An isogenic cell line panel identifies major regulators of aberrant astrocyte proliferation in Down syndrome

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Abstract

Astrocytes exert adverse effects on the brains of individuals with Down syndrome (DS). Although a neurogenic-to-gliogenic shift in the fate-specification step has been reported, the mechanisms and key regulators underlying the accelerated proliferation of astrocyte precursor cells (APCs) in DS remain elusive. Here, we established an isogenic cell line panel, based on DS-specific induced pluripotent stem cells, the *XIST*-mediated transcriptional silencing system in trisomic chromosome 21, and genome/chromosome-editing technologies to eliminate phenotypic fluctuations caused by genetic variation. The transcriptional responses of genes observed upon *XIST* induction and/or downregulation were not uniform, and only a small subset of genes showed a characteristic expression pattern, which is consistent with the proliferative phenotypes of DS APCs. Comparative analysis and experimental verification using gene modification revealed dose-dependent proliferation-promoting activity of *DYRK1A* and *PIGP* on DS APCs. Our collection of isogenic cell lines provides a comprehensive set of cellular models for DS investigations.

Introduction

Cellular and organismal homeostasis relies on a precise and delicate balance among various gene pathways¹. To maintain this intricate equilibrium, protein production is strictly controlled by chromatin structure, transcriptional regulation, or posttranscriptional modification². Alteration of gene dosage is one of the leading causes of the perturbed transcriptional network, leading to severe impacts on biological processes underlying cellular functions. Down syndrome (DS; trisomy 21) is the most common form of chromosomal aberration, which results from an extra copy of human chromosome 21^{3,4}. All individuals with DS exhibit various types of clinical features, including intellectual disability and cognitive deficits⁵. Although the healthy human brain contains almost equal numbers of neuronal and glial cells⁶, studies with DS brains showed a significantly reduced number of neurons and nearly twice as many astrocytes compared to age-matched controls^{7,8}. In general, astrocytes are mainly generated by two different processes: fate specification from multipotent progenitor cells and the local proliferation of lineage-specific (i.e., differentiated) astrocyte precursor cells (APCs)⁹. A neurogenic-to-gliogenic shift in the timing of the fate specification from radial glia or neural progenitor cells (NPCs) in DS has been reported, and several genes have been identified as causative regulators^{10,11,12,13,14}. However, a major source of astrocytes in the postnatal cortex is attributable to a local division of differentiated APCs, rather than differentiation from progenitor cells, and approximately half of all mature cortical astrocytes are produced by APC proliferation¹⁵. Because these increased astrocytes can interact negatively with neurons during neuronal maturation, synapse formation, and the release of factors that promote neuronal apoptosis^{16,17,18}, elucidating whether and how dysregulated APC proliferation is involved in DS pathophysiology is essential.

Generating systematic and precise disease models and identifying the responsible genes are crucial for investigating underlying disease mechanisms. Despite the rapid development of disease-specific, induced

pluripotent stem cells (iPSCs) and genome-editing technologies, the complexity of transcriptional dynamics affected by the extra copy of chromosome 21 and fluctuations of gene-expression profiles across individuals or cell lines hinders the identification of key regulators. An innovative and excellent model system for studying the biology of DS has been developed by integrating the human X-inactive specific transcript (*XIST*) gene, into DS-specific iPSCs¹⁹. In one such cell line, *XIST* was inserted into a copy of chromosome 21 in trisomy 21 iPSCs (Tri21 iPSCs), and a long noncoding RNA induced a series of chromatin modifications that stably silenced gene transcription across the whole chromosome in *cis*. Chromosome silencing occurred even in differentiated cells, and various pathologies observed in DS (including proliferative defects, impaired neural differentiation, and haematopoietic abnormalities) were successfully reversed by the transcriptional inactivation of the supernumerary chromosome^{20,21}. Using this genome-silencing technology, where *XIST* RNA expression is regulated by the tetracycline-inducible system, enables researchers to investigate the correlation between gene-expression changes and cellular phenotypes in DS, without limitations caused by transcriptional heterogeneity and differences among cell lines.

To eliminate the biological ‘noise’, which can result from genetic variability, we established an isogenic iPSC panel, where all cell lines share a single genetic background by combining DS-specific iPSCs, *XIST*-induced chromosome silencing, and genome/chromosome-editing technologies (Fig. 1). These cell lines were subjected to astrocytic differentiation, and comparative analysis between their gene-expression profiles and proliferative phenotypes (with a common genetic background) was performed. Once *XIST*-induced silencing was stabilised, the transcriptional levels of most genes were continuously suppressed after the removal of doxycycline (Dox). However, the enhanced proliferative phenotype of APCs in DS, which was suppressed by chromosome silencing, returned to aberrantly accelerated conditions by Dox removal. Careful analysis of this discrepancy between transcriptional and phenotypic responses enabled us to narrow down the causative genes responsible for APC overproliferation. We further established various types of systematically designed partial trisomy 21 iPSCs (Partial-Tri21 iPSCs), leading to the identification of two responsible genes, namely dual-specificity tyrosine-phosphorylation-regulated kinase 1A (*DYRK1A*) and phosphatidylinositol glycan anchor biosynthesis, class P (*PIGP*).

Results

Generation of an isogenic iPSC panel for disease modelling of trisomy 21. We previously generated a patient-derived Tri21 iPSC line that contains one paternal copy and two maternal copies of chromosome 21²². Using this DS-specific iPSC line, we further generated a corrected disomy 21 iPSC line (cDi21 iPSC), in which a single copy of chromosome 21 was artificially removed from a Tri21 iPSC line²³, and a partial trisomy 21 iPSC line (Partial-Tri21 iPSC) in which a 4-megabase (Mb) region corresponding to a ‘Down syndrome critical region’ was selectively deleted only from the paternal chromosome 21 in Tri21 iPSCs (Fig. 1)²². An *XIST*-mediated chromosome 21-silencing system was generated by inserting Dox-inducible *XIST* complementary DNA (cDNA) into one copy of chromosome 21 in Tri21 iPSCs, as described previously with a modification in terms of the Cre recombinase-mediated cassette exchange

(Supplementary Fig. 1a – c)¹⁹. The resultant iPSC clone (*XIST*-Tri21 iPSC) exhibited a typical morphology, expression of pluripotent markers, and a trisomy 21 karyotype (Supplementary Fig. 2a, b). Chromosome 21 in *XIST*-Tri21 iPSCs contained one paternal chromosome and two maternal chromosomes, which is consistent with that in the original iPSC (Supplementary Fig. 2c). Administration of Dox for 3 weeks (D+) successfully induced *XIST* RNA expression and the accumulation of H3K27me3, a hallmark of heterochromatin, which led to the transcriptional silencing of genes on chromosome 21 (Supplementary Fig. 3a – d). However, less than 45% of *XIST*-Tri21 iPSCs were H3K27me3-positive even after 3 weeks of Dox administration (Supplementary Fig. 3e). This relatively low efficiency of H3K27me3 induction was continuously observed in single-cell-derived clones of *XIST*-Tri iPSCs (Supplementary Fig. 3f). Moreover, neither *XIST* RNA expression nor H3K27me3 marks were detected in NPCs differentiated from *XIST*-Tri21 iPSCs (*XIST*-Tri21 NPCs), which was accompanied by a lack of expression of reverse tetracycline transactivator (rtTA), which was inserted in the *AAVS1* safe harbour locus on chromosome 19 (Supplementary Fig. 4a, b). To compensate for this induction failure by the tetracycline-regulated system, the rtTA was additionally transduced into NPCs differentiated from *XIST*-Tri21 iPSCs, using a *piggyBac* (PB) transposon vector and a hyperactive PB transposase²⁴ (Supplementary Fig. 4b). The insertion of PB-rtTA increased the expression levels of rtTA in NPCs, leading to a significant elevation of *XIST* RNA expression after Dox administration (Supplementary Fig. 4c, d). H3K27me3 marks were detected in approximately 90% of Dox-treated NPCs, suggesting that a sufficient amount of rtTA was required to induce *XIST* (Supplementary Fig. 4e, f).

***XIST*-mediated chromosome silencing affected the overproliferative phenotypes of DS APCs.** Among the various pathological features of DS, we focused on the astrocyte population, which is aberrantly increased in the brains of individuals with DS. *XIST*-Tri21 NPCs transfected with an rtTA-expression vector were differentiated to the astrocyte lineage, according to a previously described protocol²⁵ (Fig. 2a). Glial fibrillary acidic protein (GFAP) and S100 β (typical astrocytic markers) were detected in over 90% of the differentiated cells (Fig. 2b). Moreover, nearly all differentiated cells were positive for CD44 or vimentin (astrocyte-restricted precursor cell markers)^{10, 26}, but negative for SOX1 (an early marker for neural stem cells), indicating that the cells differentiated to APCs (*XIST*-Tri21 APCs). *XIST*-Tri21 APCs exhibited sufficient rtTA expression and Dox-dependent induction of *XIST* RNA expression and H3K27me3 marks (Fig. 2c – e, Supplementary Fig. 5a). The expression levels of genes on chromosome 21 in Dox-treated *XIST*-Tri21 APCs were lower than those in Dox-untreated cells, suggesting that *XIST*-mediated chromosome silencing was successfully induced in APCs (Fig. 2f).

Cell-proliferation assays performed using 5-ethynyl-2'-deoxyuridine (EdU) in Tri21 APCs showed a higher proliferation rate than isogenic euploid APCs (cDi21 APCs). Dox administration (D+) did not affect the basal proliferative ability of simple Tri21 APCs (i.e., those without *XIST* cDNA), but significantly decreased the proliferation rate of *XIST*-Tri21 APCs to a level similar to that of cDi21 cells (Fig. 2g – i, Supplementary Fig. 5b, c). Furthermore, removing Dox (D^{remov}) for 3 weeks after the initial 3-week Dox treatment increased the proliferation rate of *XIST*-Tri21 APCs to that of Dox-untreated cells (Fig. 2g – i). This phenotypic change was accompanied by reduced *XIST* expression (Fig. 2c), suggesting that expression

of the genes responsible for APC overexpression was suppressed by chromosome silencing, which was reversed by Dox removal.

XIST-mediated chromosome silencing was generally maintained at least 3 weeks after Dox removal.

Previous reports showed that inducible expression of murine *Xist* initially caused reversible chromosome inactivation in undifferentiated cells, and then irreversible inactivation after differentiation²⁷, whereas forced expression of human *XIST* cDNA in somatic cells resulted in reversible silencing²⁸. To assess how induction and depletion of the ectopic *XIST* affect transcriptional dynamics in human differentiated cells, gene expression and H3K27me3 histone-methylation profiles were analysed in four cell lines, i.e. cDi21 APCs or *XIST*-Tri21 APCs, with (D+), without (D-) Dox, and at 3 weeks after Dox removal (D^{remov}).

Overall, gene expression from chromosome 21 was higher in D - APCs than in cDi21 APCs, probably due to the gene-dosage effects of trisomy 21. Consistent with previous data indicating that *XIST* expression was essential for the initiation of chromosome silencing, but was not required for chromosome maintenance^{19, 27, 29}, Dox treatment effectively suppressed the expression of genes located in chromosome 21, and this suppression was maintained overall 3 weeks after Dox was removed (Fig. 3a, Supplementary Fig. 6, 7). This observation was supported by hierarchical-cluster analysis of 178 genes with positive read counts on chromosome 21, demonstrating relative similarities in the transcriptional profiles of D + and D^{remov} APC lines compared with the D - line (Fig. 3b). H3K27me3 marks were enriched along chromosome 21 in D + APCs (Fig. 3c). H3K27me3 levels were lower in D^{remov} APCs, but remained substantially more potent than those in D - cells. A clear, negative correlation between H3K27me3 enrichment and reduced expression was observed among genes on chromosome 21 in D + and D^{remov} APCs (Fig. 3c, d, Supplementary Fig. 8). These results suggest that forced expression of human *XIST* effectively inactivated chromosome 21, and the suppression was generally maintained for at least 3 weeks after Dox removal.

The 4-Mb region on chromosome 21 was critical for aberrant APC proliferation in DS. Although the results of RNA sequencing (RNA-seq) and chromatin immunoprecipitation-sequencing (ChIP-seq) analyses indicated that *XIST*-mediated chromosome silencing was preserved for 3 weeks after Dox removal and that D^{remov} APC proliferation apparently returned to the accelerated condition, similar to that of D - cells. To explore underlying mechanisms that could explain this difference, we focused on a shape change in the violin plot for the D^{remov} cells. In the violin plot, other cell lines showed uniform distribution, but the D^{remov} cells showed a bimodal distribution, as evidenced by the occurrence of an additional hump in the plot (indicated by the arrowhead in Fig. 3a), which suggested the existence of a small subgroup of genes whose expression rapidly returned after the removal of Dox-induced silencing. We speculated that this phenomenon was simulated in component 1 of our principal component analysis (PCA), in which the values of D^{remov} lines moved relatively close to those of the D - lines, while drifting away from those of the D + and cDi21 lines (Fig. 4a).

To identify causative genes among the list of genes in component 1 (Supplementary Table 2), we narrowed down the candidate genes as follows (Fig. 4b). Among 517 genes located on chromosome 21, nearly one-third of the genes (178 genes) showed positive read counts in at least one of the APC lines, and 56 genes showed > 1.5-fold increased expression, when compared with the corresponding expression levels in cDi21 APCs. The expression levels of 21 coding genes were significantly decreased in D + cells, suggesting that these genes were involved in the gene dosage-dependent phenotypic alteration found in *XIST*-Tri21 APCs.

We previously generated a genome-edited Partial-Tri21 iPSC line in which a ~ 4-Mb segment between *RUNX1* and *ETS2* was deleted from a single copy of chromosome 21 in Tri21 iPSCs (Fig. 4c)²². This 4-Mb segment corresponds to the critical region for DS pathogenesis, commonly referred to as the Down syndrome critical region. Notably, the deletion of this 4-Mb region from one chromosome 21 led to a significant decrease in the proliferation rate of APCs, down to the same level found with cDi21 APCs, indicating that this segment was responsible for the aberrant proliferative properties of DS APCs (Fig. 4d, e). Among the 32 genes located in this 4-Mb region, three genes (*DYRK1A*, *DSCR3*, and *HLCS*) were ranked in the top 10, and two genes (*MORC3* and *PIGP*) were in the 25th and 31st positions, as essential elements of PCA component 1 (Supplementary Table 2). Consistent with these results, RNA-seq data demonstrated that the gene-expression levels of these genes reverted to trisomic levels after Dox removal, except for *MORC3* (Supplementary Table 3). The gene-expression alterations were validated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis for the *DYRK1A*, *PIGP*, and *DSCR3* genes, but not for the *HLCS* gene (Fig. 4b, f). Significantly increased expression and elimination of H3K27me3 deposition were observed for these genes in D + and D^{remov} cells, respectively (Fig. 4g, Supplementary Fig. 9a, b).

***DYRK1A* potentially regulated APC proliferation in a gene dosage-dependent manner.** *DYRK1A* was identified as the most potent candidate gene in the PCA list (Supplementary Table 2). However, it is well established that *DYRK1A* exerts dose-dependent antiproliferative activity in several cell types, especially in neural precursor cells^{30, 31}. To investigate whether *DYRK1A* conversely promotes APC proliferation, and whether *DYRK1A* acts independently in DS pathophysiology or cooperates with other molecules synergistically, we prepared several genome-edited Tri21 iPSC lines, in which *DYRK1A* was targeted in one or two chromosomes in the *XIST*-Tri21 iPSC line (DY^{+/+/m}- and DY^{+/m/m}-*XIST*-Tri21 iPSC, respectively; Fig. 5a).

Single-nucleotide polymorphism (SNP) analysis of mRNAs extracted from *XIST*-Tri21 APCs (with or without Dox treatment) showed that a SNP in the *ETS2* gene (rs457705) derived from the paternal (P) allele (T→G) was conserved after *XIST*-mediated chromosome silencing. This result indicated that the *XIST* cDNA cassette was inserted in one of the two maternal copies (M1 and M2) of chromosome 21 (Supplementary Fig. 10; hereafter, the *XIST*-inserted maternal chromosome is referred to as M2). The targeting cassette was inserted into the *DYRK1A* locus in *XIST*-Tri21 iPSCs using the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9). Short-tandem repeat (STR) analysis of targeted and non-targeted alleles in isolated iPSC colonies revealed that six

types of genome-edited cell lines were obtained (single or double targeting of the M1, M2, or P alleles; Fig. 5b, c). *DYRK1A* expression in APCs decreased to similar levels found in $DY^{+/+/m}$ -*XIST*-Tri21 APCs and were even lower in $DY^{+/m/m}$ -*XIST*-Tri21 APCs than in Di21 APCs (Fig. 5d). Previous reports showed that the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway is one of the main targets of *DYRK1A* and that it is involved in gliogenic differentiation machinery in NPCs¹³. Consistent with the decreased expression of *DYRK1A*, Ser727-phosphorylated STAT3 was significantly decreased in $DY^{+/+/m}$ and $DY^{+/m/m}$ APCs, whereas no detectable changes in STAT3 protein levels were observed in these cell lines (Fig. 5e, Supplementary Fig. 11). Accelerated APC proliferation proportionally decreased in both lines, and the double-targeted ($DY^{+/m/m}$) APC line showed more severe proliferative impairment, indicating that *DYRK1A* regulated APC proliferation in an expression level-dependent manner (Fig. 5f, g).

Intriguingly, the $DY^{+/+/m}$ APC line, which contains two copies of the normal *DYRK1A* allele, still retained higher proliferation rates than the cDi21 line, suggesting that correction of the *DYRK1A* gene dose was not sufficient to fully reverse APC overproliferation.

To distinguish the roles of other genes on chromosome 21 from that of *DYRK1A*, both the $DY^{+/+/m}$ and $DY^{+/m/m}$ APC lines were subjected to *XIST*-mediated chromosome silencing. Given that *XIST* expression selectively inactivated the M2 chromosome in *cis*, these cell lines (with or without Dox treatment) exhibited various combinations of ‘*DYRK1A*-expression levels’ (zero, one, or two copies) and ‘transcriptional karyotypes of chromosome 21’ (trisomy or induced disomy), depending on the targeted alleles of the *DYRK1A* gene ($\Delta M1$, $\Delta M2$, ΔP , $\Delta M1\Delta M2$, $\Delta M1\Delta P$, or $\Delta M2\Delta P$; Fig. 5h). Dox treatment significantly reduced *DYRK1A* expression by approximately half in $\Delta M1$ - and ΔP - $DY^{+/+/m}$ APCs, as expected, whereas no significant changes occurred in $\Delta M2$ - $DY^{+/+/m}$ APCs (decreased to 48.6%, 55.0%, or 99.1% of the levels found in untreated cells, respectively), as shown in Supplementary Fig. 12a. Similarly, *DYRK1A* expression was not altered by chromosome silencing in $\Delta M1\Delta M2$ - or $\Delta M2\Delta P$ - $DY^{+/m/m}$ APCs (Supplementary Fig. 12b). These results showed that *DYRK1A* expression levels were precisely regulated in a gene dosage-dependent manner without being affected by the trisomy of other regions on chromosome 21, and that *XIST* exerted no *trans* effect on the M1 or P alleles of *DYRK1A*. The proliferation rates of APCs decreased by 22.8% and 22.5% after Dox treatment in the $\Delta M1$ - and ΔP - $DY^{+/+/m}$ lines, respectively (Fig. 5i, k). Less chromosome inactivation occurred in the $\Delta M2$ - $DY^{+/+/m}$ APC line, but cell proliferation was significantly reduced, despite the absence of a difference in *DYRK1A* expression. *XIST*-mediated silencing in the $\Delta M1\Delta P$ - $DY^{+/m/m}$ APC line, in which all *DYRK1A* alleles were lost or suppressed, showed the most severe impairment of cell proliferation (27.4% reduction compared to untreated cells, Fig. 5j, l). Similar to the results obtained with the $\Delta M2$ - $DY^{+/+/m}$ APC line, M2-inactivation in the $\Delta M1\Delta M2$ - and $\Delta M2\Delta P$ - $DY^{+/m/m}$ APC lines, in which *DYRK1A* expression was not changed by silencing, demonstrated significant proliferative decreases (10.1% and 11.9%, respectively), supporting the existence of other regulatory factors on chromosome 21 that control APC proliferation.

To confirm the effect of *DYRK1A* on proliferation, APCs and NPCs were treated with a DYRK1A inhibitor. A recently developed specific inhibitor, folding intermediate-selective inhibitor of DYRK1A (FINDY), which interferes with DYRK1A protein folding³², significantly reduced Tri21 APC proliferation (Fig. 5m, n), without effecting that of Tri21 NPCs (Fig. 5o, p). These findings indicate that *DYRK1A* potentially regulated APC proliferation in a dose-dependent manner.

PIGP, but not DSCR3, was involved in the proliferative pathophysiology of APCs in DS. Next, we used APCs to investigate whether two other candidate genes (*PIGP* and *DSCR3*) were involved in pathological features associated with DS. NPCs differentiated from Partial-Tri21 iPSCs were transfected with *piggyBac* transposon vectors containing human *PIGP* or *DSCR* cDNA, under the regulation of the Tet-inducible system, and then differentiated to the astrocyte lineage (Fig. 6a). Both *PIGP* and *DSCR3* expression increased in the corresponding Partial-Tri21 APC transfectants following Dox administration (Fig. 6b, Supplementary Fig. 13a). Proliferation rates in APCs, which were reverted to a normal level by the deletion of a 4-Mb critical region in one copy of chromosome 21, were increased by forced expression of *PIGP* (Fig. 6c, d). We confirmed this result using small-interfering RNA (siRNA)-mediated knockdown of *PIGP* in Tri21 APCs (Fig. 6e – g). However, the dose-dependent activity of *PIGP* was relatively less effective on APC proliferation, compared with that of *DYRK1A*, whose slight expression differences produced robust changes. However, *DSCR3* did not significantly impact proliferation when *DSCR3* was overexpressed in Partial-Tri21 APCs or when *DSCR3* was knocked down in Tri21 APCs (Supplementary Fig. 13b – f). Taken together, these results imply that *PIGP* is another crucial molecule for APC proliferation that is involved in DS pathology, together with *DYRK1A*.

Discussion

Emerging evidence suggests that astrocytes play crucial roles in various pathological mechanisms in the central nervous system (CNS)³³. We focused on the aberrant proliferation of DS APCs, which is a major cause of astrocyte overpopulation in the brains of DS individuals, and explored essential regulators.

Selecting a suitable model system is crucial for exploring human diseases. Although recent advances in human iPSC technologies have provided a new approach for functional disease studies, significant variations in phenotypes among different iPSC lines hinders the precise analysis of human diseases³⁴. Therefore, we developed an isogenic cell model that combines *XIST*-mediated chromosome silencing and genome-editing technology using DS-specific iPSCs, which enabled us to perform a detailed comparative analysis of genotype–phenotype correlations between dosage-sensitive genes on chromosome 21 and proliferative dynamics in DS APCs. Recent data have revealed that human iPSCs from different donors are more divergent in terms of their transcriptomes or cell phenotypes than those originating from different somatic cell types of the same donor, indicating that iPSC heterogeneity is mainly caused by genetic differences between individuals, rather than the epigenetic memory of the somatic tissue of origin^{35, 36}. These findings strongly suggest that the cell lines in our isogenic iPSC panel, all of which share a single genetic background, can provide an ideal cellular model for studying DS pathology.

Tet-regulated *XIST* was stably expressed after Dox treatment in both iPSCs and differentiated cells in previous studies^{19,20,21}. Nevertheless, *XIST* RNA was not detected in our differentiated *XIST*-Tri21 NPCs, likely due to silencing of the rtTA inserted in the *AAVS1* locus. Despite many descriptions of the *AAVS1* locus as a well-validated genomic safe harbour that enables stable expression of an inserted transgene³⁷, data from several studies have demonstrated that the *AAVS1* locus is not as a well-validated genomic 'safe' as suggested, and transgene expression varied in both iPSCs and differentiated cells, due to DNA methylation^{38,39}. Although the DNA-methylation status of the locus was not analysed in this study and other unknown mechanisms may exist, additional transduction of the rtTA transgene using the *piggyBac* transposon system in NPCs led to efficient *XIST* RNA expression, followed by chromosome silencing.

To identify critical regulators of DS APC overproliferation, we focused on the variability of genetic responses after the removal of *XIST*. In mouse embryonic stem (ES) cells, X chromosome inactivation (XCI) was reversible and depended on continued *Xist* expression, but XCI became irreversible and independent of *Xist* after differentiation²⁷. Chromosome inactivation can be introduced by ectopic expression of human *XIST* in somatic cells and maintained even after removal of *XIST*, albeit to a different extent^{19,40}. Our results were consistent with these findings in that the transcriptional levels of chromosome 21 were stably suppressed 3 weeks after Dox removal. Notably, a small subset of genes rapidly returned from silenced disomic levels to reactivated trisomic levels, which was accompanied by reoccurrence of the pathological phenotype in DS APCs. Such a multiplicity of gene reaction in XCI has been reported in several studies, indicating that 3% – 7% of mouse and 12% – 20% of human genes on the inactivated X chromosome escaped from XCI^{41,42}. Likewise, ectopically expressed *XIST* RNA on autosomes can induce silencing in *cis*, whereas 15% of genes consistently escape from XCI, and another 15% of genes vary in terms of whether they are subject to, or escape from, inactivation^{28,43}. Data from several bioinformatic studies have demonstrated that long-interspersed nuclear element repeats on the X chromosome or short-interspersed nuclear elements (such as *Alu* elements) are highly enriched around escape genes on autosomes, suggesting that genomic features contribute to the efficiency of *XIST*-mediated chromosome 21 inactivation^{43,44}.

Unexpectedly, our RNA-seq data showed a certain *XIST*-mediated reduction of gene-expression levels in several other chromosomes, especially in chromosome 18 (Supplementary Fig. 6). One possible explanation for this phenomenon is that trisomy of chromosome 21 might cause transcriptional alteration in a specific subset of genes on other chromosomes. It is known that the spatial organisation of chromosome territories in the cell nucleus is linked to genomic functions and regulation⁴⁵. Gene-rich chromosomes are located preferentially at the centre of the nucleus, whereas gene-poor chromosomes, such as chromosome 18, are located at its periphery⁴⁶. Cell-type-specific interaction patterns among chromosome territories are correlated with genome regulation at the global level, and the presence of a supernumerary chromosome 21 may perturb the physiological positioning of other chromosomes in the nucleus, leading to transcriptional dysregulation^{23,47}. *XIST*-mediated silencing of extra chromosome 21 reverted trisomy-induced transcriptional changes in other chromosomes, leading to downregulated expression levels, which was less noticeable but became evident by comparing the isogenic cell lines.

Another possible explanation of the dysregulated transcription relates to the higher expression levels of *XIST* observed in our study. We introduced an additional rtTA into NPCs using the PB transposon vector, which generally provides robust transgene integration. After introducing the *XIST* transgene into mouse ES cells, silencing of autosomal genes occurred only in cell lines with high-copy transgenes, suggesting that dose-dependent regulation by *XIST*⁴⁸ occurred. Data from transgenic experiments indicated that *XIST* expression was essential, but not sufficient, for *XIST* RNA spreading and localization^{49, 50}. Although *XIST* is a well-known *cis*-acting element, its *trans* effects on other chromosomes have not been reported. However, its robust expression, which was higher than observed physiologically, may explain the unexpected *trans* activity of *XIST*.

We identified two genes, *DYRK1A* and *PIGP*, as potent candidates responsible for the proliferative pathology of DS APCs. *DYRK1A* has been proposed to be closely involved in neural development, especially in fate specification and neuronal proliferation⁵¹. Studies conducted with human iPSCs and mouse models of DS exhibited impaired neural differentiation, which was improved by targeting *DYRK1A* pharmacologically or with short hairpin RNAs^{52, 53}. In addition, *Dyrk1a* overexpression promoted astrogliogenesis in mouse cortical progenitor cells by activating the STAT-signalling pathway¹³, suggesting that *DYRK1A* plays a key role in cell-fate switching. However, an increased *DYRK1A* gene dosage attenuated neuronal proliferation rates³⁰, while loss of *DYRK1A* function accelerated neural proliferation⁵¹. It is well established that *DYRK1A* increases the duration that cells spend in G1 phase in a dose-dependent manner by reducing cyclin D1 expression and increasing p27^{KIP1} (CDKN1B) expression^{31, 54}. Furthermore, *DYRK1A* can phosphorylate p53 and subsequently induce p53-target genes such as p21^{CIP1}, leading to impairment of the G1/G0–S-phase transition, resulting in attenuated proliferation³⁰. These antiproliferative activities of *DYRK1A* are supported by a decreased number of neurons observed in humans and mouse models of DS^{55, 56}. In contrast to its effects on neural precursors, however, we unexpectedly found a proliferation-promoting activity of *DYRK1A* on APCs, which was accompanied by an alteration in the level phosphorylated STAT3.

In the CNS, STAT3 is highly expressed in astrocytes and is activated in response to multiple pathological stimuli such as ischaemia, spinal cord injury, or neurodegenerative diseases^{57, 58}. It remains unclear whether activated JAK–STAT signalling can stimulate astrocyte proliferation, especially during the physiological-developmental stage. Nevertheless, some findings showed that the proliferation of reactive astrocytes in spinal cord injury was reduced by treatment with JAK inhibitors or conditional knockout of STAT3^{59, 60}. Although the detailed mechanism remains to be elucidated, these indirect data and our current results suggest that increased expression of *DYRK1A* can stimulate APC proliferation via the JAK–STAT pathway.

PIGP is a component of the glycosylphosphatidylinositol (GPI)–N-acetylglucosaminyltransferase (GPI–GnT) complex. More than 150 proteins have been identified as GPI-anchored proteins, which are expressed on the cell surface by being anchored to the plasma membrane⁶¹. Biosynthesis of mammalian GPIs is initiated by the transfer of N-acetylglucosamine (GlcNAc) to generate GlcNAc-PI through the

enzymatic activity of GPI–GnT. *PIGP* is one of seven subunits of GPI–GnT, and mutations in *PIGP* that lead to reduced cell-surface expression of GPI-anchored proteins have recently been linked to early infantile encephalopathy⁶². Although the specific function of *PIGP* is unclear, its overexpression has been reported to impair the membrane localisation of Wnt-signalling receptors during embryogenesis⁶³. Further studies are required to elucidate how *PIGP* accelerates APC proliferation in DS.

Astrocytes support neuronal homeostasis and regulate synaptic networks by promoting neuritogenesis and synaptogenesis⁶⁴. However, DS astrocytes exert a toxic effect on the formation and maturation of neural networks and neuron survival by reducing neuronal activity, inducing morphological alterations, and promoting neuronal apoptosis^{10, 18, 65}. In the DS brain, astrocytes may act as a primary effector in DS pathophysiology. Thus, identifying critical regulators for astrocyte overpopulation may be a critical first step for investigating disease mechanisms and developing new therapeutic strategies for DS. Our collection of isogenic iPSC lines will provide a useful resource for conducting detailed analyses of DS.

Methods

Human-iPSC generation and culture. Human iPSCs were generated and cultured as reported²². This study was approved by the Ethics Committee of Osaka University Graduate School of Medicine (approval number 13123-823). Informed consent was obtained from the patients' guardians in accordance with the Declaration of Helsinki. Briefly, iPSCs were induced from cord blood mononuclear cells of a male baby with DS using a Sendai virus (SeV) vector encoding *OCT4*, *SOX2*, *KLF4*, and *c-MYC*. iPSCs were maintained on mitomycin C (Merck)-inactivated mouse embryonic fibroblasts (MEFs) in human embryonic stem cell (hES) medium consisting of DMEM/F12 (Fujifilm Wako), KnockOut Serum Replacement (20%; Thermo Fisher Scientific), l-alanyl-l-glutamine (2 mM; Fujifilm Wako), MEM non-essential amino acid solution (1%; Fujifilm Wako), 2-mercaptoethanol (0.1 mM; Merck), and basic fibroblast growth factor (5 ng/mL; bFGF, Katayama Chemical), with or without 2 µg/mL Dox (Takara). To remove SeV, siRNA L527 (Gene Design) was mixed with Lipofectamine RNAiMAX (Thermo Fisher Scientific) and used to transfect iPSCs as described⁶⁶, until complete removal of the SeV genome was confirmed by PCR and immunostaining with an anti-SeV-NP antibody. The iPSC cultures were passaged every 6 to 9 days.

The iPSCs used in this study were karyotyped by Chromocenter, Inc. via G-band analysis or Q-band analysis. Potency assays were performed via immunocytochemistry. Briefly, iPSCs fixed in phosphate-buffer solution (PBS) containing 4% paraformaldehyde (Fujifilm Wako) were immunostained using primary antibodies against OCT4 (1:200; Santa Cruz Biotechnology) and SSEA4 (1:200; Merck). Secondary Alexa Fluor 488- or Alexa Fluor 594-conjugated antibodies were used (1:500; Thermo Fisher Scientific).

Insertion of a Dox - inducible XIST transgene. Full-length human *XIST* cDNA was kindly provided by Dr. Chikashi Obuse (Osaka University, Osaka, Japan). Introduction of a Dox-inducible *XIST* transgene was performed as described previously, with modifications¹⁹. A zinc finger nuclease (ZFN) against the *AAVS1*

locus on chromosome 19 was designed to enable insertion of the 3G rtTA transgene, as described⁶⁷. Insertion of a lox cassette into the *DYRK1A* locus was performed using the CRISPR–Cas9 system (Supplementary Table 4). Single-guide RNA (sgRNA) oligos for the CRISPR–Cas9 system were cloned into the BbsI sites of the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (Addgene, #42230). The pEF1α-3G rtTA-pA cassette was cloned from a pEF1α-Tet3G vector (Clontech, #631167). Full-length *XIST* cDNA with loxP and lox5171 sites was cloned into the pTRE3G vector (Clontech, #631168). On the day before transfection, iPSC colonies were dissociated into single cells using TrypLE Express (Thermo Fisher Scientific) in the presence of 10 μM ROCK inhibitor (Reagents Direct). Dissociated cells (1.0×10^6) were mixed with the donor vector (4 – 6 μg) and either a ZFN pair (left and right ZFNs, 0.5 μg each) or CRISPR–Cas9 (2 μg) and electroporated using the Neon Transfection System (setting: 1200 V, 20 ms, 2 pulses; Thermo Fisher Scientific). The electroporated cells were plated in 10-cm dishes with DR-4 IRR MEFs (Thermo Fisher Scientific). On day 4 post-electroporation, drug selection with G418 (150 μg/mL) or puromycin (0.5 μg/mL) was initiated according to the drug-resistance gene. The resulting colonies were selected on days 12–18. PCR-positive clones were further expanded. The lox cassette contained a positive/negative drug-selection marker (puroΔTK, encoding a fusion protein between the puromycin-resistance gene and a truncated version of herpes simplex virus type 1 thymidine kinase) between the loxP and lox5171 sites.

For Cre recombinase-mediated cassette exchange, 1.0×10^6 cells were electroporated with a Cre expression vector (4 μg) and a donor vector (8 μg), which contained *XIST* transgene with loxP and lox5171, as described above. The electroporated cells were plated, and on day 4 post-transfection, negative selection was initiated using 2 μM 1-[2-deoxy, 2-fluoro-8-d-arabinofuranosyl]-5-iodouracil. The resultant clones were analysed by PCR and positive clones were expanded.

Maintenance and differentiation of NPCs. NPC differentiation was performed according to a previously described protocol, with modifications¹⁴. Briefly, embryoid bodies (EBs) were cultured for 8 days in Costar six-well, ultra-low-attachment plates (Corning) in hES medium without bFGF, consisting of 2 μM dorsomorphin (Merck), 10 μM SB431542 (Tocris Bioscience), and 10 μM ROCK inhibitor. Next, the EBs were attached for 13 days to Matrigel (Corning)-coated dishes in neuronal medium (N2B27 medium) consisting of DMEM/F12, Neurobasal Medium (Thermo Fisher Scientific), N2 supplement (1·; Thermo Fisher Scientific), B27 without vitamin A (1·; Thermo Fisher Scientific), GlutaMAX (1·; Thermo Fisher Scientific), l-alanyl- l-glutamine (2 mM; Fujifilm Wako), MEM non-essential amino acids solution (1%; Fujifilm Wako), 2-mercaptoethanol (0.1 mM), ROCK inhibitor (10 μM), and bFGF (20 ng/mL). Neural rosettes appearing in the centres of the attached EB colonies were isolated with TrypLE Express and replated in Matrigel-coated dishes in N2B27 medium containing bFGF (20 ng/mL). The culture medium (with or without 2 μg/mL Dox) was changed every day and the cells were passaged every 3–5 days.

Transfection of the piggyBac vector into NPC. To enhance rtTA, *DSCR3*, or *PIGP* expression, NPCs were transfected with a *piggyBac* vector harbouring an additional gene (rtTA, *DSCR3*, or *PIGP*), which was generated from the PB-TA-ERN vector (Addgene, #80474). The resulting vector (2 μg) and a pCMV-hyPBBase vector (2 μg; a kind gift from the Sanger Institute) encoding transposase we co-transfected into

NPCs (4.0×10^6) using the Neon Transfection System (settings: 1200 V, 20 ms, 2 pulses). After clone selection with puromycin (0.5 $\mu\text{g/mL}$), NPCs with the additional gene were established.

Maintenance and differentiation of APCs. The protocol used for differentiating APCs from NPCs has been described in detail²⁵. NPCs were dissociated with TrypLE Express, and $2 \cdot 10^4$ cells/well were plated on Matrigel-coated 24-well plates with Astrocyte Medium (ScienCell) supplemented with 10 μM ROCK inhibitor. This medium was changed every 2 days, and the cells were passaged every 4–6 days, with dissociation using TrypLE Express. In this study, APCs were passaged 7–8 times before performing the analysis. When necessary, NPCs were differentiated into APCs via Dox administration for five days. The Dox-treated cell lines were administered Dox for approximately 6 weeks, and the D^{remov} cell lines were administered Dox for approximately 3 weeks, followed by growth in culture for 3 weeks without Dox. Two *XIST*-Tri 21 iPSC lines were generated from a male baby with trisomy 21. Both iPSC lines were differentiated into NPC lines. The NPCs were independently transfected using the *piggyBac* vector encoding an rtTA to generate three lines. The NPC-derived APCs were subjected to qRT-PCR analysis, cell-proliferation assays, RNA-seq analysis, and ChIP-seq analysis. Three cDi21 lines, which were generated using a chromosome-elimination technique, were used as controls in this study.

Genome editing of the DYRK1A gene using the CRISPR–Cas9 system. *DYRK1A* targeting was performed using the CRISPR–Cas9 system. The sgRNA sequence was designed using CRISPR Direct (<http://crispr.dbcls.jp/>; Supplementary Table 4). The sgRNA oligos were cloned into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (Addgene, #42230). On the day before transfection, iPSC colonies were dissociated into single cells using TrypLE Express with 10 μM ROCK inhibitor. Cells were dissociated with TrypLE Express, after which 1.0×10^6 cells were mixed with CRISPR–Cas9 (2 μg) and the donor vector (6 μg), and electroporated using the Neon Transfection System (settings: 1200 V, 20 ms, 2 pulses). The electroporated cells were plated in 10-cm dishes with DR-4 IRR MEFs (Thermo Fisher Scientific). On day 4, drug selection with hygromycin (75 $\mu\text{g/mL}$) was initiated. The resulting colonies were selected on days 12–18. PCR-positive clones were further expanded. The sequences of the primers used for the genome-editing experiments are listed in Supplementary Table 5.

Transfecting siRNAs into APCs. Cultured APCs were transfected with siRNAs using Lipofectamine RNAiMAX for 1 day. The final lipofectamine RNAiMAX and siRNA concentrations were 3 $\mu\text{l/mL}$ and 10 nM, respectively. siRNAs against *DSCR3* (Thermo Fisher Scientific, #s20157) and *PIGP* (Thermo Fisher Scientific, #s27717) were used. Silencer Select Negative Control siRNA #1 (Thermo Fisher Scientific, #4390843) was used as a control siRNA.

Immunocytochemistry. Immunocytochemistry was performed as described previously, with some modifications⁶⁸. Cells were fixed with PBS containing paraformaldehyde (4%) and permeabilised with PBS containing Triton X-100 (0.5%) for 15 min. After blocking with PBS containing foetal bovine serum (5%) for 30 min, the cells were incubated overnight at 4 °C with primary antibodies against H3K27me3 (1:200; Merck), PAX6 (1:100; Stemgent), SOX1 (1:100; R&D Systems), GFAP (1:1000; DakoCytomation), S100 β (1:1000; Merck), CD44 (1:200; Merck), or vimentin (1:500; Merck). The cells were washed with PBS

and incubated for 120 min with appropriate Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Thermo Fisher Scientific). The nuclei were counterstained with Hoechst dye (1:1000).

RNA fluorescence in situ hybridisation (FISH). RNA FISH was performed as previously described, with some modifications²³. *XIST*-Tri 21 iPSC colonies were dissociated into single cells and cultured on coverslips for 2 days. The cells were fixed with PBS containing 4% paraformaldehyde and incubated with ice-cold CSK buffer (100 mM NaCl, 300 mM sucrose, and 10 mM PIPES, pH 6.8) containing Triton X-100 (0.5%). After rinsing, the cells were dehydrated in ice-cold ethanol (100%) and then air-dried. The *XIST* probe was labelled using a DIG-Nick translation Mix (Merck) according to the manufacturer's protocol. Hybridisation reactions consisting of labelled products (0.1 µg), herring sperm DNA (5 µg; Fujifilm Wako), baker's yeast transfer RNA (5 µg; Thermo Fisher Scientific), human Cot-1 DNA (3 µg; Thermo Fisher Scientific), and RNase Out (4 U/µL; Thermo Fisher Scientific) in hybridisation buffer (4 × saline-sodium citrate [SSC] buffer, dextran sulphate (20%, w/v), bovine serum albumin [BSA]; 4 mg/mL) were carried out overnight in humidified incubator at 37 °C. The cells were then subjected to stringent washes at 42 °C (three times in 2 × SSC/50% formamide and three times in 2 × SSC). The cells were blocked in 4 × SSC containing 4 mg/mL BSA and 0.1% Tween-20 at 37 °C prior to detection. Detection was performed in detection buffer (4 × SSC containing 5% BSA and 0.2% Tween-20) with a mouse anti-digoxigenin antibody (Thermo Fisher Scientific) for 50 min, followed by amplification with Cy3-conjugated antibody (Jackson ImmunoResearch Labs) for 50 min. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and the slides were mounted in VECTASHIELD Antifade Mounting Medium (Vector Laboratories). Images were captured using an LSM 710 confocal scanning laser microscope (Carl Zeiss) and processed using Volocity software (PerkinElmer).

qRT-PCR analysis. Total RNA was isolated from iPSCs, NPCs, and APCs with the NucleoSpin RNA II Kit (Macherey-Nagel). Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix (Toyobo). qRT-PCR analysis was performed using Thunderbird SYBR qPCR Mix (Toyobo). Gene expression levels were normalised to the expression of β-actin (ACTB). The sequences of all primers used for qRT-PCR analysis are shown in Supplementary Table 6.

Single-cell cloning. *XIST*-Tri 21 iPSC colonies were dissociated into single cells using TrypLE Express, and then 1.0×10^3 cells were plated in a 10-cm dish with DR-4 IRR MEFs using iPSC culture medium containing 10 µM ROCK inhibitor, G418 (150 µg/ml) and Dox (2 µg/ml). On days 12–18, the resulting colonies were passaged to individual wells. These clones were expanded further, and on day 21 after Dox addition, they were fixed and assessed using an immunocytochemistry method.

Cell-proliferation assay. A Click-it EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific) was used to measure cell proliferation. At 1 or 2 days before adding the thymidine analogue EdU, APCs were dissociated with TrypLE Express, and $5 \cdot 10^3$ cells/well were plated into a Matrigel-coated 96-well plate (Greiner Bio-one). On the day of EdU treatment, the cells were cultured with EdU (10 µM) for 8 h. The cultured cells were fixed with PBS containing paraformaldehyde (4%) and then permeabilised with PBS containing TritonX-100 (0.5%). The cells were stained as instructed in the manufacturer's protocols.

Images were taken with an In Cell Analyzer 6000 (GE Healthcare), and EdU-positive cells were detected using the In Cell Developer Toolbox 1.9 (GE Healthcare). Cell-counting assays were performed according to a method similar to the protocol described above. At 1 or 2 days after plating the cells (5×10^3 /well) in a Matrigel-coated 96-well plate, they were fixed. The cell nuclei were counterstained with Hoechst 33342 dye (1:1000). Images were taken with an IN Cell Analyzer 6000, and the stained nuclei were counted using the In Cell Developer Toolbox 1.9. To study the effect of DYRK1A inhibition on cell proliferation, cells (5×10^3) were seeded into culture plates and FINDY (Merck) was added. Two days after FINDY addition, proliferation assays were performed.

RNA-seq analysis. APCs were harvested at passage 8 for RNA-seq analysis, and total RNA was isolated from each sample using the NucleoSpin RNA II Kit. RNA-seq analysis was performed by DNA Chip Research, Inc. The integrity and quantity of the total RNA were measured with an Agilent 2100 Bioanalyzer RNA 6000 Nano Kit (Agilent Technologies). Total RNA obtained from each sample was subjected to sequencing library construction using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs) with the NEBNext Poly(A) mRNA Magnetic Isolation Module, according to the manufacturer's protocols. The quality of the libraries was assessed with the Agilent 2200 TapeStation High Sensitivity D1000 ScreenTape System (Agilent Technologies). The pooled library samples were sequenced using a NextSeq 500 instrument (Illumina), with 76-base-pair (bp) single-end reads. Sequencing adaptors, low-quality reads, and bases were trimmed with the Trimmomatic-0.32 tool. The sequence reads were aligned to the human reference genome (hg19) using TopHat 2.1.1 (bowtie2-3.2.0)⁶⁹, which can adequately align reads (including splice sites) with the genome sequence. Files of the gene-model annotations and known transcripts were downloaded from the Illumina iGenomes website (http://support.illumina.com/sequencing/sequencing_software/igenome.html). These files were necessary for performing whole-transcriptome alignments with TopHat. The aligned reads were subjected to downstream analyses using StrandNGS 3.2 software (Agilent Technologies). The read counts for each gene and transcript (RefSeq Genes 2015.10.05) were quantified using the trimmed mean of M-value (TMM) method⁷⁰. Plots were created using the ggplot2 (version 3.3.2) package in R 3.6 software.

ChIP-seq analysis. For ChIP-seq analysis, APCs were harvested at passage 8. Chromatin was prepared from the cells using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer's protocols. Approximately 8×10^6 cells were used for each immunoprecipitation experiment. ChIP was performed using an antibody against H3K27me3 (Cell Signaling Technology). ChIP-seq analysis was performed by DNA Chip Research, Inc. The quality and quantity of the ChIP DNA and input DNA were measured with an Agilent 2100 Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies). For each sample, 10 ng of DNA was subjected to sequencing library construction using the NEBNext Ultra II DNA Library Prep Kit for Illumina, according to the manufacturer's protocols. The quality of each library was assessed with an Agilent 2100 Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies). Pooled sample libraries were sequenced using NextSeq 500 in 76-bp single-end reads. Sequencing adaptors, low-quality reads, and bases were trimmed with the Trimmomatic 0.32 tool. The sequence reads were aligned to the human reference genome (hg19) using

bowtie2-3.2 software. PCR duplicates were removed using Picard ver. 1.119 (<http://picard.sourceforge.net>). The aligned reads were subjected to downstream analysis using the MEDIPS (version 1.30) package in R 3.4 software. We calculated the short read coverage (extend value = 300) with genome-wide 100-bp bins using MEDIPS software. Differential coverage of the ChIP-seq data between groups of samples was detected based on the TMM method for genome-wide 100-bp sliding windows.

Short-tandem repeat (STR) analysis. To perform STR genotyping, DNA was extracted from iPSCs using a DNeasy Blood & Tissue Kit (Qiagen). To assess *DYRK1A*-targeted alleles, junctional PCR (homologous recombination+) and outside PCR (homologous recombination-) were performed using KOD FX Neo enzyme solution (Toyobo). PCR products or genomic DNA were subjected to PCR using PrimeSTAR MAX (Takara), a fluorescently labelled forward primer, and a reverse primer. The final PCR products were mixed with an internal lane standard 600 (Promega) and HiDi formamide (Thermo Fisher Scientific) and separated by capillary electrophoresis on an ABI 310 Genetic Analyzer (Thermo Fisher Scientific), per the manufacturer's instructions. The primer sequences are shown in Supplementary Table 5.

Allele-specific SNP-silencing analysis. Total RNA was isolated from *XIST*-Tri 21 APCs (Dox-untreated cell lines and Dox-treated cell lines) using the NucleoSpin RNA II Kit. Reverse transcription was performed using ReverTra Ace qPCR RT Mix. With the resulting total cDNA, PCR was performed using primers that amplified a region containing an SNP (rs457705) on exon 8 of *ETS2* on chromosome 21. The sequences of the primers used for *ETS2* are provided in Supplementary Table 7.

Western blotting. Cells were lysed with RIPA Buffer (Fujifilm Wako) containing a mixture of protease inhibitors (Merck) and phosphatase inhibitors (Nacalai Tesque). Lysates containing equal amounts of protein were mixed with an appropriate amount of Laemmli sample buffer (2×; Bio-Rad Laboratories) and 2-mercaptoethanol and denatured at 95 °C for 5 min. The samples were separated by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories). The membranes were washed with Tris-buffered saline containing Triton X-100 (0.05%) and incubated with Blocking One or Blocking One-P (Nacalai Tesque) buffer for 60 min. Mouse anti-STAT3 (1:1000; Cell Signaling Technology) and rabbit anti-Phospho-STAT3 (Ser727) (1:500; Cell Signaling Technology) were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:2500; Promega) were used as secondary antibodies. As a control, β -actin was detected with anti- β -actin pAb-HRP-Direct (1:2000; MBL). Blots were visualised using Clarity Western ECL Substrate (Bio-Rad Laboratories). The stained membranes were scanned with ImageQuant LAS 4000 Biomolecular Fluorescence Image Analyzer (GE Healthcare). When necessary, the antibody was stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific). Quantification was performed using ImageJ software (<http://imagej.nih.gov/ij/>).

Statistical analysis. All statistical analyses were performed using the EZR software. Comparisons of two groups were made using Student's t-test or Welch's two-sample t-test. We evaluated multiple comparisons using one-way analysis of variance (ANOVA) or the Kruskal–Wallis test with Bonferroni's correction. A P

value of less than 0.05 was considered to reflect a statistically significant difference. The data presented are expressed as the mean $\pm$ standard error of the mean (SEM) or standard deviation (SD).

Data availability. The RNA-seq data and ChIP-seq data reported here are available in the DDBJ Sequenced Archive under accession numbers DRA010528 and DRA010529, respectively.

Declarations

Acknowledgments

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Author contribution statement

KK and YK conceptualised and designed the experiments and wrote the manuscript. KK conducted most of the experiments and contributed to the data analysis. TN, NN, HK, HY, and KH conducted differentiation experiments. AT and KS assisted in the RNA-seq and ChIP-seq analysis. HT, HA, and YK collected clinical data to characterise patients for iPSC establishment. KO and YK supervised the entire project. All authors reviewed and commented on the manuscript.

Competing interests

The authors have read the journal's policy and have no competing interests.

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Figures

Figure 1

Generation of an isogenic iPSC panel for disease modelling of DS. Schematic overview depicting the strategy used to generate isogenic iPSC lines by combining XIST-induced chromosome silencing and genome/chromosome-editing technologies. (First row, left and middle panel) Cord blood mononuclear cells were derived from a male patient with DS. Then, a DS-specific iPSC line (Tri21) was directly established using Sendai virus. (First row, right panel) A corrected-disomy cell line (cDi21) was generated

by eliminating a single copy of chromosome 21 from a Tri21 iPSC line. (Second row) A 4-Mb region corresponding to a 'Down syndrome critical region' was selectively deleted only from the paternal chromosome 21 in Tri21 iPSCs to generate Partial-Tri21 iPSCs. NPCs differentiated from Partial-Tri21 iPSCs were transfected with piggyBac (PB) transposon vectors containing human PIGP or DSCR cDNA under the regulation of the Tet-inducible system. NPCs were differentiated into APCs for further analysis. (Third row, left panel) Dox-inducible human XIST cDNA was inserted into one copy of chromosome 21 in Tri21 iPSCs (XIST-Tri21 iPSC) for chromosome silencing. (Third row, right panel) rtTA was additionally introduced into NPCs differentiated from XIST-Tri21 iPSCs, using a PB vector. Transcriptional and phenotypic analysis was conducted in Dox-untreated (D-) and Dox-treated (D+) cells, and in NPCs or APCs at 3 weeks after Dox removal (Dremov). The location of the XIST transgene is indicated by the red rectangle. The black and striped chromosomes indicate a silenced and a reverted chromosome 21, respectively. (Fourth row) The DYRK1A gene was targeted in one (left panel) or two (right panel) chromosomes in the XIST-Tri21 iPSC line to generate DY^{+/+/m-} and DY^{+/m/m}-XIST-Tri21 iPSCs, respectively. Three types of genome-edited cell lines were generated in each single- or double-targeted clone, based on the parental origin of the three chromosomes. Black stars indicate the DYRK1A target site. Two maternal copies and one paternal copy of chromosome 21 are indicated as M1/M2, and P, respectively. (Fifth row) DYRK1A-targeted iPSC lines were differentiated to APCs and subjected to phenotypic analysis. Light yellow panels and light green panels indicate iPSC lines and NPC/APC lines, respectively.

Figure 2

XIST-mediated chromosome silencing affected the overproliferative phenotype of DS APCs. **a** Schematic depiction of the differentiation protocol for NPCs and APCs. EBs: embryoid bodies. **b** Immunocytochemistry of XIST-Tri21 APCs using GFAP and S100 β (astrocyte markers), CD44 and vimentin (APC markers), and SOX1 (NPC marker). Nuclei were stained with Hoechst 33342. Scale bars: 100 μ m. **c** Relative expression levels of XIST RNA in XIST-Tri21 APCs. Expression was normalised to that

of D- APCs (n = 3 experiments per cell line). d Immunocytochemistry of XIST-Tri21 NPCs using an H3K27me3-specific antibody. Nuclei were stained with Hoechst 33342. Scale bars: 50 μ m. e Percentage of H3K27me3-positive cells in XIST-Tri21 APCs (n = 4 differentiation experiment/cell line). f Relative expression levels of genes on chromosome 21 in XIST-Tri21 APCs. Expression levels were normalised to those of cDi21 cells (n = 5 experiments per cell line) g EdU assay in XIST-Tri21 APCs. EdU-positive cells were stained with an Alexa Fluor 488-conjugated antibody (light green). Nuclei were stained with Hoechst 33342. Scale bars: 100 μ m. h Percentage of EdU-positive APCs (n = 3 independent differentiation experiments per cell line, performed in triplicate). i Relative numbers of APCs 1 day after seeding. Cell numbers were normalized to those of cDi21 lines (n = 3 independent differentiation experiments per cell line, performed in triplicate). Error bars represent the SEM. Data were analysed by Student's t-test, Welch's two-sample t-test, or one-way ANOVA with Bonferroni's correction. *P < 0.05, **P < 0.01. EdU: 5-ethynyl-2'-deoxyuridine

Figure 3

Forced XIST expression caused whole-chromosome inactivation, which was maintained overall after Dox removal. a Violin plots showing relative log-transformed expression ratios of genes on each chromosome in XIST-Tri21 APC lines. Gene-expression levels (determined by RNA-seq) were normalised to those of cDi21 lines. The upper orange dashed lines indicate a ratio of 1.5, whereas the lower black dashed lines indicate a ratio of 1.0. Transcription from chromosome 21 was effectively suppressed in D+ cells, and inactivation was retained overall in Dremov cells. A small hump was observed in chromosome 21 of a Dremov cell line (black arrow). The plots show mean expression levels, with error bars indicating the SD. The data shown were analysed by the Kruskal–Wallis test with Bonferroni's correction. **P < 0.01. b Heatmap with a hierarchical-clustering diagram depicting the transcriptional dynamics of chromosome 21. We analysed 178 genes with positive read counts on chromosome 21. Expression levels were

normalised to those of cDi21 cell lines (Supplementary Table 1). Genes are ordered according to chromosomal position. c Overview of the RNA-seq and ChIP-seq data for chromosome 21. (Upper) The distribution of 178 genes with positive read counts (red plots), 56 genes with an D-/cDi21-expression ratio ≥ 1.5 (light green), 50 genes in which Dox-treatment decreased expression (blue), 21 genes in which Dox-treatment significantly decreased expression ($p < 0.05$, yellow), and 18 genes in which Dox removal reverted the expression levels (purple). (Lower, red bars) The distributions of regions with significantly higher accumulation of H3K27me3 are shown. False-discovery rates (FDRs) were calculated using the Benjamini–Hochberg method. P and $FDR < 0.05$ were considered to reflect significant differences. d Integrated analysis of the ChIP-seq and RNA-seq data. H3K27me3 accumulation within each gene body and 2 kb upstream of the transcription start site were analysed. The density of all paired points is shown with a blue contour line. Genes with a significant difference in H3K27me3 accumulation between two lines are indicated (red points). The green dashed ellipse (upper-middle column) emphasises the distribution of inactivated genes with increased H3K27me3 accumulation after Dox treatment. The orange dashed line indicates a two-thirds decrease in gene expression.

Figure 4

Identification of candidate marker genes for the aberrant proliferation of DS APCs using PCA and a partial-Tri21 cell line. **a** Two-dimensional PCA of the expression profiles of 178 genes with positive read counts on chromosome 21 ($n = 3$ per cell line). Note that the values of the Dremov cell lines in component 1 were relatively close to those of the D- cell lines. **b** Scheme for identifying genes responsible for APC overproliferation using RNA-seq data and PCA. **c** Schematic illustration of chromosome 21 in the Partial-

Tri21 cell line. The 4-Mb region between RUNX1 and ETS2 was deleted from a single copy of chromosome 21. d The percentage of EdU-positive APCs (n = 3 independent differentiation experiments per cell line, each performed in triplicate). e Relative numbers of APCs 1 day after seeding. The cell numbers were normalised to that of the cDi21 cell lines (n = 3 independent differentiation experiments per cell line, each performed in triplicate). f Relative expression levels of candidate genes involved in the overproliferation of DS APCs (n = 3 experiments per cell line), normalised to that of cDi21 cell lines. g Map showing the distribution of H3K27me3 modifications in DYRK1A in XIST-Tri21 APCs. (Upper) Integrative Genomics Viewer screenshot (<http://software.broadinstitute.org/software/igv/>) of H3K27me3 ChIP-seq track peaks on DYRK1A (hg19) in D-, D+, and Dremov lines. The Y-axis shows the number of fragments per base pair per million reads. (Lower, red bars) The distributions of regions with significantly higher H3K27me3 accumulation in the indicated cell lines. The error bars represent the SEM. The data shown were analysed using Student's t-test or Welch's two-sample t-test. The FDR was calculated using the Benjamini–Hochberg method. *P < 0.05, **P < 0.01

Figure 5

Targeted deletion of *DYRK1A* promoted APC proliferation in a gene dosage-dependent manner. **a** Schematic overview of the strategy used to target *DYRK1A*. **b** Comparative STR analysis of the *DYRK1A* locus was performed between a patient with DS and his parents' genomes. **c** STR analysis of *DYRK1A*-targeted iPSC lines to identify targeted alleles (F2/R3 primers) and non-targeted alleles (F2/R2 primers). **d** Relative expression levels of *DYRK1A* in DY+/+/m- and DY+/m/m- XIST-Tri21 APCs. The expression levels

were normalised to that of cDi21 cell lines (n = 3 experiments per cell line). e Immunoblot analysis of p-STAT3 (Ser 727) and STAT3 in DYRK1A-targeted XIST-Tri21 APCs. Expression levels were normalised to that of the XIST-Tri21 line (n = 3 – 7 experiments per cell line). f, g Percentage of EdU-positive cells and the relative numbers of targeted APCs. Cell numbers were normalised to those of the cDi21 lines (n = 3 experiments for the cDi21 cell lines and the other cell lines, performed in triplicate.). h Schematic illustration depicting the expected changes resulting from knocking out different DYRK1A alleles and chromosome silencing. red rectangle, XIST transgene; black stars, DYRK1A target sites; black chromosomes, inactivated chromosome 21. The expected copy numbers of active DYRK1A genes in each APC line are shown. i, j Percentage of EdU-positive, DYRK1A-targeted cell lines, with or without Dox treatment. The percentage of EdU-positive cells was normalised to that of each Dox-untreated cell line (n = 3 experiments per cell line). k, l Relative cell numbers of the DYRK1A-targeted lines, with or without Dox treatment. The cell numbers were normalised to that of each Dox-untreated line (n = 3 experiments per cell line). m – p Percentage of EdU-positive cells and the relative numbers of Tri21 APCs (m, n) or Tri21 NPCs (o, p) after a 2-day treatment with FINDY (n = 3 experiments per cell line). The cell numbers were normalised to those of Tri21 APCs or NPCs without FINDY treatment (n = 3 experiments per cell line). The error bars represent the SEM. The data shown were analysed by Student's t-test, Welch's two-sample t-test, or one-way ANOVA with Bonferroni's correction. *P < 0.05, **P < 0.01

Figure 6

PIGP promoted DS APC proliferation in an expression level-dependent manner. **a** Schematic depicting the transfection of a Dox-inducible PIGP or DSCR3 transgene using a PB transposon vector and a hyperactive PB transposase into Partial-Tri21 NPCs. Note that chromosome 21 was trisomic except for the 4-Mb segment, which corresponds to the Down syndrome critical region. Transduced NPCs were differentiated to APCs. **b** Partial-Tri21 APCs stably expressing the PIGP-overexpression vector. PIGP overexpression was induced by a 6-week treatment with Dox. Expression levels were normalised to that of untreated APCs ($n = 3$ experiments per cell line). **c, d** Percentage of EdU-positive cells (**c**) and relative cell numbers 1 day after seeding (**d**) in PIGP-overexpressing Partial-Tri21 APCs ($n = 3$ experiments per cell line). Cell numbers were normalised to that of untreated APCs. **e** Relative PIGP expression in siRNA-treated Tri21 APCs. Expression levels were normalised to those in siCtrl-treated Tri21 APCs ($n = 3$ experiments per cell line). **f, g** Percentage of EdU-positive cells (**f**) and relative cell numbers 1 day after seeding (**g**) in siRNA-treated Tri21 APCs ($n = 3$ experiments per cell line). The percentage of EdU-positive cells and cell numbers were

normalised to those of the untreated Tri21 line. siPIGP, PIGP siRNA. The error bars represent the SEM. The data shown were analysed by Student's t-test or Welch's two-sample t-test. *P < 0.05, **P < 0.01

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