Usp16 contributes to somatic stem-cell defects in Down’s syndrome

Maddalena Adorno¹, Shaheen Sikandar¹, Siddhartha S. Mitra¹, Angera Kuo¹, Benedetta Nicolì Di Robilant¹,², Veronica Haro-Acosta¹, Youcef Ouadah¹,³, Marco Quarta⁴, Jacqueline Rodriguez⁵, Dalong Qian¹, Vadiyala M. Reddy⁶, Samuel Cheshier¹,⁷, Craig C. Garner⁵ & Michael F. Clarke¹

Down’s syndrome results from full or partial trisomy of chromosome 21. However, the consequences of the underlying gene–dosage imbalance on adult tissues remain poorly understood. Here we show that in Ts65Dn mice, which are trisomic for 132 genes homologous to genes on human chromosome 21, triplication of Usp16 reduces the self-renewal of haematopoietic stem cells and the expansion of mammary epithelial cells, neural progenitors and fibroblasts. In addition, Usp16 is associated with decreased ubiquitination of Cdkn2a and accelerated senescence in Ts65Dn fibroblasts. Usp16 can remove ubiquitin from histone H2A on lysine 119, a critical mark for the maintenance of multiple somatic tissues. Downregulation of Usp16, either by mutation of a single normal Usp16 allele or by short interfering RNAs, largely rescues all of these defects. Furthermore, in human tissues overexpression of Usp16 reduces the expansion of normal fibroblasts and postnatal neural progenitors, whereas downregulation of USP16 partially rescues the proliferation defects of Down’s syndrome fibroblasts. Taken together, these results suggest that Usp16 has an important role in antagonizing the self-renewal and/or senescence pathways in Down’s syndrome and could serve as an attractive target to ameliorate some of the associated pathologies.

Down’s syndrome is a complex clinical syndrome associated with multiple pathological conditions, including deficits in motor skills and learning. It has also been associated with early onset and higher incidence of ageing-related phenomena such as Alzheimer’s disease. Recently, it has been proposed that the ageing process is correlated with an impaired or exhausted ability of stem cells to self-renew, raising the possibility that stem-cell defects have a role in Down’s syndrome.

To gain insights into the molecular mechanisms underlying abnormalities of tissue homeostasis in Down’s syndrome, we investigated haematopoietic stem cells in two Down’s syndrome mouse models, Ts65Dn and Ts1Cje (Fig. 1). Ts65Dn mice have a third copy of approximately two-thirds of the murine genes homologous to genes on human chromosome 21 and recapitulate several traits of the human disease, including learning and memory deficits. Ts1Cje mice present only 79 of the 132 trisomic genes of Ts65Dn mice (Fig. 1a) and certain defects, such as neurologic impairment, are less severe in these mice. One of the genes uniquely triplicated in Ts65Dn is Usp16, a deubiquitination enzyme involved in chromatin remodelling and cell cycle progression. Our studies implicated Usp16 as a major factor causing expansion defects and senescence in multiple somatic tissues of Ts65Dn mice.

Ts65Dn, but not Ts1Cje, mice have defective HSCs

Consistent with previous observations, transplantation of 5 × 10⁵ CD45.2⁺ Ts65Dn cells into CD45.1⁺ C57BL/6 lethally irradiated mice resulted in lower haematopoietic chimaerism in recipient mice compared to transplantation of control bone marrow cells (Extended Data Fig. 1a). To determine the trisomic genes responsible for the impaired engraftment potential of the Ts65Dn bone marrow cells, we investigated the frequency and properties of haematopoietic stem cells (HSCs) in Ts65Dn and Ts1Cje mice. Immunophenotypical analyses showed that the fraction of CD150⁻CD48⁻ or CD34 Flt3⁻ KLS (c-Kit⁺ Lineage⁻ Sca⁺) cells, both of which enrich for HSCs in mice, was reduced by more than threefold in Ts65Dn mice, but not in Ts1Cje mice (Fig. 1b, c and Extended Data Fig. 1b). A 50% reduction in in vitro colony formation by single CD34⁻CD150⁻CD48⁻ KLS cells compared to controls further suggested an impairment of Ts65Dn HSCs (Fig. 1d). To assess definitively the function of HSCs in Down’s syndrome, we performed serial dilution bone marrow transplantations with euploid, Ts65Dn and Ts1Cje cells. Wild-type and Ts1Cje bone marrow cells reconstituted the bone marrow of lethally irradiated recipient mice with a similar HSC calculated frequency (1 out of 80,338 and 1 out of 103,553, respectively). However, there was a threefold reduction in the frequency of Ts65Dn stem cells (1 out of 307,431; P = 0.0294) (Fig. 1e and Extended Data Fig. 1c). Furthermore, multi-lineage engraftment of Ts65Dn bone marrow cells was not observed in secondary transplants after 3 months (Fig. 1f and Extended Data Fig. 1d), confirming a severe impairment in the self-renewal ability of Ts65Dn haematopoietic cells.

Usp16 contributes to HSC defects in mice

Usp16 is one of the genes uniquely triplicated in Ts65Dn mice but not in Ts1Cje mice. Previous studies have reported that Usp16 can remove ubiquitin moieties from histone H2A, opposing the ubiquitination function of the Polycomb repressive complex 1 (PRC1). As Bmi1, a crucial member of the PRC1 complex, is essential for the self-renewal of stem cells in multiple tissues, we postulated that an extra copy of Usp16, an antagonist of PRC1, could impair the self-renewal ability of stem cells in Ts65Dn mice. Consistent with the presence of three alleles, Usp16 messenger RNA was expressed 1.5-fold higher in Ts65Dn HSCs compared to control HSCs (Fig. 1g). Moreover, immunofluorescence

¹Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California 94305, USA. ²International PhD School in Molecular Medicine, San Raffaele University, Milano, Italy. ³Program in Cancer Biology, Stanford University School of Medicine, Stanford, California 94305, USA. ⁴Department of Neurology, Stanford University School of Medicine, Stanford, California 94305, USA. ⁵Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, California 94305, USA. ⁶Division of Pediatric Cardiology, Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305, USA. ⁷Division of Pediatric Neurosurgery, Department of Neurosurgery, Stanford University School of Medicine, Stanford, California 94305, USA.
Usp16 is expressed in a key site of adult neurogenesis, the brain subventricular zone (SVZ) (Extended Data Fig. 4a). Because Bmi1 is known to be critical for the maintenance of neural progenitors,\textsuperscript{15,16} we proposed that an extra copy of the PRC1 antagonist Usp16 could have a role in regulating also their expansion in Ts65Dn mice. To test this hypothesis, the SVZ was micro-dissected from 8-week-old mice, and Lin\textsuperscript{−} cells (CD24\textsuperscript{−}CD31\textsuperscript{−}CD150\textsuperscript{−}Ter119\textsuperscript{−}) were enriched by flow cytometry and assayed for neurosphere formation during serial passaging in vitro. As reported previously, in vitro sphere formation by Ts65Dn neural progenitors was impaired\textsuperscript{7,8,17}. By the fourth passage, 1 out of 21 wild-type cells formed neurospheres, whereas only 1 out of 958 Ts65Dn Lin\textsuperscript{−} cells could do so (P < 0.0001) (Fig. 2a, b). To define the role of an extra copy of Usp16 in neural progenitor expansion, Ts65Dn mice were bred with mice in which one of the normal Usp16 alleles was mutated (Usp16\textsuperscript{−/−}). The offspring had a normal diploid dosage of Usp16, but retained three copies of the other genes present in the Ts65Dn parental strain (Ts65Dn/Usp16\textsuperscript{−/−} mice). Notably, Ts65Dn/Usp16\textsuperscript{−/−} neural progenitor cells maintained a normal frequency of neurosphere-initiating cells (Nsp-ICs) during serial passage (P < 0.0001) (Fig. 2a, b).

After four passages, expression of Sox2 (a known neural progenitor cell marker\textsuperscript{14}) increased twofold in control Lin\textsuperscript{−} neurospheres but was not detected in Ts65Dn Lin\textsuperscript{−} neurospheres. By contrast, Ts65Dn/Usp16\textsuperscript{−/−} cells express comparable levels of Sox2 to control cells (Fig. 2c). Next we analysed CD133\textsuperscript{+} Egfr\textsuperscript{+} Lin\textsuperscript{−} and CD15\textsuperscript{+} Egfr\textsuperscript{−} Lin\textsuperscript{−} SVZ cells\textsuperscript{19,20}, which are enriched for neural progenitor cells. These cells express high levels of nestin and Sox2 and retain the ability to expand in vitro upon serial passaging (Extended Data Fig. 5). Sphere limiting dilution analyses of either CD133\textsuperscript{+} Egfr\textsuperscript{+} Lin\textsuperscript{−} cells or CD15\textsuperscript{+} Egfr\textsuperscript{−} Lin\textsuperscript{−} cells revealed a significant decrease in the frequency of Nsp-1c in the Ts65Dn mice compared to wild-type or Ts65Dn/Usp16\textsuperscript{−/−} mice.
ARTICLE

Ts65Dn mice might also affect the expansion of mammary epithelial cells. Like HSCs, Usp16 mRNA expression was increased approximately 1.5-fold in Ts65Dn CD49f<sup>+</sup> CD24<sup>low</sup> lineage<sup>−</sup> cells (which are enriched for mammary repopulating units (MRUs))<sup>22</sup> compared to control cells (Extended Data Fig. 6a). Moreover, several Hox genes, usually repressed by PRC1, were highly expressed in Ts65Dn cells (Extended Data Fig. 6b). Immuno–phenotypic analysis of the breast tissue from Ts65Dn mice revealed a significant reduction of the overall number of CD31<sup>−</sup> CD45<sup>−</sup> Ter119<sup>−</sup> (Lineage<sup>−</sup>) cells (Fig. 3a and Extended Data Fig. 6c). Cytokeratin staining showed that compared to wild-type or Ts1Cje mice, Ts65Dn mice have an increased number of cells that co-express luminal cell cytokeratin-8 and basal cell cytokeratin-14 (Fig. 3b and Extended Data Fig. 6d), suggesting abnormalities in the development of the mammary epithelium.

To functionally test CD49f<sup>+</sup> CD24<sup>low</sup>Lin<sup>−</sup> cells, a population enriched with regeneration potential<sup>21,22</sup>, we performed three-dimensional in vitro colony assays. Ts65Dn, but not Ts1Cje, cultures showed a reduced number of colonies (Fig. 3c). Moreover, in vivo mammary transplantation assays with Lin<sup>−</sup> cells showed a significant decrease in repopulating ability of Ts65Dn Lin<sup>−</sup> cells compared to wild type or Ts65Dn/Usp16<sup>het</sup> Lin<sup>−</sup> cells (Fig. 3d).

As in HSCs and neural progenitors, lentiviral downregulation of Usp16 in Ts65Dn Lin<sup>−</sup> cells partially rescued mammary epithelium defects. There was a twofold increase in the calculated frequency of MRUs in shUsp16-infected Lin<sup>−</sup> Ts65Dn cells, and the derived outgrowths were larger compared to cells infected with control shRNA (Extended Data Fig. 6e). However, we were unable to generate mammary glands in secondary transplants of the Ts65Dn/Usp16<sup>het</sup> mammary epithelial cells, suggesting that other genes may affect the proliferation of these cells in Ts65Dn mice.

**Modulation of Ink4a/Arf by Usp16 in Ts65Dn cells**

One of the best-characterized Bmi1/PRC1 target loci is Cdkn2a<sup>23</sup>, which encodes two distinct tumour suppressors, p16<sup>Ink4a</sup> and p19<sup>Arf</sup>. Expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> normally increases with age in both rodent and human tissues and is involved in senescence-induced loss of proliferation<sup>24–26</sup>. To determine whether Usp16 trisomy affects proliferation and senescence, we used MEFs and terminal-tip fibroblasts (TTFs). Ts65Dn, but not Ts65Dn/Usp16<sup>het</sup> TTFs showed a marked proliferation deficit and high levels of senescence as shown by senescence-associated β-galactosidase staining and by p16<sup>Ink4a</sup> expression (Fig. 4a, b). These defects were partially rescued by a shRNA targeting Cdkn2a (Fig. 4b and Extended Data Fig. 7a–c). Ts65Dn MEF cultures showed high levels of senescence and a rapid increase of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> expression levels upon passing (Fig. 4c and Extended Data Fig. 7d). However, lentiviral downregulation...
of Usp16 with two different hairpins decreased p16Ink4a and p19Arf levels, and chromatin immunoprecipitation analyses demonstrated a decrease in H2AK119 ubiquitin on the Ink4a/Arf locus in Ts65Dn MEFs (Fig. 4e). These results strongly suggest that trisomy of Usp16 contributes to the cellular defects observed in Ts65Dn mice through increased removal of ubiquitin moieties from H2A, with an impact on the Cdkn2a site. However, mutational analyses of the deubiquitinase domains would be required to confirm this model.

**Potential role USP16 in human Down’s syndrome**

As reported previously, fibroblasts derived from Down’s syndrome patients showed a strong proliferation defect. Moreover, they appear to senesce prematurely (Fig. 5a). Gain-of-function experiments were performed using normal fibroblasts to determine whether USP16 overexpression might contribute to the proliferation defects. Transduction of normal foreskin fibroblasts with a vector overexpressing USP16 slowed their proliferation (Fig. 5b). Conversely, shRNA-mediated downregulation of USP16 or overexpression of BMI1 resulted in increased proliferation capacity of Down’s syndrome fibroblasts (Fig. 5c and Extended Data Fig. 8b, c). Interestingly, overexpression of USP16 in two different cultures of human neural progenitor cells (from one paediatric and one adult patient) reduced their cellular expansion potential and the formation of neurospheres.
(Fig. 5d and Extended Data Fig. 8d). These experiments suggest a role for the third copy of USP16 in the pathologies associated with Down’s syndrome.

Discussion

Our data show that the change in self-renewal capacity of HSCs and neural progenitors, as well as the proliferation defects of mammalian epithelial cells and fibroblasts in Ts65Dn mice, is linked in part to trisomy of Usp16, a negative modulator of the PRC1 activity. As Bmi1, a component of PRC1, is known to regulate senescence and self-renewal of multiple somatic stem cells, this suggests that some of the pathologies associated with Down’s syndrome may result from a stem-cell imbalance secondary to overexpression of Usp16. Clearly, other genes also have a role in traits associated with Down’s syndrome patients, such as craniofacial abnormalities and perhaps the increased incidence of leukemia and the decreased incidence of solid tumours (Extended Data Fig. 9) [29], and there are probably differences between humans with Down’s syndrome and Ts65Dn mice. Nonetheless, the defects of human fibroblasts mirror those seen in the mouse models, and the effect of overexpression of Usp16 in human neural progenitors suggest that this gene has a similar function in human Down’s syndrome and in Ts65Dn mice. Although we cannot unequivocally state that the HSCs are reduced in Down’s syndrome patients, in two children we examined they were not increased as they might be in the liver of some human Down’s syndrome fetuses (Extended Data Fig. 9) [29].

In conclusion, our data demonstrate a new axis of regulation of tissue homeostasis in multiple tissues. We believe that our study has broad implications for understanding one of the most common genetic abnormalities in humans, Down’s syndrome, and that targeting the USP16 pathway may ameliorate some of the associated pathologies.

METHODS SUMMARY

Mice. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID), Ts65Dn, Ts1Cje and euploid littermate mice were purchased from Jackson Laboratories. Usp16 Tg (FVB/N-Usp16gr1+yx2a1Pfklw/Mmjax) were obtained from the Mutant Mouse Resource Centers (MMRC).

Lentivirus. The lentiviral vector used for downregulation was pSicoR-GFP. USP16 overexpression vector was obtained by subcloning a Usp16-IRES vector (American Type Culture Collection (ATCC)) into a pCDH-MSCV-GFP vector or an pCDH-EF1A-GFP vector (System Biosciences).

Human fibroblast. Human fibroblasts (wild type: CRL-2088, CRL-2076; Down’s syndrome: CCL-54, CRL-7090, CRL-7031) were purchased by ATCC. Other fibroblast cultures (Down’s syndrome: GM2508 and normal fibroblasts) were kindly provided by J. Weimann and S. Marro.

Human neurosphere cultures. Adult SVZ neurospheres were derived from a rapid autopsy. Paediatric SVZ neurospheres were derived during a functional maturation experiment. Human fibroblast cultures (Down’s syndrome: GM2508 and normal fibroblasts) were kindly provided by J. Weimann and S. Marro.

HiPSCs. Human embryonic stem cells and their derivatives were cultivated on Matrigel (BD Biosciences) until they reached an estimated 80% confluence.


tables and figures

Supplementary information

Acknowledgements

We thank S. Marro for help in establishing MEF and TFF lines; I.L. Weissman for sharing C57BL/6 CD45.1 mice; the animal core facility of SIM1, in particular A. Valledifera and M. Alvarez. This study was supported in part by the California Institute for Regenerative Medicine (CIRM) basic biology award III, National Institutes of Health (grant numbers CA100225 and CA54209, CIRM bridges fellowship (V.H.-A.), Fondazione Umberto Veronesi (B.N.d.R.), Breast Cancer Research Program sponsored by Department of Defense (S.O.). The Breast Cancer Research Foundation (BCRF), Stanford Graduate Fellowship (Y.O.) and the Down Syndrome Research and Treatment Foundation and the Fidelity Foundation (C.G.C.).

Author Contributions

M.A. and M.F.C. conceived the project and wrote the manuscript. M.A. identified the disease-related and control exons in this study. S.S.M. performed most of the breast experiments. S.S.M. performed most of the neural experiments. M.A., B.N.d.R., D.Q., A.K., V.H.-A., J.R. and Y.O. contributed to mouse colony handling, lentivirus production and mouse fibroblast experiments. M.Q. performed the imaging studies. V.M.R. provided the bone experiments. S.S. performed most of the breast experiments. C.C.G. and S.S.M. oversaw the neural progenitor experiments. M.A. identified the disease-related and control exons in this study. S.S.M. performed most of the neural experiments. M.A., B.N.d.R., D.Q., A.K., V.H.-A., J.R. and Y.O. contributed to mouse colony handling, lentivirus production and mouse fibroblast experiments. M.Q. performed the imaging studies. V.M.R. provided the bone experiments. S.S. performed most of the breast experiments.

Author Information

Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.F.C. (mclarke@stanford.edu).
Neurosphere assays. Wild-type, Ts65Dn, Ts65Dn/Usp16het and Usp16het mice were euthanized by CO₂, decapitated, and their brains immediately removed. The SVZ was micro-dissected and stored in ice-cold PBS for further processing. The tissue was digested using TrypLE Express (Invitrogen) and Dnase I (250 U ml⁻¹) at 37 °C for 10 min followed by trituration using a fire-polished pipette. Digested tissue was washed in ice-cold PBS without calcium and magnesium, filtered through a 40-µm filter and re-suspended in neurospheres growth media that is, Neurobasal-A (Invitrogen) and Dulbecco’s modified Eagle’s medium (DMEM) F/12(1:1) supplemented with 2% B27-A (Invitrogen), 1% N2 (Invitrogen), mouse recombinant epidermal growth factor (EGF; 20 ng ml⁻¹) and basic fibroblast growth factor (bFGF; 20 ng ml⁻¹) (Shenandoah Biotechnology) and 2 µg ml⁻¹ heparin (Sigma). Lineage cells were depleted using mouse CD45, CD31 and Ter119 microbeads (Miltenyi Biotec) and the negative fraction collected. For FACS analysis the cells were stained with anti-CD15–fluorescein isothiocyanate (FITC) (MMA; BD), anti-prominin-1–APC (eBiosciences) and biotinylated EGF complexed with PE-Cy7–streptavidin (2 µg ml⁻¹; Invitrogen).

For limiting dilution analysis, cells were directly plated in 96 wells in limiting dilution down to one cell per well. Each plating dose was done in 24 wells and the number of wells with neurospheres was counted after 10 days. For serial passaging, neurospheres from each passage were collected and re-plated either as 100 cells per well in a 48-well dish or in a similar limiting dilution fashion as described above.

Micro-dissected mouse SVZ was dissected as described above and FACS-sorted into Lin CD15⁺ Egrf⁻ CD24⁻ and Lin⁺ CD15⁻ Egrf⁺ CD24⁺, or Lin Prom1⁺ Egrf⁺ CD24⁺ and Lin Prom1⁻ Egrf⁻ CD24⁻ cells. In total, 10,000 cells were sorted for each population and concentrated by centrifugation. Cells were collected on a glass slide by cytospin and air dried before methanol fixation. Fixed cells were blocked using 5% normal goat serum and incubated in rabbit anti-SOX2 antibody (ab97959, Abcam) and mouse anti-nestin antibody (307187, Abcam), followed by secondary anti-mouse Alexa 594 and anti-rabbit Alexa 488, respectively. Fluorescence was visualized using standard epifluorescence microscopy. Six random fields were imaged and data represented as ratio of Sox2- or nestin-positive cells per DAPI-positive cells in the same field.

Mouse breast analyses. Mammary glands were dissected from either wild-type, Ts65Dn or Ts1Cje mice and analysed as described previously31. In brief, the glands were dissected in collagenase/hyaluronidase followed by ACK lysis, trypsin and DNAase/dispase. The cells were then stained with the following antibodies: CD49F APC, CD24 PE-Cy7, CD45 PacBlue, Ter119 PacBlue, CD31 PacBlue (Biolegend).

Cells were gated on the basis of forward- and side-scatter profiles, and live/dead discrimination was obtained with 7-amino-actinomycin D (7-AAD) or DAPI. Analyses and sorting was performed using a FACs Aria II (BD Biosciences). Data were also analysed with FlowJo software.

**In vitro mammary colony-forming assays.** 96-well ultra-low attachment plates (BD Biosciences) were prepared with a feeder layer of irradiated L-WNT3a mixed with 60 µl of growth factor reduced Matrigel (BD) per well. 1000 sorted MRUs from WT, Ts65Dn or Ts1Cje mice were then plated into liquid media as previously described32. 10% FBS and 2.5% growth factor reduced Matrigel were added as supplements.

Mammary transplants. Lin⁺ CD45⁺ CD31⁻ Ter119⁺ cell populations were isolated from 12-week-old mice in staining media and re-suspended in 10 µl of sterile PBS + 30% Matrigel per transplant before being injected into the cleared fat pads of 21–28-day-old recipient NOD/SCID mice as described previously33. All transplants were allowed to grow for at least 6 weeks but not more than 10 weeks before analysis. For knockdown of Usp16, Lin⁺ cells were infected with either control lentivirus or shRNA against Usp16 in DMEM/F12 plus 10% FBS overnight. The cells were then washed and re-suspended in sterile PBS plus 30% Matrigel for transplant.

For mammary transplant outgrowth area calculation, NIH Image J software was used. In brief, GFP-positive mammary ducts were measured with the freehand polygon by drawing a shape around the duct. Measurements were performed in a ‘blind’ fashion and at the same magnification for all samples. The entire fat pad was used to determine the maximum area coverage (100%). Only positive outgrowths were used in the measurement.

**Immunofluorescence of mammary tissue.** 12-week-old mice were euthanized and mammary glands were surgically removed. Glands were fixed in formalin overnight and then transferred to 70% ethanol. They were then embedded in paraffin and sectioned for histology. For staining the slides were de-paraffinized in xylene and alcohol grades. Antigen retrieval was carried out in Tris-EDTA buffer by heating in a microwave for 20 min. Primary antibodies CK14 A546 (Covance) and mouse anti-nestin antibody (Developmental Studies Hybridoma Bank, University of Iowa) were applied overnight. Secondary antibodies were anti-rat DyLight 488 and anti-rabbit DyLight 594 (both from Jackson Laboratories). Sections were then mounted using ProLong Antifade reagent (Invitrogen). Images were taken with a NIKON inverted

**METHODS**

**Statistical analyses.** In all the graphs, bars show average as central values and ± s.e.m. as error bars, unless otherwise specified. P-values were calculated using two-tailed t-test analyses. For limiting dilution analyses, ELDA software or L-Calc were used to test inequality between multiple groups. Expected frequencies are reported, as well as the 95% confidence intervals (lower and upper values are indicated). *P < 0.05, **P < 0.01, ***P < 0.001

**Bone marrow and peripheral blood analyses.** Isolation and analyses of bone marrow cells were performed as described previously30. In brief, bone marrow cells were isolated by crushing long bones and hips with mortar and pestle in calcium and magnesium-free Hank’s balanced salt solution (HBSS) with 2% heat-inactivated bovine serum. The cells were drawn by passing through a 25G needle several times, treated with ammonium chloride potassium (ACK) for 1 min and filtered with a 40-µm nylon mesh. Before sorting, progenitor cells were enriched through magnetic isolation with mouse lineage depletion kit (Miltenyi Biotec) using an autoMACS pro Separator. Markers used for identification of lineage cells were CD3, CD5, CD8, Gr-1, B20 and Ter119.

For peripheral blood analysis, red blood cells were lysed with hypotonic buffer, and nucleated cells were stained with antibodies against CD45.1, CD45.2, Ter119, Gr-1, Mac-1, CD3 and B20.

**Details of antibodies used for analyses and sorting of hematopoietic cells:** c-Kit APC780, CD45 (B20) Phycoerythrin (PE)-Cy5, CD45R (B20) PE (Becton), Ly5G (Gr-1) APC, CD8a PE-Cy5, CD3e Cy5, CD3e PE, CD135 (Flk) APC, CD34 FITC, CD45.1 PE, CD45.2 FITC (eBioscience); CD150 PE, CD48 APC, Sca1 PacBlue, Ter119 PacBlue, Ter119 PE-Cy5, CD5 PE-Cy5, CD11b (Mac-1) APC (Biolegend).

Cells were gated on the basis of forward- and side-scatter profiles, and live/dead discrimination was obtained with 7-amino-actinomycin D (7-AAD) or DAPI. Analyses and sorting was performed using a FACs Aria II (BD Biosciences). Data were also analysed with FlowJo software.

**Bone marrow transplants.** Recipient C57BL/6 CD45.1 mice (8–12 weeks old) were lethally irradiated (1,140 rad), with two doses of radiations delivered 3 h apart. Bone marrow single-cell suspensions were obtained from long bones and hips of Ts65Dn, Ts1Cje and wild-type mice (8–12 weeks old) and treated for 1 min with ACK lysis, trypsin and DNAase/dispase. The cells were then stained with the following antibodies: CD49F APC, CD24 PE-Cy7, CD45 PacBlue, Ter119 PacBlue, CD31 PacBlue (Biolegend).

Cells were gated on the basis of forward- and side-scatter profiles, and live/dead discrimination was obtained with DAPI. Analyses and sorting was performed using a FACs Aria II (BD Biosciences).

**In vitro mammary colony-forming assays.** 96-well ultra-low attachment plates (BD Biosciences) were prepared with a feeder layer of irradiated L-WNT3a mixed with 60 µl of growth factor reduced Matrigel (BD) per well. 1000 sorted MRUs from WT, Ts65Dn or Ts1Cje mice were then plated into liquid media as previously described32. 10% FBS and 2.5% growth factor reduced Matrigel were added as supplements.

**Mammary transplants.** Lin⁺ CD45⁺ CD31⁻ Ter119⁺ cell populations were isolated from 12-week-old mice in staining media and re-suspended in 10 µl of sterile PBS + 30% Matrigel per transplant before being injected into the cleared fat pads of 21–28-day-old recipient NOD/SCID mice as described previously33. All transplants were allowed to grow for at least 6 weeks but not more than 10 weeks before analysis. For knockdown of Usp16, Lin⁺ cells were infected with either control lentivirus or shRNA against Usp16 in DMEM/F12 plus 10% FBS overnight. The cells were then washed and re-suspended in sterile PBS plus 30% Matrigel for transplant.

For mammary transplant outgrowth area calculation, NIH Image J software was used. In brief, GFP-positive mammary ducts were measured with the freehand polygon by drawing a shape around the duct. Measurements were performed in a ‘blind’ fashion and at the same magnification for all samples. The entire fat pad was used to determine the maximum area coverage (100%). Only positive outgrowths were used in the measurement.

**Immunofluorescence of mammary tissue.** 12-week-old mice were euthanized and mammary glands were surgically removed. Glands were fixed in formalin overnight and then transferred to 70% ethanol. They were then embedded in paraffin and sectioned for histology. For staining the slides were de-paraffinized in xylene and alcohol grades. Antigen retrieval was carried out in Tris-EDTA buffer by heating in a microwave for 20 min. Primary antibodies CK14 A546 (Covance) and mouse anti-nestin antibody (Developmental Studies Hybridoma Bank, University of Iowa) were applied overnight. Secondary antibodies were anti-rat DyLight 488 and anti-rabbit DyLight 594 (both from Jackson Laboratories). Sections were then mounted using ProLong Antifade reagent (Invitrogen). Images were taken with a NIKON inverted
Western blot and ChIP. For western blot analyses, chromatin extracts were prepared with subcellular protein fractionation kit (Thermo Scientific). H2AK19 antibody (rabbit) was purchased from Cell Signaling; H2A antibody was purchased from Millipore.

ChIP was performed essentially as described previously37 using the polyclonal antibody for H2AK19 (Cell Signaling).

Lentivirus preparation. The lentiviral vector that we used for downregulation was vector pSicoR-GFP. We cloned the following hairpins: shC (5'-TTCTCCGAACGTTACGTCAT-3'), shUp16 no. 1 (5'-CGAGTGCTGTATCTACCGTTAT-3'), shUp16 no. 2 (5'-TTCTCCGGAATACCACTATG-3'), shp19 (5'-CATCAAGACATCGTGATAT-3'), sh19 (5'-GCCCATTAAACGTTACGTTT-3'), human shUp16 (5'-TATATACGTACCCGTTAT-3'). A lentivirus construct expressing mCherry and Bmi1 (pEIZ-HIV-mCherry-Bmi1) was kindly provided by Y. Shimono32. USP16 overexpression vector was obtained by subcloning USP16 clone (ATCC) in pCDH-MSCV-GFP vector or pCDH-EF1A-GFP vector (System Biosciences).

Viral stocks produced in 293T cells with a second-generation lentivirus system. Supernatants were collected at 48 h and 72 h, and concentrated through ultracentrifugation39. Viral titres were calculated by FACS analyses of 293T cells clone (ATCC) in pCDH-MSCV-GFP vector or pCDH-EF1A-GFP vector (System Biosciences).

Viruses were produced in 293T cells with a second-generation lentivirus system. Supernatants were collected at 48 h and 72 h, and concentrated through ultracentrifugation39. Viral titres were calculated by FACS analyses of 293T cells infected with serial dilution of concentrated virus.

MEFs, TTFS and human fibroblasts. MEFs were generated from E14.5 embryos obtained from Ts65Dn mothers. Genotype was verified by real-time PCR. Cells were passaged 1:4 when almost confluent. To culture mouse primary TTFs, the skin obtained from Ts65Dn mothers. Genotype was verified by real-time PCR. Cells infected with serial dilution of concentrated virus.

For SA-βgal and p16 staining. In total, 5 x 10⁴ fibroblasts were seeded in a 24-well plate and viable cells were counted by trypan blue exclusion at the indicated time points.

For SA-βgal staining of senescent cells, the Senescence Detection Kit (Abcam, ab65351) was used according to manufacturer’s protocol.

For p16 staining, fibroblasts were permeabilized with 0.2% Triton-PBS, blocked in 3% BSA-PBS and stained with a mouse anti-human p16 (C8, Santa Cruz Biotechnology) or rabbit anti-mouse p16. Specific secondary antibodies (Alexa Fluor 488 anti-mouse and Alexa Fluor 647 anti-rabbit, Invitrogen) were used (1:1,000).

SA-βGal and p16 staining were detected by microscope at ×10 and the positive cells were evaluated in three different fields per well. Three technical replicates were performed.

For real-time analyses, cells were collected in Trizol (Invitrogen) following manufacturer's directions. Samples were then mounted and imaged.

Imaging was performed using a Zeiss Observer Z1 fluorescent microscope (Zeiss) equipped with a Hamamatsu Orca-ER camera or a Zeiss confocal system LSM710 (Zeiss). Data acquisition and foci measurements were performed using Imaris software.

The primary antibody used in this study was anti ubiquityl-histone H2A D27C4 (Cell Signaling). Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen).

Human bone marrow samples. Down’s syndrome bone marrow samples were retrieved from sternal biopsies obtained from children undergoing corrective heart surgery at Stanford University. The families were consented according to IRB approved protocols. Control bone marrow samples were obtained from AllCells, LLC. All samples were processed and stained as described previously36.

For lentiviral infection, neurospheres were dissociated into single cells and 50,000 cells were infected with pCDH-EF1a-GFP-Luc or pCDH-EF1a-GFP-USP16 at a m.o.i. of 25 and followed for GFP expression. Live GFP+ cells were counted at a count of 100 cells per well in a 96-well plate and number of neurospheres was counted after 7 days in culture. For secondary sphere forming ability, neurospheres formed during the previous experiment were re-dissociated and again plated at 100 cells per well in a 96-well plate and number of spheres per well were counted. The experiment was repeated at two different passages.

RNA expression analyses. For real-time analyses, cells were collected in Trizol (Invitrogen), and RNA was extracted following the manufacturer’s protocol. Complementary DNA was obtained using Superscript III First Strand Synthesis (Invitrogen).

Real-time reactions were assembled using Taqman probes (Applied Biosystems) in accordance with the manufacturer’s directions. Expression data were normalized by the expression of housekeeping genes ActB and Gapdh. Probes used in this study: Up16 (mouse; Mm_00470393, Mm_00470406; human; Hs01062191_m1, Hs01062191_m1, p16Ink4a (Mm_01257348, Mm_00494449), p19Arf (Mm_00486943), Actb (Mm_00607939), Gapdh (Mm_99999915). Hoxa1 (Mm_00439359_m1). Hoxa3 (Mm_01326402_m1), Bmi1 (Hs00995191_g1), Sox2 (NM_011443, System Biosciences).

Immunofluorescence of histones in MEFS and HSCs. Cells were cultured for 48 h (for MEFS) or directly cytosponed on glass slides after purification (for HSCs). Cells were fixed in PFA 2% for 10 min and washed in Trition-PBS 0.1%. We performed a blocking incubation in PBS with donkey serum 10% for 1 h at room temperature (16–22°C) before incubation with primary antibody at 4°C overnight. Incubation with secondary antibody and DAPI was set for 45 min at room temperature. Alternatively, antibody staining was performed with Zenon kit (Invitrogen) following manufacturer’s directions. Samples were then mounted and imaged.

31. Reinholdt, L. G. et al. Molecular characterization of the translocation breakpoints in the Down syndrome mouse model Ts65Dn. Mamm. Genome 22, 685–691 (2011).

32. Hu, Y. & Smyth, G. K. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J. Immunol. Methods 347, 70–78 (2009).

33. Dalerba, P. et al. Single-cell dissection of transcriptional heterogeneity in human colon tumors. Nature Biotechnol. 29, 1120–1127 (2011).

34. Zeng, Y. A. & Nusse, R. Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell Stem Cell 6, 568–577 (2010).

35. Negishi, M. et al. A novel zinc finger protein Zfp277 mediates transcriptional repression of the myk4/airf locus through polycomb repressive complex 1. PloS ONE 5, e12373 (2010).

36. Ventura, A. et al. Cre-lox-regulated conditional RNA interference from transgenes. Proc. Natl Acad. Sci. USA 101, 10380–10385 (2004).

37. Shimono, Y. et al. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 138, 592–603 (2009).

38. Tiscornia, G., Singer, O. & Verma, I. M. Production and purification of lentiviral vectors. Nature Protocols 1, 241–245 (2006).

39. Majeti, R., Park, C. Y. & Weissman, I. L. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 1, 635–645 (2007).

©2013 Macmillan Publishers Limited. All rights reserved
Extended Data Figure 1 | Ts65Dn, but not Ts1Cje, bone marrow show a decreased number of functional HSCs. a, Impaired engraftment ability of Ts65Dn bone marrow (BM) cells in transplantation experiments. The percentage of donor cells (CD45.2^+^) was evaluated at the indicated time points (five mice per group, repeated twice). b, Representative fluorescence-activated cell sorting (FACS) plots of KLS cells show a reduced number of CD34^-^Flt3^-^ KLS cells in Ts65Dn marrows (four mice of each genotype were examined). c, Peripheral blood analyses four months after bone marrow transplant revealed multi-lineage engraftment in Ts65Dn mice with only 500,000 donor cells; lower doses of Ts65Dn donor bone marrow failed to reconstitute haematopoietic lineages. d, Multi-lineage analyses of peripheral blood 3 months after bone marrow transplantation showed that Ts65Dn bone marrow cells failed to reconstitute secondary recipients. Representative FACS plots are shown.
Extended Data Figure 2 | HSCs in Ts65Dn mice have lower levels of H2A ubiquitination. a, Immunofluorescence for H2A ubiquitination on Lys119 shows a decrease in the number of positive foci in MEFs derived from Ts65Dn embryos compared to controls. Each dot represents a different cell and each column a different mouse. One hundred cells per group were analysed and the experiment was repeated twice. b, H2AK119\(^+\) staining is decreased in Ts65Dn compared to control MEFs. c, Western blot analyses of chromatin extracts from MEFs. H2AK119\(^+\) levels are decreased in Ts65Dn (quantification performed using ImageJ software). H2A western blotting verifies equal loading of extracts.
Extended Data Figure 3 | Downregulation of Usp16 improves engraftment of Ts65Dn KLS cells in primary and secondary transplants. a, Usp16 mRNA quantification after infection of KLS cells with the indicated lentivirus. b, Peripheral blood analyses revealed multi-lineage engraftment from Ts65 KLS bone marrow cells infected with a shUsp16 hairpin. Representative FACS plots are shown. c, Two months after transplantation in secondary recipients, shC Ts65Dn bone marrow cells fail to engraft, whereas shUsp16 Ts65Dn cells show multi-lineage reconstitution. Representative FACS plots are shown.
Extended Data Figure 4 | Analyses of Nsp-IC frequency in neurospheres cultures. 

a, Usp16 mRNA quantification in murine neurospheres cultures (P4). 
b, c, Raw data used for ELDA analyses of Nsp-ICs derived from Lin^- SVZ cells or for the indicated sorted population. For each cell dilution, 24 replicates were tested. The table indicates the number of positive wells in each condition.
Extended Data Figure 5 | CD15⁺ Egfr⁺ and Prom1⁺ Egfr⁺ populations are enriched for neuronal progenitors in mice. a, Representative FACS plots are shown for viable Lin⁻ cells derived from SVZ preparations. Double-positive cells were sorted and used for testing neurosphere-formation potential. b, Representative pictures of immunofluorescence staining for Sox2 and nestin on the indicated sorted populations. The arrows indicate cells scored positive for Sox2 (green) or nestin (red). For this analysis, the indicated Lin⁻ cell populations were FACS sorted and collected by cytospin. On the right, twelve fields were randomly selected for analyses from four wild-type mice from different litters. The percentage of positive cells is given by the ratio of cells positive for Sox2 or nestin among the DAPI (4',6-diamidino-2-phenylindole)⁺ cells. c, Neurosphere expansion in vitro during passaging by different sorted populations derived from mouse SVZ. CD15⁺ Egfr⁺ cells are able to expand upon passaging.
Extended Data Figure 6 | Defects in mammary glands in Down’s syndrome mice models. a, b, mRNA quantification of Usp16 and different Hox genes in CD49<sup>high</sup>CD24<sup>med</sup> mammary cells. Hox1, Hox3 and Hox5 are expressed at higher levels in Ts65Dn cells. c, Representative FACS plot of mammary cells gated on live cells (first row) or live Lin<sup>−</sup> cells. We observed a perturbation in the overall FACS profile with reduction of basal and luminal cells (indicated gates) in Ts65Dn mice but not in Ts1Cje mice. These experiments were repeated five times for each group. d, Quantification of overlap between staining for the basal cytokeratin CK14 (red) and the luminal cytokeratin CK8 (green). Pearson’s correlation analyses (lumosity software) showed a marked increase in cells that co-stain for both cytokeratins in Ts65Dn mammary epithelium. Each experiment was repeated with three mice per group. e, Downregulation of Usp16 by shRNA lentiviral infection partially rescues the in vivo defects shown by Ts65Dn mammary cells (P = 0.03). Three independent transplantation experiments were performed. Right, the percentage of fat pad filled by GFP outgrowths is significantly higher upon downregulation of Usp16 (P = 0.007).
Extended Data Figure 7 | Senescence in Ts65Dn fibroblasts is affected by levels of Usp16 and Cdkn2a. a, Western blot analyses verifies knockdown of p16. β-actin was used as a loading control. b, Proliferation of Ts65Dn TTFs increased upon infection with a hairpin targeting Cdkn2a. Control TTFs proliferate more upon downregulation of both p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf}. The experiment was repeated three times with similar results. c, Representative pictures of p16\textsuperscript{Ink4a} immunostaining (left) and quantification of the percentage of positive cells (right). Each dot represents a TTF culture derived from a different mouse. The hairpin effectively ablates p16\textsuperscript{Ink4a} expression. d, SA-βgal staining in control and Ts65Dn MEFs at P4. Representative pictures are shown on the left. The percentages of positive cells are shown on the right. Experiments were replicated with three different MEF lines per genotype.
Extended Data Figure 8 | Human fibroblast cultures. a, b, mRNA quantification of USP16 upon lentiviral overexpression or downregulation. c, BMI1 overexpression significantly increases the proliferation of fibroblasts derived from a Down’s syndrome carrier. The effect on control fibroblasts is not significant. On the right, the levels of expression of BMI1 mRNA were quantified by real-time PCR. The experiment was repeated twice with similar results. d, Overexpression of USP16 reduces the formation of neurospheres derived from human paediatric SVZ cells. On the right, the quantification of the number of spheres obtained in the first and in the second passage. All the experiments were replicated twice.
Extended Data Figure 9 | HSCs in human bone marrow derived from Down's syndrome patients. Left, representative plot of Lin^− cells derived from human bone marrow. Right, a plot of the percentage of CD34^+ CD38^− CD90^+ observed between the Lin^− cells (Down’s syndrome: two samples; healthy donors: three samples). Down’s syndrome bone marrow samples were retrieved from sternal biopsies obtained from children undergoing corrective heart surgery at Stanford University. The families were consented according to IRB approved protocols. Control bone marrow samples were obtained from AllCells, LLC. Although we cannot unequivocally state that the HSCs are reduced in Down’s syndrome patients, in these children they are not increased as they might be in the liver of some human Down’s syndrome fetuses. Down’s syndrome is associated with increased rates of childhood leukaemia and decreased rates of some adult solid tumours. We can only speculate as to why Down’s syndrome predisposes individuals to one type of malignancy and protects from another, and whether USP16 has a role in either observation. Other syndromes causing bone marrow failure, such as Fanconi anaemia, predispose to leukaemia. Lymphoid leukaemias in Down’s syndrome patients frequently involve mutation of CDKN2A. Because CDKN2A appears to have a role in the proliferation defects caused by trisomy of USP16, mutations of CDKN2A could give Down’s syndrome HSCs a strong selection advantage. Differences between fetal liver and adult bone marrow HSCs could provide another explanation for an increased incidence of leukaemia in Down’s syndrome. An expansion of Down’s syndrome fetal liver HSCs but not of adult bone marrow HSCs could also explain why Down’s syndrome leukaemia appears to originate more often in fetal blood cells.