing the Notch signaling pathway. EMBO Rep. *19*.

King, M.C., and Wilson, A.C. (1975). Evolution at two levels in humans and chimpanzees. Science *188*, 107–116.

Kish, S., Karlinsky, H., Becker, L., Gilbert, J., Rebbetoy, M., Chang, L.J., DiStefano, L., and Hornykiewicz, O. (1989). Down's syndrome individuals begin life with normal levels of brain cholinergic markers. J. Neurochem. *52*, 1183–1187.

Kleinstiver, B.P., Pattanayak, V., Prew, M.S., Tsai, S.Q., Nguyen, N.T., Zheng, Z., and Joung, J.K. (2016). High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. Nature *529*, 490–495.

Kleschevnikov, A.M., Belichenko, P.V., Villar, A.J., Epstein, C.J., Malenka, R.C., and Mobley, W.C. (2004). Hippocampal long-term potentiation suppressed by increased inhibition in the Ts65Dn mouse, a genetic model of Down syndrome. J. Neurosci. *24*, 8153–8160.

Koenderink, M.J., Uylings, H.B., and Mrzljak, L. (1994). Postnatal maturation of the layer III pyramidal neurons in the human prefrontal cortex: a quantitative Golgi analysis. Brain Res. *653*, 173–182.

Kovall, R.A., Gebelein, B., Sprinzak, D., and Kopan, R. (2017). The Canonical Notch Signaling Pathway: Structural and Biochemical Insights into Shape, Sugar, and Force. Dev. Cell *41*, 228–241.

Kowalczyk, T., Pontious, A., Englund, C., Daza, R.A.M., Bedogni, F., Hodge, R., Attardo, A., Bell, C., Huttner, W.B., and Hevner, R.F. (2009). Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex. Cereb. Cortex *19*, 2439–2450.

Koyanagi-Aoi, M., Ohnuki, M., Takahashi, K., Okita, K., Noma, H., Sawamura, Y., Teramoto, I., Narita, M., Sato, Y., Ichisaka, T., et al. (2013). Differentiation-defective phenotypes revealed by large-scale analyses of human pluripotent stem cells. Proc. Natl. Acad. Sci. U.S.A. *110*, 20569–20574.

Krencik, R., Weick, J.P., Liu, Y., Zhang, Z.-J., and Zhang, S.-C. (2011). Specification of transplantable astroglial subtypes from human pluripotent stem cells. Nat. Biotechnol. *29*, 528–534.

Kristoffersson, U., and Bergwall, B. (1984). Partial trisomy 15(q25qter) in two brothers. Hereditas *100*, 7–10.

Kunkiel, J., Gödecke, N., Ackermann, M., Hoffmann, D., Schambach, A., Lachmann, N., Wirth, D., and Moritz, T. (2017). The CpG-sites of the CBX3 ubiquitous chromatin opening element are critical structural determinants for the anti-silencing function. Scientific Reports *7*, 7919.

Kwan, K.Y., Lam, M.M.S., Johnson, M.B., Dube, U., Shim, S., Rašin, M.-R., Sousa, A.M.M., Fertuzinhos, S., Chen, J.-G., Arellano, J.I., et al. (2012). Species-dependent posttranscriptional regulation of NOS1 by FMRP in the developing cerebral cortex. Cell *149*, 899–911.

Lachmann, R. (2004). Herpes simplex virus-based vectors. Int J Exp Pathol *85*, 177–190.

Laker, C., Meyer, J., Schopen, A., Friel, J., Heberlein, C., Ostertag, W., and Stocking, C. (1998). Host cis-mediated extinction of a retrovirus permissive for expression in embryonal stem cells during differentiation. J. Virol. *72*, 339–348.

Lancaster, M.A., and Knoblich, J.A. (2014). Generation of cerebral organoids from human pluripotent stem cells. Nat Protoc *9*, 2329–2340.

Lancaster, M.A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., and Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. Nature *501*, 373–379.

Larsen, K.B., Laursen, H., Graem, N., Samuelsen, G.B., Bogdanovic, N., and Pakkenberg, B. (2008). Reduced cell number in the neocortical part of the human fetal brain in Down syndrome. Ann. Anat. *190*, 421–427.

Lee, J.T., and Jaenisch, R. (1997). Long-range cis effects of ectopic X-inactivation centres on a mouse autosome. Nature *386*, 275–279.

Leisti, J.T., Kaback, M.M., and Rimoin, D.L. (1975). Human X-autosome translocations: differential inactivation of the X chromosome in a kindred with an X-9 translocation. Am. J. Hum. Genet. *27*, 441–453.

Lejeune, J., Gautier, M., and Turpin, R. (1959). [Study of somatic chromosomes from 9 mongoloid children]. C. R. Hebd. Seances Acad. Sci. *248*, 1721–1722.

Letinic, K., Zoncu, R., and Rakic, P. (2002). Origin of GABAergic neurons in the human neocortex. Nature *417*, 645–649.

Letourneau, A., Santoni, F.A., Bonilla, X., Sailani, M.R., Gonzalez, D., Kind, J., Chevalier, C., Thurman, R., Sandstrom, R.S., Hibaoui, Y., et al. (2014). Domains of genome-wide gene expression dysregulation in Down's syndrome. Nature *508*, 345–350.

Levy, Y., and Eilam, A. (2013). Pathways to language: a naturalistic study of children with Williams syndrome and children with Down syndrome. J Child Lang *40*, 106–138.

Li, L.B., Chang, K.-H., Wang, P.-R., Hirata, R.K., Papayannopoulou, T., and Russell, D.W. (2012). Trisomy Correction in Down Syndrome Induced Pluripotent Stem Cells. Cell Stem Cell *11*, 615–619.

Li, X.-J., Du, Z.-W., Zarnowska, E.D., Pankratz, M., Hansen, L.O., Pearce, R.A., and Zhang, S.-C. (2005). Specification of motoneurons from human embryonic stem cells. Nat. Biotechnol. *23*, 215–221.

Li, X.-J., Zhang, X., Johnson, M.A., Wang, Z.-B., Lavaute, T., and Zhang, S.-C. (2009). Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. Development *136*, 4055–4063.

Li, Z., Yu, T., Morishima, M., Pao, A., LaDuca, J., Conroy, J., Nowak, N., Matsui, S.-I., Shiraishi, I., and Yu, Y.E. (2007). Duplication of the entire 22.9 Mb human chromosome

21 syntenic region on mouse chromosome 16 causes cardiovascular and gastrointestinal abnormalities. Hum. Mol. Genet. *16*, 1359–1366.

Liang, G., and Zhang, Y. (2013). Genetic and epigenetic variations in iPSCs: potential causes and implications for application. Cell Stem Cell *13*, 149–159.

Lipsky, R.H., and Goldman, D. (2003). Genomics and variation of ionotropic glutamate receptors. Ann. N. Y. Acad. Sci. *1003*, 22–35.

Liu, Y., Weick, J.P., Liu, H., Krencik, R., Zhang, X., Ma, L., Zhou, G., Ayala, M., and Zhang, S.-C. (2013). Medial ganglionic eminence-like cells derived from human embryonic stem cells correct learning and memory deficits. Nat. Biotechnol. *31*, 440–447.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods *25*, 402–408.

Lockstone, H.E., Harris, L.W., Swatton, J.E., Wayland, M.T., Holland, A.J., and Bahn, S. (2007a). Gene expression profiling in the adult Down syndrome brain. Genomics *90*, 647–660.

Lockstone, H.E., Harris, L.W., Swatton, J.E., Wayland, M.T., Holland, A.J., and Bahn, S. (2007b). Gene expression profiling in the adult Down syndrome brain. Genomics *90*, 647–660.

Louvi, A., and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. Nat. Rev. Neurosci. *7*, 93–102.

Lowes, L.P., Alfano, L.N., Arnold, W.D., Shell, R., Prior, T.W., McColly, M., Lehman, K.J., Church, K., Sproule, D.M., Nagendran, S., et al. (2019). Impact of age and motor function in a phase 1/2A study of infants with SMA Type 1 receiving single-dose gene replacement therapy. Pediatric Neurology.

Lu, H.-E., Yang, Y.-C., Chen, S.-M., Su, H.-L., Huang, P.-C., Tsai, M.-S., Wang, T.-H., Tseng, C.-P., and Hwang, S.-M. (2013). Modeling neurogenesis impairment in Down syndrome with induced pluripotent stem cells from Trisomy 21 amniotic fluid cells. Exp. Cell Res. *319*, 498–505.

Lu, J., Esposito, G., Scuderi, C., Steardo, L., Delli-Bovi, L.C., Hecht, J.L., Dickinson, B.C., Chang, C.J., Mori, T., and Sheen, V. (2011). S100B and APP promote a gliocentric shift and impaired neurogenesis in Down syndrome neural progenitors. PLoS ONE *6*, e22126.

Lui, J.H., Hansen, D.V., and Kriegstein, A.R. (2011). Development and evolution of the human neocortex. Cell *146*, 18–36.

Lukaszewicz, A., Savatier, P., Cortay, V., Giroud, P., Huissoud, C., Berland, M., Kennedy, H., and Dehay, C. (2005). G1 phase regulation, area-specific cell cycle control, and cytoarchitectonics in the primate cortex. Neuron *47*, 353–364.

Lyon, M.F. (1961). Gene action in the X-chromosome of the mouse (Mus musculus L.). Nature *190*, 372–373.

Ma, D.K., Jang, M.-H., Guo, J.U., Kitabatake, Y., Chang, M.-L., Pow-Anpongkul, N., Flavell, R.A., Lu, B., Ming, G.-L., and Song, H. (2009). Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science *323*, 1074–1077.

Ma, T., Wang, C., Wang, L., Zhou, X., Tian, M., Zhang, Q., Zhang, Y., Li, J., Liu, Z., Cai, Y., et al. (2013). Subcortical origins of human and monkey neocortical interneurons. Nat. Neurosci. *16*, 1588–1597.

Majumdar, D., Gao, Y., Li, D., and Webb, D.J. (2011). Co-culture of neurons and glia in a novel microfluidic platform. J. Neurosci. Methods *196*, 38–44.

Mak, W., Nesterova, T.B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A.P., and Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. Science *303*, 666–669.

Malatesta, P., Hartfuss, E., and Götz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development *127*, 5253–5263.

Malik, S., Vinukonda, G., Vose, L.R., Diamond, D., Bhimavarapu, B.B.R., Hu, F., Zia, M.T., Hevner, R., Zecevic, N., and Ballabh, P. (2013). Neurogenesis continues in the third trimester of pregnancy and is suppressed by premature birth. J. Neurosci. *33*, 411–423.

Mangeot, P.E., Risson, V., Fusil, F., Marnef, A., Laurent, E., Blin, J., Mournetas, V., Massouridès, E., Sohier, T.J.M., Corbin, A., et al. (2019). Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. Nature Communications *10*, 45.

Mann, D.M., and Esiri, M.M. (1989). The pattern of acquisition of plaques and tangles in the brains of patients under 50 years of age with Down's syndrome. J. Neurol. Sci. *89*, 169–179.

Marahrens, Y., Panning, B., Dausman, J., Strauss, W., and Jaenisch, R. (1997). Xist-deficient mice are defective in dosage compensation but not spermatogenesis. Genes Dev. *11*, 156–166.

Mariani, J., Simonini, M.V., Palejev, D., Tomasini, L., Coppola, G., Szekely, A.M., Horvath, T.L., and Vaccarino, F.M. (2012). Modeling human cortical development in

vitro using induced pluripotent stem cells. Proc. Natl. Acad. Sci. U.S.A. *109*, 12770–12775.

Mariani, J., Coppola, G., Zhang, P., Abyzov, A., Provini, L., Tomasini, L., Amenduni, M., Szekely, A., Palejev, D., Wilson, M., et al. (2015). FOXG1-Dependent Dysregulation of GABA/Glutamate Neuron Differentiation in Autism Spectrum Disorders. Cell *162*, 375–390.

Marín, O. (2012). Interneuron dysfunction in psychiatric disorders. Nat. Rev. Neurosci. *13*, 107–120.

Marin-Padilla, M. (1976). Pyramidal cell abnormalities in the motor cortex of a child with Down's syndrome. A Golgi study. J. Comp. Neurol. *167*, 63–81.

Maroof, A.M., Keros, S., Tyson, J.A., Ying, S.-W., Ganat, Y.M., Merkle, F.T., Liu, B., Goulburn, A., Stanley, E.G., Elefanty, A.G., et al. (2013). Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. Cell Stem Cell *12*, 559–572.

Matzke, M.A., Mette, M.F., and Matzke, A.J. (2000). Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. Plant Mol. Biol. *43*, 401–415.

McHugh, C.A., Chen, C.-K., Chow, A., Surka, C.F., Tran, C., McDonel, P., Pandya-Jones, A., Blanco, M., Burghard, C., Moradian, A., et al. (2015). The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. Nature *521*, 232–236.

Mégarbané, A., Ravel, A., Mircher, C., Sturtz, F., Grattau, Y., Rethoré, M.-O., Delabar, J.-M., and Mobley, W.C. (2009). The 50th anniversary of the discovery of trisomy 21: the past, present, and future of research and treatment of Down syndrome. Genet. Med. *11*, 611–616.

Meyer, G. (2007). Genetic control of neuronal migrations in human cortical development. Adv Anat Embryol Cell Biol *189*, 1 p preceding 1, 1–111.

Mi, H., and Thomas, P. (2009). PANTHER pathway: an ontology-based pathway database coupled with data analysis tools. Methods Mol. Biol. *563*, 123–140.

Mi, H., Huang, X., Muruganujan, A., Tang, H., Mills, C., Kang, D., and Thomas, P.D. (2017). PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res. *45*, D183–D189.

Migeon, B.R. (1994). X-chromosome inactivation: molecular mechanisms and genetic consequences. Trends Genet. *10*, 230–235.

Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T.-K., Koche, R.P., et al. (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553–560.

Miller, D.J., Duka, T., Stimpson, C.D., Schapiro, S.J., Baze, W.B., McArthur, M.J., Fobbs, A.J., Sousa, A.M.M., Sestan, N., Wildman, D.E., et al. (2012). Prolonged myelination in human neocortical evolution. Proc. Natl. Acad. Sci. U.S.A. *109*, 16480–16485.

Minks, J., Baldry, S.E., Yang, C., Cotton, A.M., and Brown, C.J. (2013). XIST-induced silencing of flanking genes is achieved by additive action of repeat a monomers in human somatic cells. Epigenetics Chromatin *6*, 23.

Mito, T., and Becker, L.E. (1993). Developmental changes of S-100 protein and glial fibrillary acidic protein in the brain in Down syndrome. Exp. Neurol. *120*, 170–176.

Miyake, N., Miyake, K., Yamamoto, M., Hirai, Y., and Shimada, T. (2011). Global gene transfer into the CNS across the BBB after neonatal systemic delivery of single-stranded AAV vectors. Brain Research *1389*, 19–26.

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T., and Ogawa, M. (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development *131*, 3133–3145.

Moindrot, B., Cerase, A., Coker, H., Masui, O., Grijzenhout, A., Pintacuda, G., Schermelleh, L., Nesterova, T.B., and Brockdorff, N. (2015). A Pooled shRNA Screen Identifies Rbm15, Spen, and Wtap as Factors Required for Xist RNA-Mediated Silencing. Cell Rep *12*, 562–572.

Molliver, M.E., Kostović, I., and van der Loos, H. (1973). The development of synapses in cerebral cortex of the human fetus. Brain Res. *50*, 403–407.

Monfort, A., Di Minin, G., Postlmayr, A., Freimann, R., Arieti, F., Thore, S., and Wutz, A. (2015). Identification of Spen as a Crucial Factor for Xist Function through Forward Genetic Screening in Haploid Embryonic Stem Cells. Cell Rep *12*, 554–561.

Monzel, A.S., Smits, L.M., Hemmer, K., Hachi, S., Moreno, E.L., van Wuellen, T., Jarazo, J., Walter, J., Brüggemann, I., Boussaad, I., et al. (2017). Derivation of Human Midbrain-Specific Organoids from Neuroepithelial Stem Cells. Stem Cell Reports *8*, 1144–1154.

Moreno-Jiménez, E.P., Flor-García, M., Terreros-Roncal, J., Rábano, A., Cafini, F., Pallas-Bazarra, N., Ávila, J., and Llorens-Martín, M. (2019). Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. Nature Medicine 1.

Morrow, T., Song, M.R., and Ghosh, A. (2001). Sequential specification of neurons and glia by developmentally regulated extracellular factors. Development *128*, 3585–3594.

Mowery, C.T., Reyes, J.M., Cabal-Hierro, L., Higby, K.J., Karlin, K.L., Wang, J.H., Kimmerling, R.J., Cejas, P., Lim, K., Li, H., et al. (2018). Trisomy of a Down Syndrome Critical Region Globally Amplifies Transcription via HMGN1 Overexpression. Cell Rep *25*, 1898-1911.e5.

Mugford, J.W., Yee, D., and Magnuson, T. (2012). Failure of extra-embryonic progenitor maintenance in the absence of dosage compensation. Development *139*, 2130–2138.

Mullard, A. (2016). Alzheimer amyloid hypothesis lives on. Nat Rev Drug Discov *16*, 3–5.

Mundy, P., Kasari, C., Sigman, M., and Ruskin, E. (1995). Nonverbal communication and early language acquisition in children with Down syndrome and in normally developing children. J Speech Hear Res *38*, 157–167.

Muotri, A.R., Nakashima, K., Toni, N., Sandler, V.M., and Gage, F.H. (2005). Development of functional human embryonic stem cell-derived neurons in mouse brain. Proc. Natl. Acad. Sci. U.S.A. *102*, 18644–18648.

Naldini, L. (2011). Ex vivo gene transfer and correction for cell-based therapies. Nature Reviews Genetics *12*, 301–315.

Nami, F., Basiri, M., Satarian, L., Curtiss, C., Baharvand, H., and Verfaillie, C. (2018). Strategies for In Vivo Genome Editing in Nondividing Cells. Trends in Biotechnology *36*, 770–786.

Namihira, M., Kohyama, J., Semi, K., Sanosaka, T., Deneen, B., Taga, T., and Nakashima, K. (2009). Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. Dev. Cell *16*, 245–255.

de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M., et al. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev. Cell *7*, 663–676.

Nechanitzky, R., Dávila, A., Savarese, F., Fietze, S., and Grosschedl, R. (2012). Satb1 and Satb2 are dispensable for X chromosome inactivation in mice. Dev. Cell *23*, 866–871.

Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., and Kriegstein, A.R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature *409*, 714–720.

Noctor, S.C., Martínez-Cerdeño, V., and Kriegstein, A.R. (2008). Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J. Comp. Neurol. *508*, 28–44.

O'Doherty, A., Ruf, S., Mulligan, C., Hildreth, V., Errington, M.L., Cooke, S., Sesay, A., Modino, S., Vanes, L., Hernandez, D., et al. (2005). An aneuploid mouse strain carrying human chromosome 21 with Down syndrome phenotypes. Science *309*, 2033–2037.

Ogura, A., Morizane, A., Nakajima, Y., Miyamoto, S., and Takahashi, J. (2013). γ-secretase inhibitors prevent overgrowth of transplanted neural progenitors derived from human-induced pluripotent stem cells. Stem Cells Dev. *22*, 374–382.

Ohno, S., and Hauschka, T.S. (1960). Allocycly of the X-chromosome in tumors and normal tissues. Cancer Res. *20*, 541–545.

Okamoto, I., Patrat, C., Thépot, D., Peynot, N., Fauque, P., Daniel, N., Diabangouaya, P., Wolf, J.-P., Renard, J.-P., Duranthon, V., et al. (2011). Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development. Nature *472*, 370–374.

Olmos-Serrano, J.L., Kang, H.J., Tyler, W.A., Silbereis, J.C., Cheng, F., Zhu, Y., Pletikos, M., Jankovic-Rapan, L., Cramer, N.P., Galdzicki, Z., et al. (2016). Down Syndrome Developmental Brain Transcriptome Reveals Defective Oligodendrocyte Differentiation and Myelination. Neuron *89*, 1208–1222.

Olson, M.I., and Shaw, C.M. (1969). Presenile dementia and Alzheimer's disease in mongolism. Brain *92*, 147–156.

Olson, L.E., Roper, R.J., Baxter, L.L., Carlson, E.J., Epstein, C.J., and Reeves, R.H. (2004). Down syndrome mouse models Ts65Dn, Ts1Cje, and Ms1Cje/Ts65Dn exhibit variable severity of cerebellar phenotypes. Dev. Dyn. *230*, 581–589.

Ormel, P.R., Vieira de Sá, R., van Bodegraven, E.J., Karst, H., Harschnitz, O., Sneeboer, M.A.M., Johansen, L.E., van Dijk, R.E., Scheefhals, N., Berdenis van Berlekom, A., et al. (2018). Microglia innately develop within cerebral organoids. Nat Commun *9*, 4167.

Oromendia, A.B., Dodgson, S.E., and Amon, A. (2012). Aneuploidy causes proteotoxic stress in yeast. Genes Dev. *26*, 2696–2708.

Ottesen, A.M., Aksglaede, L., Garn, I., Tartaglia, N., Tassone, F., Gravholt, C.H., Bojesen, A., Sørensen, K., Jørgensen, N., Rajpert-De Meyts, E., et al. (2010). Increased number of sex chromosomes affects height in a nonlinear fashion: a study of 305 patients with sex chromosome aneuploidy. Am. J. Med. Genet. A *152A*, 1206–1212.

Ovchinnikov, D.A., Korn, O., Virshup, I., Wells, C.A., and 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*, 32–42.

Oyer, J.A., Chu, A., Brar, S., and Turker, M.S. (2009). Aberrant epigenetic silencing is triggered by a transient reduction in gene expression. PLoS ONE *4*, e4832.

Paina, S., Garzotto, D., DeMarchis, S., Marino, M., Moiana, A., Conti, L., Cattaneo, E., Perera, M., Corte, G., Calautti, E., et al. (2011). Wnt5a is a transcriptional target of Dlx homeogenes and promotes differentiation of interneuron progenitors in vitro and in vivo. J. Neurosci. *31*, 2675–2687.

Papapetrou, E.P., and Schambach, A. (2016). Gene Insertion Into Genomic Safe Harbors for Human Gene Therapy. Molecular Therapy *24*, 678–684.

Paredes, M.F., James, D., Gil-Perotin, S., Kim, H., Cotter, J.A., Ng, C., Sandoval, K., Rowitch, D.H., Xu, D., McQuillen, P.S., et al. (2016). Extensive migration of young neurons into the infant human frontal lobe. Science *354*.

Park, W.W. (1957). The occurrence of sex chromatin in early human and macaque embryos. J. Anat. *91*, 369–373.

Park, I.-H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., and Daley, G.Q. (2008). Disease-specific induced pluripotent stem cells. Cell *134*, 877–886.

Park, J., Wetzel, I., Marriott, I., Dréau, D., D'Avanzo, C., Kim, D.Y., Tanzi, R.E., and Cho, H. (2018). A 3D human triculture system modeling neurodegeneration and neuroinflammation in Alzheimer's disease. Nat. Neurosci. *21*, 941–951.

Paşca, A.M., Sloan, S.A., Clarke, L.E., Tian, Y., Makinson, C.D., Huber, N., Kim, C.H., Park, J.-Y., O'Rourke, N.A., Nguyen, K.D., et al. (2015). Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. Nat. Methods *12*, 671–678.

Patil, D.P., Chen, C.-K., Pickering, B.F., Chow, A., Jackson, C., Guttman, M., and Jaffrey, S.R. (2016). m(6)A RNA methylation promotes XIST-mediated transcriptional repression. Nature *537*, 369–373.

Payer, B., and Lee, J.T. (2008). X chromosome dosage compensation: how mammals keep the balance. Annu. Rev. Genet. *42*, 733–772.

Pedersen, C. (1976). Letter: Partial trisomy 15 as a result of an unbalanced 12/15 translocation in a patient with a cloverleaf skull anomaly. Clin. Genet. *9*, 378–380.

Pehrson, J.R., Changolkar, L.N., Costanzi, C., and Leu, N.A. (2014). Mice without macroH2A histone variants. Mol. Cell. Biol. *34*, 4523–4533.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for Xist in X chromosome inactivation. Nature *379*, 131–137.

Pérez-Cremades, D., Hernández, S., Blasco-Ibáñez, J.M., Crespo, C., Nacher, J., and Varea, E. (2010). Alteration of inhibitory circuits in the somatosensory cortex of Ts65Dn mice, a model for Down's syndrome. J Neural Transm (Vienna) *117*, 445–455.

Perry, G.H., Dominy, N.J., Claw, K.G., Lee, A.S., Fiegler, H., Redon, R., Werner, J., Villanea, F.A., Mountain, J.L., Misra, R., et al. (2007). Diet and the evolution of human amylase gene copy number variation. Nat. Genet. *39*, 1256–1260.

Petanjek, Z., Berger, B., and Esclapez, M. (2009). Origins of cortical GABAergic neurons in the cynomolgus monkey. Cereb. Cortex *19*, 249–262.

Petanjek, Z., Judaš, M., Šimic, G., Rasin, M.R., Uylings, H.B.M., Rakic, P., and Kostovic, I. (2011). Extraordinary neoteny of synaptic spines in the human prefrontal cortex. Proc. Natl. Acad. Sci. U.S.A. *108*, 13281–13286.

Petropoulos, S., Edsgärd, D., Reinius, B., Deng, Q., Panula, S.P., Codeluppi, S., Plaza Reyes, A., Linnarsson, S., Sandberg, R., and Lanner, F. (2016a). Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos. Cell *165*, 1012–1026.

Petropoulos, S., Edsgärd, D., Reinius, B., Deng, Q., Panula, S.P., Codeluppi, S., Plaza Reyes, A., Linnarsson, S., Sandberg, R., and Lanner, F. (2016b). Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos. Cell *165*, 1012–1026.

Pham, M.T., Pollock, K.M., Rose, M.D., Cary, W.A., Stewart, H.R., Zhou, P., Nolta, J.A., and Waldau, B. (2018). Generation of human vascularized brain organoids. Neuroreport *29*, 588–593.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131–135.

Plona, K., Kim, T., Halloran, K., and Wynshaw-Boris, A. (2016). Chromosome therapy: Potential strategies for the correction of severe chromosome aberrations. Am. J. Med. Genet. n/a-n/a.

Poirier, J., Bertrand, P., Poirier, J., Kogan, S., Gauthier, S., Poirier, J., Gauthier, S., Davignon, J., Bouthillier, D., and Davignon, J. (1993). Apolipoprotein E polymorphism and Alzheimer's disease. The Lancet *342*, 697–699.

Powell, S.K., Rivera-Soto, R., and Gray, S.J. (2015). Viral Expression Cassette Elements to Enhance Transgene Target Specificity and Expression in Gene Therapy. Discov Med *19*, 49–57.

Prasher, V.P., Farrer, M.J., Kessling, A.M., Fisher, E.M., West, R.J., Barber, P.C., and Butler, A.C. (1998). Molecular mapping of Alzheimer-type dementia in Down's syndrome. Ann. Neurol. *43*, 380–383.

Qian, X., Nguyen, H.N., Song, M.M., Hadiono, C., Ogden, S.C., Hammack, C., Yao, B., Hamersky, G.R., Jacob, F., Zhong, C., et al. (2016). Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. Cell *165*, 1238–1254.

Qian, X., Jacob, F., Song, M.M., Nguyen, H.N., Song, H., and Ming, G.-L. (2018). Generation of human brain region-specific organoids using a miniaturized spinning bioreactor. Nat Protoc *13*, 565–580.

Quadrato, G., Nguyen, T., Macosko, E.Z., Sherwood, J.L., Min Yang, S., Berger, D.R., Maria, N., Scholvin, J., Goldman, M., Kinney, J.P., et al. (2017). Cell diversity and network dynamics in photosensitive human brain organoids. Nature *545*, 48–53.

Raja, W.K., Mungenast, A.E., Lin, Y.-T., Ko, T., Abdurrob, F., Seo, J., and Tsai, L.-H. (2016). Self-Organizing 3D Human Neural Tissue Derived from Induced Pluripotent Stem Cells Recapitulate Alzheimer's Disease Phenotypes. PLoS ONE *11*, e0161969.

Rakic, P. (1971). Guidance of neurons migrating to the fetal monkey neocortex. Brain Res. *33*, 471–476.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. J. Comp. Neurol. *145*, 61–83.

Raz, N., Torres, I.J., Briggs, S.D., Spencer, W.D., Thornton, A.E., Loken, W.J., Gunning, F.M., McQuain, J.D., Driesen, N.R., and Acker, J.D. (1995). Selective neuroanatomic abnormalities in Down's syndrome and their cognitive correlates: evidence from MRI morphometry. Neurology *45*, 356–366.

Redmond, L., Oh, S.R., Hicks, C., Weinmaster, G., and Ghosh, A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. Nat. Neurosci. *3*, 30–40.

Redon, R., Ishikawa, S., Fitch, K.R., Feuk, L., Perry, G.H., Andrews, T.D., Fiegler, H., Shapero, M.H., Carson, A.R., Chen, W., et al. (2006). Global variation in copy number in the human genome. Nature *444*, 444–454.

Reeves, R.H., Irving, N.G., Moran, T.H., Wohn, A., Kitt, C., Sisodia, S.S., Schmidt, C., Bronson, R.T., and Davisson, M.T. (1995). A mouse model for Down syndrome exhibits learning and behaviour deficits. Nat. Genet. *11*, 177–184.

Ricciardi, A.S., Bahal, R., Farrelly, J.S., Quijano, E., Bianchi, A.H., Luks, V.L., Putman, R., López-Giráldez, F., Coşkun, S., Song, E., et al. (2018). In utero nanoparticle delivery for site-specific genome editing. Nat Commun *9*, 2481.

Riddell, D.R., Zhou, H., Atchison, K., Warwick, H.K., Atkinson, P.J., Jefferson, J., Xu, L., Aschmies, S., Kirksey, Y., Hu, Y., et al. (2008). Impact of Apolipoprotein E (ApoE) Polymorphism on Brain ApoE Levels. J. Neurosci. *28*, 11445–11453.

Roessmann, U., and Gambetti, P. (1986). Astrocytes in the developing human brain. An immunohistochemical study. Acta Neuropathol. *70*, 308–313.

Roizen, N.J., and Patterson, D. (2003). Down's syndrome. Lancet *361*, 1281–1289.

Roper, R.J., and Reeves, R.H. (2006). Understanding the basis for Down syndrome phenotypes. PLoS Genet. *2*, e50.

Roper, R.J., Baxter, L.L., Saran, N.G., Klinedinst, D.K., Beachy, P.A., and Reeves, R.H. (2006). Defective cerebellar response to mitogenic Hedgehog signaling in Down syndrome mice. Proc. Natl. Acad. Sci. U.S.A. *103*, 1452–1456.

Ropper, A.H., and Williams, R.S. (1980). Relationship between plaques, tangles, and dementia in Down syndrome. Neurology *30*, 639–644.

Rosin, M.M., Swift, E., Bless, D., and Kluppel Vetter, D. (1988). Communication Profiles of Adolescents With Down Syndrome. Journal of Childhool Communication Disorders *12*, 49–64.

Ross, M.H., Galaburda, A.M., and Kemper, T.L. (1984). Down's syndrome: is there a decreased population of neurons? Neurology *34*, 909–916.

Rouaux, C., and Arlotta, P. (2013). Direct lineage reprogramming of post-mitotic callosal neurons into corticofugal neurons in vivo. Nat. Cell Biol. *15*, 214–221.

Rovelet-Lecrux, A., Hannequin, D., Raux, G., Le Meur, N., Laquerrière, A., Vital, A., Dumanchin, C., Feuillette, S., Brice, A., Vercelletto, M., et al. (2006). APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. Nat. Genet. *38*, 24–26.

Rovelet-Lecrux, A., Frebourg, T., Tuominen, H., Majamaa, K., Campion, D., and Remes, A.M. (2007). APP locus duplication in a Finnish family with dementia and intracerebral haemorrhage. J. Neurol. Neurosurg. Psychiatry *78*, 1158–1159.

Rowe, J., Lavender, A., and Turk, V. (2006). Cognitive executive function in Down's syndrome. Br J Clin Psychol *45*, 5–17.

Russell, L.B. (1963). Mammalian X-chromosome action: inactivation limited in spread and region of origin. Science *140*, 976–978.

Sahakyan, A., Kim, R., Chronis, C., Sabri, S., Bonora, G., Theunissen, T.W., Kuoy, E., Langerman, J., Clark, A.T., Jaenisch, R., et al. (2017). Human Naive Pluripotent Stem Cells Model X Chromosome Dampening and X Inactivation. Cell Stem Cell *20*, 87–101.

Sahakyan, A., Yang, Y., and Plath, K. (2018). The Role of Xist in X-Chromosome Dosage Compensation. Trends Cell Biol. *28*, 999–1013.

Salloway, S., Sperling, R., Fox, N.C., Blennow, K., Klunk, W., Raskind, M., Sabbagh, M., Honig, L.S., Porsteinsson, A.P., Ferris, S., et al. (2014). Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. N. Engl. J. Med. *370*, 322–333.

Satgé, D., Sommelet, D., Geneix, A., Nishi, M., Malet, P., and Vekemans, M. (1998). A tumor profile in Down syndrome. Am. J. Med. Genet. *78*, 207–216.

Savarese, F., Flahndorfer, K., Jaenisch, R., Busslinger, M., and Wutz, A. (2006). Hematopoietic precursor cells transiently reestablish permissiveness for X inactivation. Mol. Cell. Biol. *26*, 7167–7177.

Savelsbergh, G., van der Kamp, J., Ledebt, A., and Planinsek, T. (2000). Information-movement coupling in children with Down syndrome. Perceptual-Motor Behavior in Down Syndrome 251–276.

Schmidt-Sidor, B., Wisniewski, K.E., Shepard, T.H., and Sersen, E.A. (1990). Brain growth in Down syndrome subjects 15 to 22 weeks of gestational age and birth to 60 months. Clin. Neuropathol. *9*, 181–190.

Selkoe, D., and Kopan, R. (2003). Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration. Annu. Rev. Neurosci. *26*, 565–597.

Shaffer, L.G., and Lupski, J.R. (2000). Molecular mechanisms for constitutional chromosomal rearrangements in humans. Annu. Rev. Genet. *34*, 297–329.

Shechner, D.M., Hacisuleyman, E., Younger, S.T., and Rinn, J.L. (2015). Multiplexable, locus-specific targeting of long RNAs with CRISPR-Display. Nature Methods *12*, 664–670.

Sheltzer, J.M., Torres, E.M., Dunham, M.J., and Amon, A. (2012). Transcriptional consequences of aneuploidy. Proc. Natl. Acad. Sci. U.S.A. *109*, 12644–12649.

Shi, Y., Kirwan, P., and Livesey, F.J. (2012a). Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. Nat Protoc *7*, 1836–1846.

Shi, Y., Kirwan, P., Smith, J., MacLean, G., Orkin, S.H., and Livesey, F.J. (2012b). A human stem cell model of early Alzheimer's disease pathology in Down syndrome. Sci Transl Med *4*, 124ra29.

Shim, K.S., and Lubec, G. (2002). Drebrin, a dendritic spine protein, is manifold decreased in brains of patients with Alzheimer's disease and Down syndrome. Neurosci. Lett. *324*, 209–212.

Sidman, R.L., and Rakic, P. (1973). Neuronal migration, with special reference to developing human brain: a review. Brain Res. *62*, 1–35.

Silbereis, J.C., Pochareddy, S., Zhu, Y., Li, M., and Sestan, N. (2016). The Cellular and Molecular Landscapes of the Developing Human Central Nervous System. Neuron *89*, 248–268.

Silva, S.S., Rowntree, R.K., Mekhoubad, S., and Lee, J.T. (2008). X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells. PNAS *105*, 4820–4825.

Sleegers, K., Brouwers, N., Gijselinck, I., Theuns, J., Goossens, D., Wauters, J., Del-Favero, J., Cruts, M., van Duijn, C.M., and Van Broeckhoven, C. (2006). APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy. Brain *129*, 2977–2983.

Smart, I.H.M., Dehay, C., Giroud, P., Berland, M., and Kennedy, H. (2002). Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. Cereb. Cortex *12*, 37–53.

Smith, B.L., and Oller, D.K. (1981). A comparative study of pre-meaningful vocalizations produced by normally developing and Down's syndrome infants. J Speech Hear Disord *46*, 46–51.

Smith, C., Abalde-Atristain, L., He, C., Brodsky, B.R., Braunstein, E.M., Chaudhari, P., Jang, Y.-Y., Cheng, L., and Ye, Z. (2015). Efficient and allele-specific genome editing of disease loci in human iPSCs. Mol. Ther. *23*, 570–577.

Soldner, F., and Jaenisch, R. (2012). Medicine. iPSC disease modeling. Science *338*, 1155–1156.

Song, J.K., and Giniger, E. (2011). Noncanonical Notch function in motor axon guidance is mediated by Rac GTPase and the GEF1 domain of Trio. Dev. Dyn. *240*, 324–332.

Sorrells, S.F., Paredes, M.F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K.W., James, D., Mayer, S., Chang, J., Auguste, K.I., et al. (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. Nature *555*, 377–381.

Stankiewicz, P., Kuechler, A., Eller, C.D., Sahoo, T., Baldermann, C., Lieser, U., Hesse, M., Gläser, C., Hagemann, M., Yatsenko, S.A., et al. (2006). Minimal phenotype in a girl with trisomy 15q due to t(X;15)(q22.3;q11.2) translocation. Am. J. Med. Genet. A *140*, 442–452.

Stern, C.D. (2005). Neural induction: old problem, new findings, yet more questions. Development *132*, 2007–2021.

Stoodley, C.J. (2016). The Cerebellum and Neurodevelopmental Disorders. Cerebellum *15*, 34–37.

Stühmer, T., Anderson, S.A., Ekker, M., and Rubenstein, J.L.R. (2002). Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. Development *129*, 245–252.

Suetsugu, M., and Mehraein, P. (1980). Spine distribution along the apical dendrites of the pyramidal neurons in Down's syndrome. A quantitative Golgi study. Acta Neuropathol. *50*, 207–210.

Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E.J., Hatanaka, F., Yamamoto, M., Araoka, T., Li, Z., et al. (2016). In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature *540*, 144–149.

Sweeney, N.P., Meng, J., Patterson, H., Morgan, J.E., and McClure, M. (2017). Delivery of large transgene cassettes by foamy virus vector. Scientific Reports *7*, 8085.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

Takahashi, T., Nowakowski, R.S., and Caviness, V.S. (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J. Neurosci. *15*, 6046–6057.

Takashima, S., Becker, L.E., Armstrong, D.L., and 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 Res. *225*, 1–21.

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev. Cell *1*, 749–758.

Tao, Y., and Zhang, S.-C. (2016). Neural Subtype Specification from Human Pluripotent Stem Cells. Cell Stem Cell *19*, 573–586.

Tartaglia, N.R., Howell, S., Sutherland, A., Wilson, R., and Wilson, L. (2010). A review of trisomy X (47,XXX). Orphanet J Rare Dis *5*, 8.

Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., et al. (2012). RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. Cell *148*, 664–678.

Theisen, A., and Shaffer, L.G. (2010). Disorders caused by chromosome abnormalities. Appl Clin Genet *3*, 159–174.

Tirosh, I., Izar, B., Prakadan, S.M., Wadsworth, M.H., Treacy, D., Trombetta, J.J., Rotem, A., Rodman, C., Lian, C., Murphy, G., et al. (2016). Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science *352*, 189–196.

Valledor, M., Myers, R.S., and Schiller, P.C. (2018). Herpes ICP8 protein stimulates homologous recombination in human cells. PLoS ONE *13*, e0200955.

Vallot, C., Huret, C., Lesecque, Y., Resch, A., Oudrhiri, N., Bennaceur-Griscelli, A., Duret, L., and Rougeulle, C. (2013). XACT, a long noncoding transcript coating the active X chromosome in human pluripotent cells. Nat. Genet. *45*, 239–241.

Vallot, C., Patrat, C., Collier, A.J., Huret, C., Casanova, M., Liyakat Ali, T.M., Tosolini, M., Frydman, N., Heard, E., Rugg-Gunn, P.J., et al. (2017). XACT Noncoding RNA Competes with XIST in the Control of X Chromosome Activity during Human Early Development. Cell Stem Cell *20*, 102–111.

van der Kant, R., and Goldstein, L.S.B. (2015). Cellular Functions of the Amyloid Precursor Protein from Development to Dementia. Developmental Cell *32*, 502–515.

Velasco, S., Kedaigle, A.J., Simmons, S.K., Nash, A., Rocha, M., Quadrato, G., Paulsen, B., Nguyen, L., Adiconis, X., Regev, A., et al. (2019). Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. Nature.

Vilardell, M., Rasche, A., Thormann, A., Maschke-Dutz, E., Pérez-Jurado, L.A., Lehrach, H., and Herwig, R. (2011). Meta-analysis of heterogeneous Down Syndrome data reveals consistent genome-wide dosage effects related to neurological processes. BMC Genomics *12*, 229.

Vukšić, M., Petanjek, Z., Rasin, M.R., and Kostović, I. (2002). Perinatal growth of prefrontal layer III pyramids in Down syndrome. Pediatr. Neurol. *27*, 36–38.

Walton, R.M. (2012). Postnatal Neurogenesis: Of Mice, Men, and Macaques. Vet Pathol *49*, 155–165.

Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J.C., Nagy, A., and Magnuson, T. (2001). Imprinted X inactivation maintained by a mouse Polycomb group gene. Nat. Genet. *28*, 371–375.

Wang, Y., Chan, S.L., Miele, L., Yao, P.J., Mackes, J., Ingram, D.K., Mattson, M.P., and Furukawa, K. (2004). Involvement of Notch signaling in hippocampal synaptic plasticity. Proc. Natl. Acad. Sci. U.S.A. *101*, 9458–9462.

Waterhouse, P.M., Wang, M.-B., and Lough, T. (2001). Gene silencing as an adaptive defence against viruses. Nature *411*, 834.

Weick, J.P., Held, D.L., Bonadurer, G.F., Doers, M.E., Liu, Y., Maguire, C., Clark, A., Knackert, J.A., Molinarolo, K., Musser, M., et al. (2013). Deficits in human trisomy 21 iPSCs and neurons. Proc. Natl. Acad. Sci. U.S.A. *110*, 9962–9967.

Welshons, W.J., and Russell, L.B. (1959). The Y-chromosome as the bearer of male determining factors in the mouse. Proc. Natl. Acad. Sci. U.S.A. *45*, 560–566.

Williams, B.R., Prabhu, V.R., Hunter, K.E., Glazier, C.M., Whittaker, C.A., Housman, D.E., and Amon, A. (2008). Aneuploidy affects proliferation and spontaneous immortalization in mammalian cells. Science *322*, 703–709.

Wiseman, F.K., Al-Janabi, T., Hardy, J., Karmiloff-Smith, A., Nizetic, D., Tybulewicz, V.L.J., Fisher, E.M.C., and Strydom, A. (2015). A genetic cause of Alzheimer disease: mechanistic insights from Down syndrome. Nat. Rev. Neurosci. *16*, 564–574.

Wisniewski, K.E. (1986). Neuronal density and synaptogenesis in the postnatal stage of brain maturation in Down syndrome. The Neurobiology of Down Syndrome 29–44.

Wisniewski, K.E. (1990). Down syndrome children often have brain with maturation delay, retardation of growth, and cortical dysgenesis. Am J Med Genet Suppl *7*, 274–281.

Wisniewski, K.E., and Schmidt-Sidor, B. (1989). Postnatal delay of myelin formation in brains from Down syndrome infants and children. Clin. Neuropathol. *8*, 55–62.

Wisniewski, H.M., Silverman, W., and Wegiel, J. (1994). Ageing, Alzheimer disease and mental retardation. J Intellect Disabil Res *38 (Pt 3)*, 233–239.

Wisniewski, K.E., Wisniewski, H.M., and Wen, G.Y. (1985a). Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. Ann. Neurol. *17*, 278–282.

Wisniewski, K.E., Dalton, A.J., McLachlan, C., Wen, G.Y., and Wisniewski, H.M. (1985b). Alzheimer's disease in Down's syndrome: clinicopathologic studies. Neurology *35*, 957–961.

Workman, A.D., Charvet, C.J., Clancy, B., Darlington, R.B., and Finlay, B.L. (2013). Modeling transformations of neurodevelopmental sequences across mammalian species. J. Neurosci. *33*, 7368–7383.

Wutz, A., and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol. Cell *5*, 695–705.

Wutz, A., Rasmussen, T.P., and Jaenisch, R. (2002). Chromosomal silencing and localization are mediated by different domains of Xist RNA. Nat. Genet. *30*, 167–174.

Xia, X., Zhang, Y., Zieth, C.R., and Zhang, S.-C. (2007). Transgenes Delivered by Lentiviral Vector Are Suppressed in Human Embryonic Stem Cells in a Promoter-Dependent Manner. Stem Cells Dev *16*, 167–176.

Xing, Y., Johnson, C.V., Dobner, P.R., and Lawrence, J.B. (1993). Higher level organization of individual gene transcription and RNA splicing. Science *259*, 1326–1330.

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., et al. (2019). OLIG2 Drives Abnormal Neurodevelopmental Phenotypes in Human iPSC-Based Organoid and Chimeric Mouse Models of Down Syndrome. Cell Stem Cell *24*, 908-926.e8.

Yakovlev, P. (1967). The myelogenetic cycles of regional maturation of the brain. Regional Development of the Brain in Early Life 3–70.

Yamamoto, Y., Bliss, J., and Gerbi, S.A. (2015). Whole Organism Genome Editing: Targeted Large DNA Insertion via ObLiGaRe Nonhomologous End-Joining in Vivo Capture. G3: Genes, Genomes, Genetics *5*, 1843–1847.

Yang, Y., Conners, F.A., and Merrill, E.C. (2014). Visuo-spatial ability in individuals with Down syndrome: is it really a strength? Res Dev Disabil *35*, 1473–1500.

Ye, Z., Mostajo-Radji, M.A., Brown, J.R., Rouaux, C., Tomassy, G.S., Hensch, T.K., and Arlotta, P. (2015). Instructing Perisomatic Inhibition by Direct Lineage Reprogramming of Neocortical Projection Neurons. Neuron *88*, 475–483.

Yeung, M.S.Y., Zdunek, S., Bergmann, O., Bernard, S., Salehpour, M., Alkass, K., Perl, S., Tisdale, J., Possnert, G., Brundin, L., et al. (2014). Dynamics of oligodendrocyte generation and myelination in the human brain. Cell *159*, 766–774.

Yildirim, E., Kirby, J.E., Brown, D.E., Mercier, F.E., Sadreyev, R.I., Scadden, D.T., and Lee, J.T. (2013). Xist RNA is a potent suppressor of hematologic cancer in mice. Cell *152*, 727–742.

Yin, H., Song, C.-Q., Dorkin, J.R., Zhu, L.J., Li, Y., Wu, Q., Park, A., Yang, J., Suresh, S., Bizhanova, A., et al. (2016). Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components *in vivo*. Nature Biotechnology *34*, 328–333.

Yoon, S.-J., Elahi, L.S., Pașca, A.M., Marton, R.M., Gordon, A., Revah, O., Miura, Y., Walczak, E.M., Holdgate, G.M., Fan, H.C., et al. (2019). Reliability of human cortical organoid generation. Nat. Methods *16*, 75–78.

Young-Pearse, T.L., and Morrow, E.M. (2016). Modeling developmental neuropsychiatric disorders with iPSC technology: challenges and opportunities. Curr. Opin. Neurobiol. *36*, 66–73.

Yu, X., and Zecevic, N. (2011). Dorsal radial glial cells have the potential to generate cortical interneurons in human but not in mouse brain. J. Neurosci. *31*, 2413–2420.

Yu, T., Li, Z., Jia, Z., Clapcote, S.J., Liu, C., Li, S., Asrar, S., Pao, A., Chen, R., Fan, N., et al. (2010). A mouse model of Down syndrome trisomic for all human chromosome 21 syntenic regions. Hum. Mol. Genet. *19*, 2780–2791.

Zawerton, A., Yao, B., Yeager, J.P., Pippucci, T., Haseeb, A., Smith, J.D., Wischmann, L., Kühl, S.J., Dean, J.C.S., Pilz, D.T., et al. (2019). De Novo SOX4 Variants Cause a Neurodevelopmental Disease Associated with Mild Dysmorphism. Am. J. Hum. Genet. *104*, 777.

Zdaniuk, G., Wierzba-Bobrowicz, T., Szpak, G.M., and Stępień, T. (2011). Astroglia disturbances during development of the central nervous system in fetuses with Down's syndrome. Folia Neuropathol *49*, 109–114.

Zecevic, N., Hu, F., and Jakovcevski, I. (2011). Interneurons in the developing human neocortex. Dev Neurobiol *71*, 18–33.

Zhang, S.C., Wernig, M., Duncan, I.D., Brüstle, O., and Thomson, J.A. (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol. *19*, 1129–1133.

Zhao, J., Sun, B.K., Erwin, J.A., Song, J.-J., and Lee, J.T. (2008). Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science *322*, 750–756.

Zigman, W.B., Schupf, N., Sersen, E., and Silverman, W. (1996). Prevalence of dementia in adults with and without Down syndrome. Am J Ment Retard *100*, 403–412.

Zimmerman, L.B., De Jesús-Escobar, J.M., and Harland, R.M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell *86*, 599–606.

Zipursky, A., Poon, A., and Doyle, J. (1992). Leukemia in Down syndrome: a review. Pediatr Hematol Oncol *9*, 139–149.

Zuchero, J.B., and Barres, B.A. (2015). Glia in mammalian development and disease. Development *142*, 3805–3809.

Zugates, G.T., Peng, W., Zumbuehl, A., Jhunjhunwala, S., Huang, Y.-H., Langer, R., Sawicki, J.A., and Anderson, D.G. (2007). Rapid optimization of gene delivery by parallel end-modification of poly(beta-amino ester)s. Mol. Ther. *15*, 1306–1312.

Żylicz, J.J., Bousard, A., Žumer, K., Dossin, F., Mohammad, E., da Rocha, S.T., Schwalb, B., Syx, L., Dingli, F., Loew, D., et al. (2019). The Implication of Early Chromatin Changes in X Chromosome Inactivation. Cell *176*, 182-197.e23.