ARTICLE

Translating dosage compensation to trisomy 21

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Down's syndrome is a common disorder with enormous medical and social costs, caused by trisomy for chromosome 21. We tested the concept that gene imbalance across an extra chromosome can be *de facto* corrected by manipulating a single gene, *XIST* (the X-inactivation gene). Using genome editing with zinc finger nucleases, we inserted a large, inducible *XIST* transgene into the *DYRK1A* locus on chromosome 21, in Down's syndrome pluripotent stem cells. The *XIST* non-coding RNA coats chromosome 21 and triggers stable heterochromatin modifications, chromosome-wide transcriptional silencing and DNA methylation to form a 'chromosome 21 Barr body'. This provides a model to study human chromosome inactivation and creates a system to investigate genomic expression changes and cellular pathologies of trisomy 21, free from genetic and epigenetic noise. Notably, deficits in proliferation and neural rosette formation are rapidly reversed upon silencing one chromosome 21. Successful trisomy silencing *in vitro* also surmounts the major first step towards potential development of 'chromosome therapy'.

In the United States, about 1 in 300 live births carry a trisomy, half of which are for chromosome 21, which causes Down's syndrome. Down's syndrome is the leading genetic cause of intellectual disabilities and the millions of Down's syndrome patients across the world also face multiple other health issues, including congenital heart defects, haematopoietic disorders and early-onset Alzheimer's disease[1,2]. Down's syndrome researchers have sought to define the genes on chromosome 21 most closely associated with Down's syndrome, but this has proven difficult due to high genetic complexity and phenotypic variability of Down's syndrome, confounded by normal variation between individuals[1–3]. Despite progress with mouse models for Down's syndrome[4,5], there remains a need for better ways to understand the underlying cell and developmental pathology of human Down's syndrome, key to therapeutic design of any kind[2].

The last decade has seen great advances in strategies to correct single-gene defects of rare monogenic disorders, beginning with cells *in vitro* and in several cases advancing to *in vivo* and clinical trials[6]. In contrast, genetic correction of the over-dose of genes across a whole extra chromosome in trisomic cells has remained outside the realm of possibility. Our effort was motivated by the idea that functional correction of living trisomic cells may be feasible by inserting a single gene that can epigenetically silence a whole chromosome. An inducible system for such 'trisomy silencing' would have immediate translational relevance as a resource to investigate the cellular pathology and gene pathways affected in Down's syndrome, in a setting free from pervasive genetic or epigenetic variation that exists between individuals, sub-clones, or even isogenic cell isolates[3,7,8].

There is a natural mechanism to compensate the difference in dosage of X-linked gene copies between mammalian females (XX) and males (XY). This is driven by a large (~17 kilobases (kb) in human), non-coding RNA, *XIST*, which is produced exclusively from the inactive X chromosome[9], and 'paints' (accumulates across) the interphase chromosome structure[10]. During early development, the *XIST* RNA induces numerous heterochromatin modifications and architectural changes which transcriptionally silence the inactive X chromosome and manifest cytologically

as a condensed Barr body (reviewed in refs 11, 12). There is evidence for some DNA sequence specificity to *XIST* function, as certain human genes escape X-inactivation[13]; however, autosomal chromatin has substantial capacity to be silenced[14–16]. Understanding the full potential of an autosome to be silenced, however, requires examination under conditions that avoid creating a deleterious functional monosomy. The strategy pursued here meets that requirement and creates a tractable model to study the distinct biology of human chromosome inactivation.

As outlined in Fig. 1a, we set out to determine whether the human X-inactivation gene, *XIST*, could be inserted into one copy of chromosome 21, and enact a chromosome-wide change in its epigenetic state. We pursued zinc finger nuclease (ZFN)-driven targeted addition[17] of an inducible *XIST* transgene to the gene-rich core of chromosome 21 in induced pluripotent stem (iPS) cells derived from a Down's syndrome patient. If accomplished, this milestone would provide a system to study Down's syndrome cell pathology and the first step towards a potential genetic/epigenetic approach to 'chromosome therapy'.

Insertion of *XIST* into a trisomic chromosome 21

Given its large size, neither the *XIST* gene nor its cDNA has previously been integrated in a targeted fashion. Thus, our first goal was to demonstrate that ZFNs could accurately insert the largest transgene to date, substantially larger than sequences commonly used for genome editing[18]. We first attempted this with a ~16-kb *XIST* transgene in a transformed cell line (HT1080), using established ZFNs to the *AAVS1* locus on chromosome 19 (ref. 19). This proved highly successful (our unpublished data). To extend this to chromosome 21, we engineered ZFNs to a 36-base pair (bp) sequence in intron 1 of the *DYRK1A* locus at chromosome 21q22 (as in Fig. 1b), and validated their robust activity (Supplementary Fig. 1a, b). We tested an even larger (~21 kb) construct containing near full-length *XIST* cDNA in HT1080 cells and demonstrated efficient, accurate addition to this gene-rich region (Supplementary Fig. 2a, b).

We next determined whether this was achievable in technically challenging iPS cells, which have unique therapeutic and developmental

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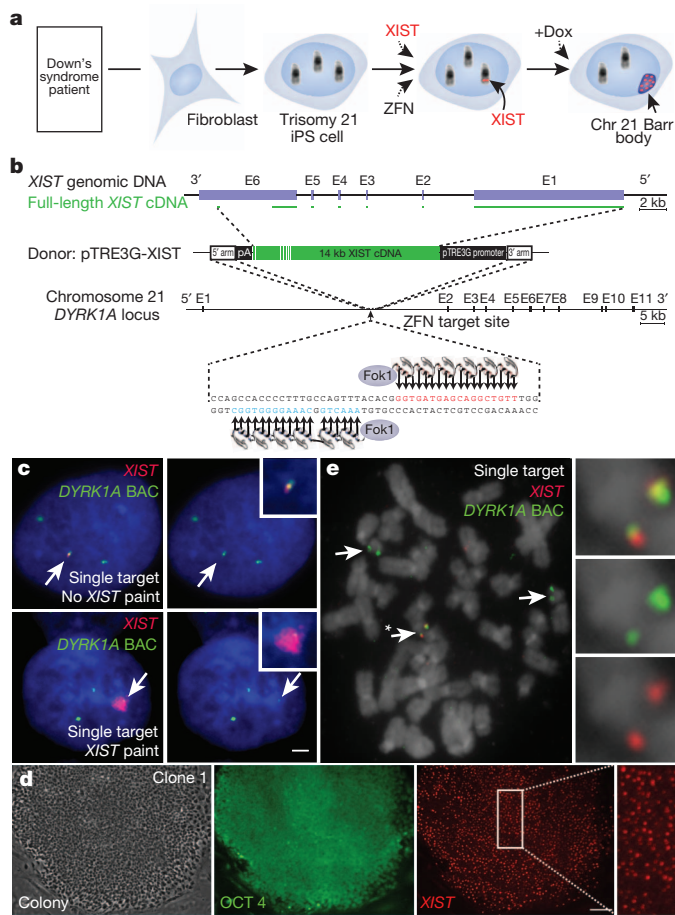
Figure 1 | Genome editing integrates *XIST* into chromosome 21 in trisomic iPS cells. a, Concept for translating dosage compensation to trisomy 21. **b**, *XIST* construct (19 kb): two homologous arms and 14-kb *XIST* cDNA with inducible pTRE3G promoter. **c**, DNA/RNA FISH in interphase Down's syndrome iPS cells shows that *XIST* overlaps one of three *DYRK1A* genes (left panels and insets) in a non-expressing cell (top, arrows), and a cell induced to express a large *XIST* RNA territory over the *DYRK1A* locus after 3 days in doxycycline (bottom, arrows). Right panels show green channel (*DYRK1A*) alone. Nuclear DNA is stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 2 μm. **d**, OCT4 immunostaining and *XIST* RNA FISH in a transgenic colony: highly consistent *XIST* expression throughout the colony. Scale bar, 100 μm. **e**, Metaphase DNA FISH shows one targeted chromosome 21. *XIST* gene (asterisk and close-up) overlaps one of three *DYRK1A* genes (arrows).

potential to form various cell types, and thus would be important for any future *ex vivo* cellular therapy efforts. We used a male Down's syndrome iPS cell line[20] which we confirmed maintains pluripotency markers and trisomy 21. Although a constitutively transcribed transgene could be used, we engineered an inducible system to maximize utility for investigating Down's syndrome biology. In one step, we integrated both the doxycycline-controlled *XIST* transgene into chromosome 21 (Fig. 1b and Supplementary Fig. 3a) and a transgene carrying the doxycycline control component (rtTA) into the *AAVS1* chromosome 19 safe harbour, disruption of which creates no known adverse effects[19] (Supplementary Fig. 3b).

We analysed 245 colonies from the pooled transformants by interphase RNA/DNA fluorescence *in situ* hybridization (FISH) (Fig. 1c) to determine whether *XIST* was present and overlapped one of three *DYRK1A* alleles. Notably, 98.5% of *XIST* RNA-positive colonies carried *XIST* at this location on chromosome 21, and also contained the rtTA/selection transgene (Supplementary Table 1). Efficiency was sufficiently high that, through modifications to editing conditions, we obtained a few sub-clones with *XIST* integrated into two or even

all three alleles of *DYRK1A* (see Methods and Supplementary Fig. 3c and Supplementary Table 1). Six independent sub-clones were chosen for further study based on: an *XIST* transgene on one of three chromosome 21 copies, pluripotent colony morphology, OCT4 (also called POU5F1) staining (Fig. 1d and Supplementary Fig. 4a), and formation of embryoid bodies. FISH to metaphase chromosomes (Fig. 1e and Supplementary Fig. 3d) and Southern blotting (Supplementary Fig. 1c–e) confirmed the gene addition accuracy, with 47 chromosomes, for all six clones. High-resolution cytogenetic banding and/or array comparative genomic hybridization (CGH) on selected clones showed no significant abnormalities other than full chromosome 21 trisomy (Supplementary Fig. 4c–e).

XIST RNA induces a chromosome 21 Barr body

In the panel of six independent genome-edited clones, we induced transgene expression and detected *XIST* RNA by FISH 3 days later. A localized *XIST* RNA 'territory' over one chromosome 21 (Fig. 1c) was seen in over 85% of cells in all six clones (Fig. 1d and Supplementary Fig. 4a, b). This mirrored the unique behaviour of endogenous *XIST* RNA which 'paints' the inactive X chromosome nuclear territory[10].

The natural inactivated X chromosome forms a condensed Barr body which carries repressive histone marks[11]. Similarly, 5 days after *XIST* induction, the edited chromosome 21 became markedly enriched in all heterochromatin marks examined, including H3K27me3, UbH2A and H4K20me in 90–100% of cells and, later, with macroH2A (Fig. 2a, b and Supplementary Fig. 5a). Supplementary Fig. 5b illustrates that H3K27me spreads across the whole metaphase chromosome 21. Moreover, chromosome 21 DNA in many nuclei became notably condensed, further evidence that we successfully generated a heterochromatic chromosome 21 Barr body (Fig. 2c).

Allele-specific silencing across chromosome 21

To measure overall transcription across the *XIST*-targeted chromosome 21, we used an approach that we developed to broadly assay heterogeneous nuclear RNA (hnRNA) expression and to distinguish inactive from active X chromosome[16], on the basis of *in situ* hybridization to CoT-1 repeat RNA. This showed that the chromosome 21 *XIST* RNA territory was depleted for hnRNA detected by CoT-1 (Supplementary Fig. 5c), similar to the inactive X chromosome[16].

We next used multi-colour RNA FISH to determine the presence of transcription foci at each allele for six specific chromosome 21 genes, an established approach that we earlier showed[10,15] discriminates active versus silenced genes on inactive X chromosome. Without *XIST* expression, there are three bright transcription foci from each *DYRK1A* allele (Fig. 1c, top), but after *XIST* expression, the targeted allele becomes
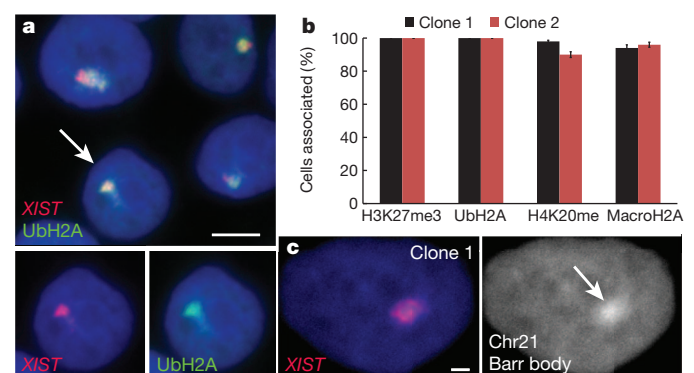

Figure 2 | *XIST* induces heterochromatin modifications and condensed chromosome 21 Barr body. a, *XIST* RNA recruits heterochromatic epigenetic marks (for example, UbH2A). Channels are separated for cell indicated with an arrow. Scale bar, 5 μm. **b**, Percentage of *XIST* territories with heterochromatin marks. Mean ± standard error, 100 nuclei in ~5 colonies. **c**, *XIST* RNA induces chromosome 21 Barr body visible by DAPI stain (arrow). Scale bar, 2 μm.

weaker or undetectable, indicating repression of *DYRK1A* (Fig. 1c, bottom).

The *APP* gene on chromosome 21 encodes β-amyloid precursor protein; mutations in *APP* which cause accumulation of β-amyloid lead to early-onset familial Alzheimer's disease, and *APP* overexpression is linked to the Alzheimer's disease characteristic of Down's syndrome[1]. Initially, three bright RNA transcription foci are apparent (Fig. 3a, top). Short-term *XIST* expression resulted in incomplete repression of the targeted allele (Fig. 3a, middle), which after 20 days was completely silenced, as shown in two independent clones (Fig. 3a, bottom, and Fig. 3b).

We examined four more loci, 3–21 megabases (Mb) from *XIST*: *ITSN1*, *USP25*, *CXADR* and *COL18A1*. Complete silencing of each allele on the edited chromosome 21 was seen in ~100% of cells accumulating *XIST* RNA (Fig. 3c, d and Supplementary Fig. 6a). Allele-specific silencing was further validated using single nucleotide polymorphism (SNP) analysis. PCR with reverse transcription (RT–PCR) products for eight known polymorphic sites (in four genes) were sequenced (*ADAMTS1*, *ETS2*, *TIAM1* and *HSPA13*) (Supplementary Fig. 6b, c). Interestingly, clones 2 and 3 showed an identical pattern of eight SNP alleles repressed, whereas clone 1 showed an alternative pattern of SNPs repressed. As summarized in Supplementary Fig. 6c, this chromosome-wide pattern allows extrapolation of the haplotype for each of the three chromosome 21 homologues, and indirectly identifies for each clone which chromosome 21 was silenced by an *XIST* transgene.

We also examined clones carrying *XIST* on two or all three copies of chromosome 21 and found that after 20 days in doxycycline, most or all cells lost *XIST* localization or expression, and the targeted chromosomes did not silence the *APP* gene (Supplementary Fig. 7a, b). Thus, there is *in vitro* selection and epigenetic adaptation to circumvent creating a functional monosomy or nullisomy, consistent with observations that monosomic cells do not persist in mosaic patients.

Genome-wide silencing and methylation

Having demonstrated allele-specific repression for the ten genes examined above, we extended this to genome-wide expression profiling. We treated three transgenic clones and the parental line with doxycycline for 3 weeks, and compared their transcriptomes to parallel cultures without *XIST* transcription, all in triplicate. Only on chromosome 21 is there overwhelming change, in all three clones (Fig. 4a), with ~95% of significantly expressed genes becoming repressed (Supplementary Table 2).
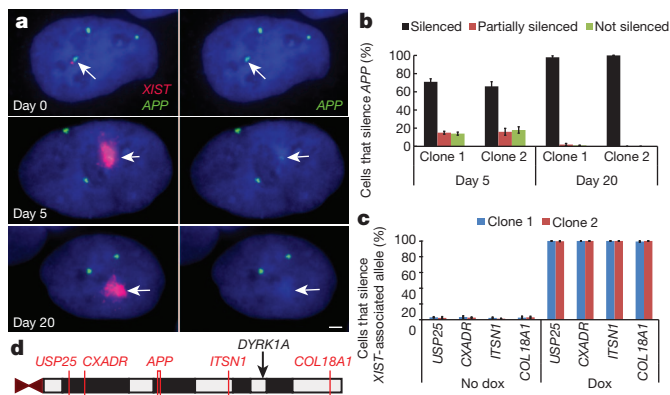
Dosage compensation corrects chromosome 21 expression to near normal disomic levels, based on the change in total output of expressed genes per chromosome after *XIST* is induced. Because evidence suggests that many chromosome 21 genes are not increased the theoretical 1.5-fold in trisomy[21,22], we also directly compared trisomic to disomic cells. This provides a baseline for evaluating the degree to which chromosome 21 overexpression is corrected by *XIST*. After *XIST* induction, overall chromosome 21 expression is reduced by 20%, 15% and 19% for clones 1, 2 and 3, respectively; this mirrors very well the 22% reduction for disomic iPS cells that lack the third chromosome 21 altogether (Fig. 4a). This disomic line is representative, as a similar difference (21%) was seen for an isogenic disomic sub-clone that we isolated from the trisomic parental iPS cells (not shown). Individual genes repressed by *XIST* are distributed throughout chromosome 21, as do genes overexpressed in trisomic versus disomic cells (Fig. 4b). In addition, qRT–PCR confirmed repression for individually examined genes (Supplementary Fig. 7c). Clearly, *XIST* induces robust dosage compensation of most chromosome 21 genes overexpressed in trisomy.

Trisomy 21 may have an impact on genome-wide expression pathways, but differences attributable to trisomy 21 are confounded by genetic and epigenetic variability[21]. This inducible trisomy silencing system provides a new foothold into this important question. For example, even the three isogenic transgenic sub-clones show many expression differences (>1,000), but upon *XIST* induction, ~200 genes throughout the genome change in all three clones (but not the doxycycline-treated parental), most probably directly due to chromosome 21 trisomy. Therefore, 'trisomy correction in a dish' has promise as a means to identify genome-wide pathways perturbed by trisomy 21.

In addition to transcriptional silencing, X-inactivation is stabilized by hypermethylation of promoter CpG islands[23,24], which occurs late

Figure 3 | *XIST* induces long-range silencing in targeted iPS cells. a, RNA FISH. *APP* RNA transcribes from three loci in uninduced cells (day 0), and is progressively silenced after induction (targeted chromosome 21, arrows). Scale bar, 2 μm. **b**, Quantification of *APP* silencing. Mean ± standard error, 100 nuclei. **c**, Silencing for four more chromosome-21-linked genes by RNA FISH. Mean ± standard error from 100 nuclei. **d**, Long-range silencing of chromosome 21 genes by *XIST* RNA. *USP25* is ~21 Mb from the *XIST* integration site (black arrow).
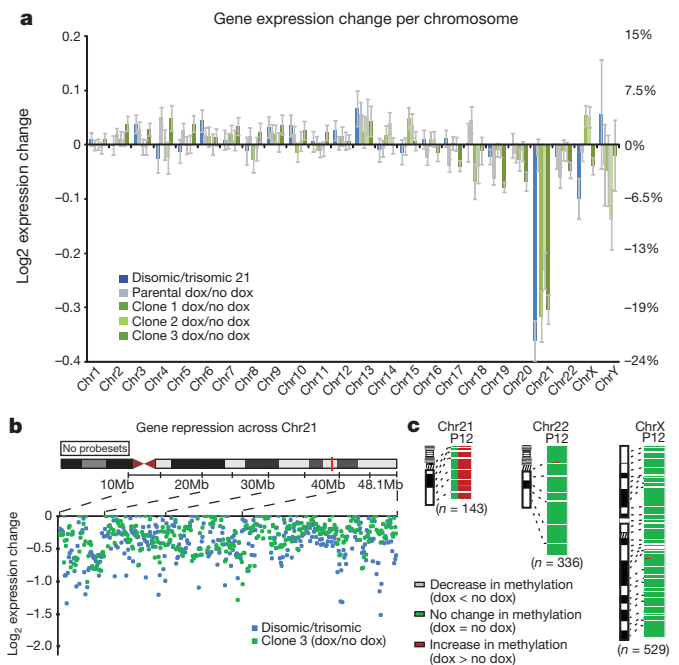

Figure 4 | Genomic expression and methylation reveal widespread silencing of chromosome 21. a, Microarray: expression difference for three transgenic clones in doxycycline (dox) versus no doxycycline, compared to disomic line versus trisomic parental line. Total change in gene expression (*n* = 3) per chromosome shows chromosome 21 'correction' near disomic levels, with only limited changes on other chromosomes. The right *y* axis is scaled for per cent gene expression change. Mean ± standard deviation, in triplicate. **b**, Distribution of individual gene repression across chromosome 21. **c**, Methylation of CpG island promoters. In treated clones, 97% of chromosome 21 genes increased by at least 5% (2-fold greater than average), compared to none in the parental line. P, parental line; 1, clone 1; 2, clone 2.

in the silencing process. Therefore, we also examined the promoter methylome in two genome-edited clones 3 weeks after *XIST* induction and found it largely unaltered, with one striking exception, genes on chromosome 21 (*P* value $<2.2 \times 10^{-16}$) (Fig. 4c). Here, 97% of CpG-island-containing genes exhibited a robust increase in promoter DNA methylation, within the range of that seen for the inactive X chromosome[24] (adjusted for active/inactive chromosomes; see Methods). This change swept the entire chromosome, with the interesting exception of a few genes that 'escape' methylation in both clones.

In summary, data from eight different approaches demonstrate impressive competence of most chromosome 21 genes to undergo epigenetic modification and silencing in response to an RNA that evolved to silence the X chromosome.

Phenotypic correction *in vitro*

Dosage compensation of chromosome imbalance presents a new paradigm, with opportunities to advance Down's syndrome research in multiple directions, including a new means to investigate human Down's syndrome cellular pathologies, which are largely unknown. Inducing trisomy silencing in parallel cultures of otherwise identical cells may reveal cellular pathologies due to trisomy 21, which could be obscured by differences between cell isolates. We examined cell proliferation and neural rosette formation to look for an impact on cell phenotype.

There is some evidence of proliferative impairment in Down's syndrome brains[4,25]; however, we observed that this varied *in vitro* between our Down's syndrome fibroblast samples, and this would be highly sensitive to culture history. A clear answer emerged from comparing identical cell cultures, grown with or without doxycycline for 1 week. *XIST* induction in six independent transgenic sub-clones rapidly and consistently resulted in larger, more numerous and tightly packed colonies in just 7 days (Fig. 5a and Supplementary Fig. 8a), with 18–34% more cells (Fig. 5b). Doxycycline did not enhance growth of the parental Down's syndrome cells or sub-clone (Fig. 5b and Supplementary Fig. 8a). Thus, a proliferative impairment linked to chromosome 21 overexpression can be rapidly ameliorated by dosage compensation.

We next examined differentiation of targeted Down's syndrome iPS cells into neural progenitor cells. In 11–12 days after neural induction of already confluent cultures, all three *XIST*-expressing cultures began to form neural rosettes, and in 1–2 days were replete with

neural rosettes (Fig. 5c), a signature of neural progenitors (confirmed by PAX6 and SOX1 staining) (Supplementary Fig. 8b). Notably, even at day 14, parallel uninduced cultures remained devoid of rosettes (Fig. 5c). Uncorrected cultures required 4–5 more days in neural-induction media to fill with neural rosettes of similar size and number, which they did on day 17 (Fig. 5d and Supplementary Fig. 8d). There was no effect of doxycycline on neurogenesis in the parental line (Fig. 5c, d and Supplementary Fig. 8d). This marked delay in neural differentiation seems to be primarily independent of cell proliferation (Methods). Variability in the kinetics of neural differentiation between various iPS cell lines can obscure differences due to trisomy 21 (ref. 26). We circumvented this using parallel cultures and on-demand chromosome 21 silencing, which made clear these important phenotypic differences. This highlights the potential of this new experimental model to illuminate cellular pathologies directly attributable to chromosome 21 overexpression in iPS cells and their differentiated progeny.

Towards future applications

The Supplementary Information summarizes two significant points relevant to potential applications and therapeutic strategies. First, we show that heterochromatic silencing is stably maintained, even upon removal of doxycycline and *XIST* expression (Supplementary Fig. 9a, b), consistent with previous studies[23]. Second, although not investigated extensively, we targeted *XIST* in non-immortalized fibroblasts from a female Down's syndrome patient, which generated many cells carrying *XIST* (and some heterochromatin marks) on chromosome 21 (Supplementary Fig. 9c, d). Finally, we note that our *XIST* transgene lacks X-chromosome 'counting' sequences, and thus is compatible with natural female X inactivation.

Discussion

We set out to bridge the basic biology of X-chromosome dosage compensation with the pathology of chromosomal dosage disorders, particularly Down's syndrome. In so doing, the present work yields advances that have an impact on three important areas: one basic and two translational.

Although not our primary focus here, a significant impact of this work is that we have created a tractable, inducible system to study human chromosome silencing. Importantly, unlike random integration into a diploid cell, silencing a trisomic autosome avoids selection against full autosomal silencing, and this demonstrated remarkably robust competence of chromosome 21 to be silenced. Thus, *XIST* RNA evolved for the X chromosome uses epigenome-wide mechanisms[12]. The ability to insert a single *XIST* transgene in any locus provides a more powerful tool to study *XIST* function, and our effort also almost triples the size of transgenes that can be thus targeted for a host of other applications.

From a translational perspective, trisomy silencing has immediate impact as a new means to define the poorly understood cellular pathways deregulated in Down's syndrome, and creates the opportunity to derive and study various patient-compatible cell types potentially relevant to Down's syndrome therapeutics. Inducible trisomy silencing *in vitro* compares otherwise identical cultures, allowing greater discrimination of differences directly due to chromosome 21 overexpression distinct from genetic and epigenetic variation between transgenic sub-clones, or potentially even rare disomic sub-clones isolated from a trisomic population (refs 27, 28 and this study). *XIST* expression triggers not only chromosome 21 repression, but a defined effect on the genomic expression profile, and reverses deficits in cell proliferation and neural progenitors, which has implications for hypocellularity in the Down's syndrome brain[4,25]. This new approach can illuminate the cohort of genes and cognate pathways most consistently impacted in Down's syndrome, to inform the search for drugs that may rebalance those pathways and cell pathologies. This general strategy could be extended to study other chromosomal disorders, such as trisomy 13 and 18, often fatal in the first 1–2 years.
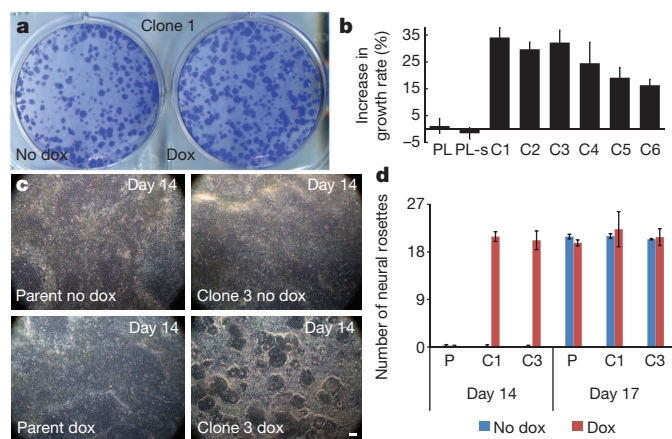

Figure 5 | 'Trisomy correction' affects cell proliferation and neurogenesis. a, One week of *XIST* expression resulted in larger, more numerous colonies (representative sample). **b**, Changes in cell number for parental line (PL), parental line subclone (PL-s), and six transgenic clones (C1–C6). Mean ± s.e. (*n* = 4–6). **c**, Corrected cultures formed neural rosettes by day 14; trisomic (parental and non-induced) cultures took longer (17 days). Scale bar, 100 μm. **d**, Number of rosettes formed on day 14 and day 17. Mean ± standard error, 10–12 random fields in triplicate. P, parental; C1, clone 1; C3, clone 3.

Finally, the more forward-looking implication of this work is to bring Down's syndrome into the realm of consideration for future gene therapy research. Although development of any clinical gene therapy is a multi-step process, any prospect requires that the first step, functional correction of the underlying genetic defect in living cells, is achievable. We have demonstrated that this step is no longer insurmountable for chromosomal imbalance in Down's syndrome. Our hope is that for individuals and families living with Down's syndrome, the proof-of-principle demonstrated here initiates multiple new avenues of translational relevance for the 50 years of advances in basic X-chromosome biology.

METHODS SUMMARY

ZFNs against the *DYRK1A* locus on chromosome 21 were designed and validated by established procedures[18]. These and previously identified ZFNs to chromosome 19 *AAVS1* (ref. 19) were used to deliver the *XIST* gene and rtTA/puro to chromosome 21 and chromosome 19, respectively. All constructs were simultaneously electroporated into a Down's syndrome iPS cell line (DS1-iPS4) (G. Q. Daley, Children's Hospital Boston)[20]. Over 100 clones were isolated and 6 chosen for more analysis. Silencing of the targeted chromosome 21 was demonstrated by eight different approaches as detailed in Methods (RNA microarray, DNA methylation array, RNA FISH to chromosome 21 genes, heterochromatin hallmarks, qRT–PCR, gene SNP analysis, Barr body formation, RNA FISH to hnRNA). Down's syndrome iPS cells were assessed for phenotypic differences (proliferation and neural rosette formation) before and after trisomy correction, as detailed in Methods.

Full Methods and any associated references are available in the online version of the paper.

1. Mégarbané, A. *et al.* 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 (2009).
2. Gardiner, K. J. Molecular basis of pharmacotherapies for cognition in Down syndrome. *Trends Pharmacol. Sci.* **31,** 66–73 (2010).
3. Prandini, P. *et al.* Natural gene-expression variation in Down syndrome modulates the outcome of gene-dosage imbalance. *Am. J. Hum. Genet.* **81,** 252–263 (2007).
4. Haydar, T. F. & Reeves, R. H. Trisomy 21 and early brain development. *Trends Neurosci.* **35,** 81–91 (2012).
5. O'Doherty, A. *et al.* An aneuploid mouse strain carrying human chromosome 21 with Down syndrome phenotypes. *Science* **309,** 2033–2037 (2005).
6. Lee, B. & Davidson, B. L. Gene therapy grows into young adulthood: special review issue. *Hum. Mol. Genet.* **20,** R1 (2011).
7. Hall, L. L. *et al.* X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected. *J. Cell. Physiol.* **216,** 445–452 (2008).
8. Nazor, K. L. *et al.* Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. *Cell Stem Cell* **10,** 620–634 (2012).
9. Brown, C. J. *et al.* 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 (1992).
10. Clemson, C. M., McNeil, J. A., Willard, H. F. & Lawrence, J. B. 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 (1996).
11. Heard, E. Delving into the diversity of facultative heterochromatin: the epigenetics of the inactive X chromosome. *Curr. Opin. Genet. Dev.* **15,** 482–489 (2005).
12. Hall, L. L. & Lawrence, J. B. XIST RNA and architecture of the inactive X chromosome: implications for the repeat genome. *Cold Spring Harb. Symp. Quant. Biol.* **75,** 345–356 (2010).
13. Carrel, L. & Willard, H. F. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* **434,** 400–404 (2005).
14. Lee, J. T., Strauss, W. M., Dausman, J. A. & Jaenisch, R. A 450 kb transgene displays properties of the mammalian X-inactivation center. *Cell* **86,** 83–94 (1996).
15. Hall, L. L., Clemson, C. M., Byron, M., Wydner, K. & Lawrence, J. B. Unbalanced X;autosome translocations provide evidence for sequence specificity in the association of XIST RNA with chromatin. *Hum. Mol. Genet.* **11,** 3157–3165 (2002).
16. Hall, L. L. *et al.* An ectopic human *XIST* gene can induce chromosome inactivation in postdifferentiation human HT-1080 cells. *Proc. Natl Acad. Sci. USA* **99,** 8677–8682 (2002).
17. Moehle, E. A. *et al.* Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proc. Natl Acad. Sci. USA* **104,** 3055–3060 (2007).
18. Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nature Rev. Genet.* **11,** 636–646 (2010).
19. DeKelver, R. C. *et al.* Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res.* **20,** 1133–1142 (2010).
20. Park, I. H. *et al.* Disease-specific induced pluripotent stem cells. *Cell* **134,** 877–886 (2008).
21. Aït Yahya-Graison, E. *et al.* Classification of human chromosome 21 gene-expression variations in Down syndrome: impact on disease phenotypes. *Am. J. Hum. Genet.* **81,** 475–491 (2007).
22. Biancotti, J. C. *et al.* Human embryonic stem cells as models for aneuploid chromosomal syndromes. *Stem Cells* **28,** 1530–1540 (2010).
23. Csankovszki, G., Nagy, A. & Jaenisch, R. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. *J. Cell Biol.* **153,** 773–784 (2001).
24. Cotton, A. M. *et al.* Chromosome-wide DNA methylation analysis predicts human tissue-specific X inactivation. *Hum. Genet.* **130,** 187–201 (2011).
25. Guidi, S., Ciani, E., Bonasoni, P., Santini, D. & Bartesaghi, R. Widespread proliferation impairment and hypocellularity in the cerebellum of fetuses with down syndrome. *Brain Pathol.* **21,** 361–373 (2011).
26. Shi, Y. *et al.* A human stem cell model of early Alzheimer's disease pathology in Down syndrome. *Sci. Transl. Med.* **4,** 124ra29 (2012).
27. Lavon, N. *et al.* Derivation of euploid human embryonic stem cells from aneuploid embryos. *Stem Cells* **26,** 1874–1882 (2008).
28. Li, L. B. *et al.* Trisomy correction in down syndrome induced pluripotent stem cells. *Cell Stem Cell* **11,** 615–619 (2012).

Supplementary Information is available in the online version of the paper.

METHODS

Cell culture. HT1080 TetR cells (Invitrogen) and female Down's syndrome human primary fibroblast line (Coriell) (AG13902) were cultured as recommended by the supplier. Down's syndrome iPS cell parental line (DS1-iPS4) was provided by G. Q. Daley (Children's Hospital Boston)[20] and maintained on irradiated mouse embryonic fibroblasts (iMEFs) (R&D Systems, PSC001) in hiPSC medium containing DMEM/F12 supplemented with 20% knockout serum replacement (Invitrogen), 1 mM glutamine (Invitrogen), 100 μM non-essential amino acids (Invitrogen), 100 μM β-mercaptoethanol (Sigma) and 10 ng ml^{-1} FGF-β (Invitrogen, PHG0024). Cultures were passaged every 5–7 days with 1 mg ml^{-1} of collagenase type IV (Invitrogen).

ZFN design. ZFNs against the human *AAVS1* locus on chromosome 19 have been previously described[19]. ZFNs against the *DYRK1A* locus were designed using an archive of pre-validated zinc finger modules[18,29], and validated for genome editing activity by transfection into K562 cells and Surveyor endonuclease-based measurement of endogenous locus disruption ('Cel1'[30,31]) exactly as described[29]. Southern blotting for targeted gene addition was performed exactly as described[17,32] on SphI-digested genomic DNA probed with a fragment corresponding to positions Chr21:38825803+38826056 (hg19).

XIST and rtTA/puro plasmid construction. Fourteen-kilobase human *XIST* cDNA, a splicing isoform of full-length *XIST* cDNA, was subcloned into pTRE3G (Clontech, catalogue no. 631167). Two homologous arms (left arm, 690 bp; right arm, 508 bp) of *DYRK1A* gene on chromosome 21 were amplified by PCR from primary Down's syndrome fibroblasts (AG13902) (Coriell) and cloned into the pTRE3G vector (human chromosome 21 *DYRK1A* left arm primers: forward 5′-GCCGTATACCATTAACTCTTTACTGTTC-3′, reverse 5′-TCTGTATACGTAAACTGGCAAAGGGGTGG-3′; human chromosome 21 *DYRK1A* right arm primers: forward 5′-ATTTCGCGAACGGGTGATGAGCAGGCTGT-3′, reverse 5′-CCGTCGCGAAAACCAGAAAGTATTCTCAG-3′). The pEF1α-3G rtTA-pA cassette from pEF1α-Tet3G vector (Clontech) was subcloned into a plasmid for targeted gene addition to the *PPP1R12C/AAVS1* locus[19], which contains a unique HindIII site flanked by two 800-bp stretches of homology to the ZFN-specified position in the genome.

Dual-targeted addition of human Down's syndrome iPS cells and generation of stable targeted clones. The Down's syndrome iPS cell line was cultured in 10 μM of Rho-associated protein kinases (ROCK) inhibitor (Calbiochem; Y27632) 24 h before electroporation. Single cells (1×10^7) were collected using TryPLE select (Invitrogen), re-suspended in 1× PBS and electroporated with a total of 55 μg DNA including five plasmids (*XIST*, *DYRK1A* ZFN1, *DYRK1A* ZFN2, rtTA/puro and *AAVS1* ZFN) with both 3:1 and 5:1 ratios of *XIST*:rtTA/ puro. The electroporation conditions were 220 V and 750 μF (BioRad Gene Pulser II System). Cells were subsequently plated on puromycin-resistant DR4 MEF feeders (Open Biosystems, catalogue no. MES3948) in hiPSC medium supplemented with ROCK inhibitor for the first 24 h. Over 300 colonies remained after 12 days of 0.4 μg ml^{-1} puromycin selection and 245 randomly chosen individual colonies across 36 pooled wells were examined by interphase DNA/RNA FISH for the presence and expression of *XIST*, correct targeting and retention of trisomy (because some subclones lacked *XIST* or showed just two *DYRK1A* DNA signals). Over 100 individual clones were isolated and characterized, and those of interest, containing targeted *XIST* on one of three *DYRK1A* loci, were frozen. Six single target clones with good pluripotent morphology, OCT4 positive staining, correct targeting to one trisomic chromosome, and good *XIST* RNA paint were expanded for further characterization. One double and one triple target line, two non-target clones, and one disomic clone were also isolated and frozen. Targeting and correct chromosome number (47) was confirmed by interphase and metaphase FISH and genome integrity was confirmed by high-resolution G-band karyotype and CGH array.

Chromosome preparation. iPS cells were treated with 100 ng ml^{-1} KaryoMAX colcemid (Invitrogen) for 2–4 h at 37 °C in a 5% CO_2 incubator. Cells were trypsinized, treated with hypotonic solution, and fixed with methanol:acetic acid (3:1). Metaphases were spread on microscope slides, and at least 20 analysed per clone. Karyotype analysis was done on pro-metaphase chromosomes using Standard Giemsa-trypsin G band methods.

CGH array. CGH was performed in the Cytogenetics Laboratory at University of Massachusetts Medical School. Genomic Microarray analysis using University of Massachusetts Genomic Microarray platform (Human Genome Build hg19) was performed with 1 μg of DNA. The array contains approximately 180,000 oligonucleotides (60-mers) that represent coding and non-coding human sequences and high-density coverage for clinically relevant deletion/duplication syndromes and the telomeric and pericentromeric regions of the genome. Data were analysed by BlueFuse Multi, v3.1 (BlueGnome, Ltd).

DNA/RNA FISH and immunostaining. DNA and RNA FISH were carried out as previously described[10,15,16,33]. The *XIST* probe is a cloned 14-kb *XIST* cDNA

(the same sequence as *XIST* transgene in Fig. 1b) in pGEM-7Zf(+) (Promega). Six chromosome 21 gene probes are BACs from BACPAC Resources (*DYRK1A*, Rp11-105O24; *APP*, RP11-910G8; *USP25*, RP11-840D8; *CXADR*, RP11-1150I14; *ITSN1*, RP11-1033C16; *COL18A1*, RP11-867O18). DNA probes were labelled by nick translation with either biotin-11-dUTP or digoxigenin-16-dUTP (Roche). In simultaneous DNA/RNA FISH (interphase targeting assay), cellular DNA was denatured and hybridization performed without eliminating RNA and also treated with 2 U μl^{-1} of RNasin Plus RNase inhibitor (Promega). For immunostaining with RNA FISH, cells were immunostained first with RNasin Plus and fixed in 4% paraformaldehyde before RNA FISH. Antibodies were as follows: H3K27me3 (Millipore, 07-449), UbH2A (Cell Signaling, 8240), H4K20me (Abcam, ab9051), macroH2A (Millipore, 07-219), OCT4 (Santa Cruz, sc-9081), PAX6 (Stemgent, 09-0075), SOX1 (R&D Systems, AF3369).

Allele-specific SNP analysis. Primers were designed to amplify 3′ untranslated regions of chromosome 21 genes reported to contain SNPs (Supplementary Table 3). Total cDNA was used from three transgenic clones with and without *XIST* induction for 22 days. RT–PCR products were sequenced by GENEWIZ. Of ~10 genes examined, four were heterozygous and informative in the patient Down's syndrome iPS cell line used here.

Microarray analysis. Three independently targeted subclones plus the parental chromosome 21 trisomic (non-targeted) iPS cell line were grown with or without doxycycline (2 μg ml^{-1}) for 22 d. Normal male iPS cell and disomic isogenic lines were also cultured for 22 d and total RNA was extracted with a High Pure RNA extraction kit (Roche) in triplicate for each, processed with a Gene Chip 3′ IVT express kit (Affymetrix), and hybridized to Affymetrix human gene expression PrimeView arrays. Array normalization was performed with Affymetrix Expression Console Software with Robust Multichip Analysis (RMA)[34]. Probe sets with the top 60% of signal values were considered present and 'expressed' and were used for all further analysis. Data in Fig. 4 has no other threshold applied. When designated, a gene expression change significance threshold was applied using a two-tailed *t*-test comparing samples with or without doxycycline in triplicate ($n = 3$) (Supplementary Table 2, $P < 0.01$). For the ~200 genes found to significantly change in all three clones (in text), a *t*-test with $P < 0.001$ was applied.

Microarray data interpretation. Using extraction-based methods, changes on just one of three alleles (from the *XIST*-bearing chromosome) will be diluted by the other two. If all three chromosomes are fully expressed, this would predict a 33% reduction in chromosome 21 expression levels per cell when one chromosome 21 is fully silenced. However, 33% would apply only if chromosome 21 genes are fully overexpressed to start, and previous evidence and results in this study show this is not the case for many genes. Previous microarray studies have analysed expression levels of chromosome 21 in Down's syndrome patient cells, although such analyses are hampered by the extensive genetic and epigenetic differences between any two individuals[3]. The fraction of chromosome 21 genes detected as overexpressed varies with the study and tissue, but generally is in the 19–36% range[3,22], with individual gene increases often in the ~1.2–1.4 range (less than the theoretical 1.5). For example, one study of Down's syndrome embryoid bodies showed that only 6–15% of genes appeared significantly upregulated, but this was comparing non-isogenic samples of different ES cell isolates[22].

Our trisomy correction system allows direct comparison of the same cells grown in identical parallel cultures, with and without *XIST*-mediated chromosome silencing. Our data show a ~20% reduction in chromosome 21 expression overall; importantly, this level of reduction is seen either when the third chromosome is silenced in trisomic cells, or when disomic and trisomic cells are compared. This 20% reduction represents an average per cell for all three chromosomes, but corresponds to a 60% reduction in expression for just one chromosome 21 (the one silenced by *XIST* RNA, as shown here).

Apart from our goal here of trisomy dosage compensation, these results add significantly to understanding the extent of chromosome 21 overexpression in Down's syndrome, by providing a more comprehensive analysis that shows that expression of most genes is increased, but less than the theoretical 1.5 fold.

qRT–PCR. qRT–PCR was performed for eight downregulated chromosome 21 genes determined by microarray on a Bio-Rad MyiQ real-time PCR detection system in triplicate for clone 3 with/without doxycycline treatment for 22 d. The β-actin gene was used as an internal standard for calculation of expression levels. Primers for eight chromosome 21 genes and β-actin were described in Supplementary Table 4.

DNA methylation analysis. The parental line and two independent targeted lines were grown with and without doxycycline for 22 d, in duplicate cultures. Genomic DNA was extracted using PureLink Genomic DNA mini kit (Invitrogen) and 750 ng bisulphite modified with the Alternative Incubation Conditions from the EZ DNA methylation kit (Zymo Research). 160 ng of bisulphite DNA was amplified, fragmented and hybridized to Illumina Infinium HumanMethylation450

array following the standard protocol as outlined in the user guide. CpG islands were defined as high and intermediate CpG densities using the CpG density classifications based on those used previously[35]. The program CpGIE was used to locate HC and IC islands on the X chromosome and chromosomes 21 and 22. When multiple probes in CpG islands were associated with the same TSS, an average genic methylation value was calculated. These average genic values were compared before and after doxycycline induction using the Mann–Whitney U-test. Analysis was based on CpG islands within promoters of 143 chromosome 21 genes (Fig. 4c).

The average methylation value was 6% on chromosome 21 before *XIST* induction, and increased to 20–21% in both subclones after induction. Because any methylation increase on the transgenic chromosome would be diluted by the presence of three chromosome 21 copies, this suggests the range of 60% methylation on the one *XIST*-coated chromosome, which is within the range seen for the inactive X chromosome[24].

Cell proliferation analysis. Eight different iPS cell lines (parental line, one non-targeted subclone, and six independent targeted subclones) were passaged onto 6-well plates at equal cell densities per well of each line and grown with or without doxycycline for 7 d. At least four replicates of each line were analysed in two independent experiments. Rigorous measures were taken to minimize and control for any minor variations in seeding densities of iPS cells, which cannot be plated as single cell suspensions. First, the analysis was done twice for six different transgenic clones, in each case comparing triplicate plates of corrected versus not corrected (doxycycline versus no doxycycline). To avoid differences in plating efficiencies of doxycycline and no doxycycline cells, we performed the experiments over a time course that did not require passage. For each of the six transgenic clones, the parental line and one negative control (non-targeted) subclone, a single well of Down's syndrome iPS cells (without doxycycline) was used to generate a cell suspension (cells and small disaggregated clumps). Next, equal aliquots of the cell suspension were plated into each of six wells four times (not relying on one measurement but the average of four for seeding each well). After plating, doxycycline was added to three of the six wells, and the cultures were maintained for 7 d. For images, plates were fixed, stained with 1 mg ml^{-1} crystal violet (Sigma) in 70% ethanol for 30 min and scanned to generate TIFF images. For cell counts, single cells were collected by TryPLE select and counted using Beckman Coulter Z1 Particle Counter.

Differentiation of neural progenitors and irreversibility in cortical neurons. For differentiation, independent *XIST*-transgenic iPS cell clones and the parental Down's syndrome iPS cell line were dissociated with Accutase (Innovative Cell Technologies) and 4×10^5 single cells were plated on Matrigel-coated 6-well plates in mTeSR1 medium (Stemcell technologies). Once the cell culture reached 90–100% confluence, neural induction was initiated by changing the culture medium to neural induction medium, a 1:1 mixture of N2- and B27-containing media supplemented with 500 ng ml^{-1} noggin (R&D Systems), 10 µM SB431542 (Tocris Bioscience), and 1 µM retinoic acid (Sigma, catalogue no. R2625), with/without treatment of doxycycline for the specified times. The neural rosettes were counted and their diameter measured for at least 300 rosettes (sampled in random areas from triplicate dishes). At day 14, the doxycycline-induced culture had an average rosette diameter of 142 µm ± 0.55 µm in clone 1 and 141 µm ± 3.49 µm in clone 3. Rosettes could not be measured at the same time point in the uncorrected culture, as they had not formed. At day 17, the uncorrected culture had

neural rosettes of similar number and size for both clones 1 (140 µm ± 0.87 µm) and 3 (140 µm ± 1.09 µm). The corrected culture could not be accurately compared for day 17 because the rosettes had become so mature and often had merged. After 17 d, neural rosettes were collected by dissociation with dispase and replated on poly-ornithine and laminin-coated plastic dishes in N2- and B27-containing media including 20 ng ml^{-1} FGF2. After a further 2 d, FGF2 was withdrawn to promote differentiation of cortical neurons. To test for the irreversibility of silencing, two independent clones were differentiated to cortical neurons in the presence of doxycycline for 70 days to initiate silencing. They were then split into parallel cultures grown with and without doxycycline for another 30 days, and *XIST* and *APP* expression analysed by RNA FISH.

Targeted addition to primary fibroblasts. We used non-immortalized primary human female Down's syndrome fibroblasts, which like all primary fibroblasts have a limited lifespan in culture (potentially more limited for Down's syndrome fibroblasts). We reasoned that the robustness of ZFN-driven editing, combined with reduction to disomy for the *DRYK1A* gene, may make it possible to observe some edited cells before they senesce. We used a transgene carrying near full-length (~14 kb) *XIST* cDNA under a TetO$_2$ inducible promoter, and a selectable marker on the same construct, with ~600-bp homology arms to the *DYRK1A* gene (vector is ~21 kb, with a ~17-kb insert) (data not shown). When introduced without the Tet-repressor construct, the TetO$_2$ CMV promoter is constitutively active. Two ZFN-containing vectors and the 21-kb *XIST* transgene were transfected into primary DS fibroblasts (Coriell AG13902) using Stemfect polymer (Stemgent) (10:1 ratio of *XIST* to ZFN, and 13 µg DNA to 1.3 µl Stemfect per well of 6-well plate). The frequency of stable integrants was such that a sparse monolayer of transgenic fibroblasts emerged, rather than a few individual colonies following selection with hygromycin (75 µg ml^{-1}). The pooled population of selected cells was analysed by FISH and immunostaining for targeting, *XIST* expression and heterochromatin marks. *XIST* RNA was observed over the *DYRK1A* locus in ~74% of cells, indicating accurate transgene targeting, which was also verified by metaphase FISH (Supplementary Fig. 9c). In many cells there was notable enrichment of H3K27me, UbH2A and H4K20me heterochromatic marks (Supplementary Fig. 9d). Owing to the limited lifespan of primary cells and the progressive silencing of the CMV promoter used in this construct, these cells were not more fully characterized.

29. Doyon, J. B. *et al.* Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. *Nature Cell Biol.* **13,** 331–337 (2011).
30. Miller, J. C. *et al.* An improved zinc-finger nuclease architecture for highly specific genome editing. *Nature Biotechnol.* **25,** 778–785 (2007).
31. Guschin, D. Y. *et al.* A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649,** 247–256 (2010).
32. Urnov, F. D. *et al.* Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435,** 646–651 (2005).
33. Byron, M., Hall, L. L. & Lawrence, J. B. A multifaceted FISH approach to study endogenous RNAs and DNAs in native nuclear and cell structures. *Curr. Protoc. Hum. Gen.* **Chapter 4,** Unit 4 15 (2013).
34. Irizarry, R. A. *et al.* Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* **31,** e15 (2003).
35. Weber, M. *et al.* Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature Genet.* **39,** 457–466 (2007).