$\Delta 133p53$ represses p53-inducible senescence genes and enhances the generation of human induced pluripotent stem cells

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p53 functions to induce cellular senescence, which is incompatible with self-renewal of pluripotent stem cells such as induced pluripotent stem cells (iPSC) and embryonic stem cells (ESC). However, p53 also has essential roles in these cells through DNA damage repair for maintaining genomic integrity and high sensitivity to apoptosis for eliminating severely damaged cells. We hypothesized that $\Delta 133p53$, a physiological inhibitory p53 isoform, is involved in the balanced regulation of self-renewing capacity, DNA damage repair and apoptosis. We examined 12 lines of human iPSC and their original fibroblasts, as well as three ESC lines, for endogenous protein levels of $\Delta 133p53$ and full-length p53 (FL-p53), and mRNA levels of various p53 target genes. While FL-p53 levels in iPSC and ESC widely ranged from below to above those in the fibroblasts, all iPSC and ESC lines expressed elevated levels of $\Delta 133p53$. The p53-inducible genes that mediate cellular senescence (p21^{WAF1}, miR-34a, PAI-1 and IGFBP7), but not those for apoptosis (BAX and PUMA) and DNA damage repair (p53R2), were downregulated in iPSC and ESC. Consistent with these endogenous expression profiles, overexpression of $\Delta 133p53$ in human fibroblasts preferentially repressed the p53-inducible senescence mediators and significantly enhanced their reprogramming to iPSC. The iPSC lines derived from $\Delta 133p53$ -overexpressing fibroblasts formed well-differentiated, benign teratomas in immunodeficient mice and had fewer numbers of somatic mutations than an iPSC derived from p53-knocked-down fibroblasts, suggesting that $\Delta 133p53$ overexpression is non- or less oncogenic and mutagenic than total inhibition of p53 activities. Overexpressed $\Delta 133p53$ prevented FL-p53 from binding to the regulatory regions of p21^{WAF1} and miR-34a promoters, providing a mechanistic basis for its dominant-negative inhibition of a subset of p53 target genes. This study supports the hypothesis that upregulation of $\Delta 133p53$ is an endogenous mechanism that facilitates human somatic cells to become self-renewing pluripotent stem cells with maintained apoptotic and DNA repair activities. *Cell Death and Differentiation* (2017) 24, 1017–1028; doi:10.1038/cdd.2017.48; published online 31 March 2017

p53 regulates a variety of biological processes, including cellular senescence, apoptosis and DNA damage response.^{1–3} Cellular pluripotency and differentiation potential are critical to tissue homeostasis and regeneration and thus contribute to healthy lifespan in humans.⁴ p53 functions to regulate pluripotency and differentiation through the transcriptional regulation of its target genes.^{5,6} The ability of p53 to induce cellular senescence may be incompatible with the self-renewing potential of iPSC and ESC, since p53 and cellular senescence act as a barrier to iPSC reprogramming *in vitro* in a cell-autonomous manner.^{7–11} Although p16^{INK4A}/ARF-mediated cellular senescence promotes *in vivo* reprogramming through secretory cytokines, p53 still functions to limit reprogramming *in vivo* as well.¹² On the other hand, the activity of p53 in DNA damage response and repair plays an essential role in maintaining genomic stability and preventing malignant transformation in iPSC and ESC.^{13,14} High rates of apoptosis in human iPSC and ESC^{15,16} contributes to elimination of damaged cells and is also regulated by p53. It is important to identify a regulator of p53 that possibly

coordinates these different functions of p53 in human pluripotent stem cells.

The human *TP53* gene encodes N-terminally or C-terminally truncated isoforms, in addition to the full-length p53 protein.¹⁷ Among those natural p53 isoforms, an N-terminally truncated $\Delta 133p53$ (which lacks the N-terminal 132 amino acids) inhibits the activity of wild-type, full-length p53 (FL-p53).^{17–20} Unlike FL-p53 that is subject to proteasome-mediated degradation, $\Delta 133p53$ is degraded via chaperone-assisted selective autophagy,^{21,22} which leads to its downregulation during replicative cellular senescence in normal human fibroblasts, astrocytes and CD8⁺ T lymphocytes.^{18–20} The specific knockdown of $\Delta 133p53$, mimicking its senescence-associated downregulation, relieves FL-p53 from inhibition by this isoform and results in the induction of cellular senescence in these normal human cells.^{18–20} Conversely, the overexpression of $\Delta 133p53$ delays the onset of replicative cellular senescence and extends the replicative lifespan, while it does not lead to cellular immortalization or malignant transformation by itself.^{18,19} It should also be noted that

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$\Delta 133p53$ is likely to exist only in humans and primates, since any other organisms examined, including mice, do not have a methionine codon at the amino acid position corresponding to human codon 133 (ref. 20). These characteristics of $\Delta 133p53$ prompted us to hypothesize that the expression of this p53 isoform may play a unique role in human pluripotent stem cells. In this study we show expression, functional and genetic data supporting this hypothesis.

Results

Human pluripotent stem cells express abundant levels of endogenous $\Delta 133p53$ protein. We first investigated the expression levels of endogenous FL-p53 and $\Delta 133p53$ protein in human pluripotent stem cells. Twelve lines of human iPSC (named i14 through i25), a normal human fibroblast strain (CRL-2097) from which all these iPSC lines were derived, and 3 human ESC lines (WA01, WA07 and WA09) were examined in western blot analysis (Figure 1). The pluripotent status of these human iPSC and ESC lines was confirmed by the expression of Oct-4 (Figure 1) and Nanog (Supplementary Figure S1a). The expression levels of FL-p53 protein widely varied among these pluripotent stem cell lines, ranging from below (e.g., i17, i22, i23 and the three ESC lines) to above (e.g., i18, i21, i24 and i25) the level in CRL-2097 fibroblasts (Figure 1), which may be associated with two distinct p53-related states of human pluripotent stem cells.²³ In contrast, all the iPSC and ESC lines were revealed to express elevated levels of $\Delta 133p53$ protein, which were at least 10-fold higher than the level in CRL-2097 (Figure 1). The expression levels of $\Delta 133p53$ mRNA were 1.9–3.6-fold higher in iPSC and ESC than in CRL-2097, but to a lesser degree compared with the protein levels (Supplementary Figure S1b). Inhibition of autophagy by treatment with bafilomycin A1 in an iPSC line only slightly increased $\Delta 133p53$ protein, in contrast to marked stabilization of another autophagy substrate p62/SQSTM1 (ref. 22; Supplementary Figure S1c), suggesting that elevated expression of $\Delta 133p53$ in human pluripotent stem cells is attributed in part to decreased degradation of this selective autophagy substrate. When CRL-2097 transduced with the retroviral vector

of the Yamanaka factors (i.e., Oct-4, Sox-2, Klf-4 and c-Myc) was examined for $\Delta 133p53$ protein expression prior to the iPSC reprogramming protocol, these factors by themselves had only a minimal effect on $\Delta 133p53$ (Supplementary Figure S1d), suggesting that the upregulation of endogenous $\Delta 133p53$ occurs largely during the reprogramming process.

A subset, but not all, of p53-inducible genes are repressed in human pluripotent stem cells. To investigate the transcriptional activity of p53 in human pluripotent stem cells, we next examined the mRNA expression levels of p53-inducible genes of different functions in the above set of human iPSC, ESC and fibroblasts (Figure 2). Although each of the p53-inducible genes could be involved in multiple functions of p53, four of the genes examined (p21^{WAF1}, PAI-1, IGFBP7 and miR-34a) are reported to mediate p53-induced cellular senescence,^{1,24–27} while BAX and PUMA regulate apoptosis,^{1,28,29} and p53R2 is involved in nuclear and mitochondrial DNA repair and homeostasis.^{1,30,31} All of the four p53-inducible genes mediating cellular senescence were significantly repressed in all the iPSC and ESC lines compared with CRL-2097 fibroblasts (Figure 2a–d), although the repression of miR-34a in two iPSC (i14 and i18) was less clear than the others (Figure 2d). The mRNA expression levels of BAX (Figure 2e) and PUMA (Figure 2f) were higher in all iPSC and ESC but one (WA09), which expressed similar levels to CRL-2097. All the iPSC and ESC lines showed elevated levels of mRNA expression of p53R2 (Figure 2g) compared with CRL-2097. These results indicate that human pluripotent stem cells are associated with the repression of a subset of p53-inducible genes, which are involved in the induction of cellular senescence.

Overexpression of $\Delta 133p53$ in normal human fibroblasts preferentially represses p53-inducible genes involved in cellular senescence. We reproduced an iPSC/ESC-like upregulation of $\Delta 133p53$ in normal human fibroblasts (BJ strain) by retrovirally overexpressing $\Delta 133p53$ (Figure 3a). As a control, shRNA (short-hairpin RNA) -mediated knockdown of p53 was also performed (Figure 3a). We examined mRNA expression levels of the above seven p53-inducible genes in

Figure 1 Human pluripotent stem cells express elevated levels of $\Delta 133p53$. Western blot analysis was performed using protein lysates prepared from a normal human fibroblast strain CRL-2097, 12 iPSC lines derived from CRL-2097 (i14 through i25) and three human ESC lines (WA01, WA07 and WA09). Oct-4 was a pluripotency marker positive in all iPSC and ESC lines. Full-length p53 was detected using the DO-1 antibody and $\Delta 133p53$ using the MAP4 antibody.^{18–21} β -actin was a loading control for normalization in the quantitative image analysis. Results of three separate blots processed in parallel are shown, all of which contain WA09 and two of which (middle and right) contain CRL-2097 for cross-comparison. The expression levels of full-length p53 and $\Delta 133p53$ in iPSC and ESC lines are shown in relative to those in CRL-2097 (defined as 1.0)

Figure 2 A subset of p53-inducible genes are repressed in human pluripotent stem cells. qRT-PCR analyses of mRNA expression of p21^{WAF1} (a), PAI-1 (plasminogen activator inhibitor-1) (b), IGFBP7 (insulin-like growth factor binding protein 7) (c), miR-34a (microRNA-34a) (d), BAX (Bcl-2-associated X protein) (e), PUMA (p53 upregulated modulator of apoptosis) (f) and p53R2 (p53 inducible ribonucleotide reductase) (g) were performed in CRL-2097 human fibroblasts, their derived iPSC and human ESC (the same set of cells as in Figure 1; their names are below d and g). Expression data are means $\pm$ S.D. ($n = 3$) and shown in relative to CRL-2097 (defined as 1.0). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, as determined by unpaired two-tailed Student's t -test

these $\Delta 133p53$ -overexpressing and p53-knocked-down fibroblasts, along with their controls. All the seven p53-inducible genes were remarkably downregulated by p53 knockdown as expected (Figure 3b, left bars). The overexpression of $\Delta 133p53$ repressed p21^{WAF1}, PAI-1, IGFBP7 and miR-34a, but not BAX, PUMA and p53R2 (Figure 3b, right bars). When $\Delta 133p53$ was overexpressed in CRL-2097 fibroblasts (Supplementary Figure S2a), similar results were obtained: p21^{WAF1}, PAI-1, IGFBP7 and miR-34a were repressed, while the other three genes were either unchanged (PUMA) or modestly downregulated (BAX and p53R2) (Supplementary Figure S2b). These results suggest that $\Delta 133p53$ overexpression preferentially represses a subset of p53-inducible genes mediating cellular senescence in normal human fibroblasts, being coincident with the expression profiles in iPSC and ESC. To examine whether the three less responsive genes (BAX, PUMA and p53R2) are still activated by p53 in the presence of abundant levels of $\Delta 133p53$, we transfected a siRNA targeting the N-terminal region of p53

(therefore knocking down FL-p53 but not $\Delta 133p53$) to BJ and CRL-2097 fibroblasts with overexpressed $\Delta 133p53$ and to an iPSC line (Supplementary Figure S3a). We found that the expression of these three genes in these cells was largely or at least in part dependent on FL-p53 levels (Supplementary Figure S3b–d).

Overexpression of $\Delta 133p53$ enhances reprogramming from normal human fibroblasts to iPSC. Since $\Delta 133p53$ overexpression in normal human fibroblasts reproduced the repression of the p53-inducible senescence-mediating genes observed in human pluripotent stem cells (Figure 3 and Supplementary Figure S2), we examined whether it also enhances reprogramming from normal human fibroblasts to iPSC. BJ fibroblasts with and without overexpressed $\Delta 133p53$, along with those with p53 knockdown as a positive control (the same cells as in Figure 3), were used in an iPSC reprogramming protocol with retroviral transduction of three (Oct-4, Sox-2 and Klf-4; Figure 4a) or four Yamanaka factors

Figure 3 Overexpression of $\Delta 133p53$ in human fibroblasts represses a subset of p53-inducible genes. Normal human fibroblasts (BJ strain) were retrovirally transduced with an shRNA knockdown vector against p53 and its scrambled control (Scr), as well as with an overexpression vector of $\Delta 133p53$ and its empty vector control (Vec). (a) Western blot analysis confirming p53 knockdown and $\Delta 133p53$ overexpression. $\Delta 133p53$, full-length p53 (FL-p53) and β -actin were detected as in Figure 1. $\Delta 133p53$ overexpression also increased FL-p53 as in previous publications.⁴⁰ (b) mRNA expression of p53-inducible genes. The qRT-PCR analyses were performed as in Figure 2. The expression levels in p53-knocked-down and $\Delta 133p53$ -overexpressing cells are shown in relative to those in Scr and Vec controls (each defined as 1.0), respectively. Means $\pm$ S.D. are shown, $n = 3$. *** $P < 0.001$, **** $P < 0.0001$, as determined by unpaired two-tailed Student's t -test

(the former three plus c-Myc; Figure 4b).³² In both cases, significantly increased numbers of iPSC colonies were generated from the $\Delta 133p53$ -overexpressing fibroblasts compared with the vector control cells, although to a lesser degree than from p53-knocked-down cells (Figure 4a and b). We also used another pair of normal human fibroblasts, CRL-2097 with and without overexpressed $\Delta 133p53$ (the same cells as in Supplementary Figure S2), in a separate reprogramming protocol based on transfection of synthetic mRNA for the four Yamanaka factors,³³ which again showed that $\Delta 133p53$ overexpression increased the frequency of generation of iPSC colonies (Figure 4c and Supplementary Figure S4a). The mRNA transfection-based protocol was also carried out using BJ fibroblasts with inducible $\Delta 133p53$ expression (Supplementary Figure S4b), and the induction of $\Delta 133p53$ was shown to enhance iPSC reprogramming (Figure 4d).

iPSC lines generated from $\Delta 133p53$ -overexpressing fibroblasts form benign teratomas, have normal chromosomes and stable microsatellite repeats, and are similar to original fibroblasts or other iPSC lines in mitochondrial DNA copy number. We isolated and established iPSC lines that were generated from $\Delta 133p53$ -overexpressing, p53-knocked-down, control vector-transduced and untransduced fibroblasts (all cell lines established in this study are listed in Supplementary Table S1). In a teratoma formation assay, two iPSC lines from $\Delta 133p53$ -overexpressing CRL-2097 fibroblasts (Ci133-3 and Ci133-5), along with two iPSC lines from control vector-transduced CRL-2097 (CiV1 and CiV4) and one from untransduced CRL-2097 (CiC1), were subcutaneously injected into NOD/SCID mice. All of the five iPSC lines formed teratomas with no malignant histology and with all three germ layer-derived tissues (Supplementary Table S1 and Figure 4e). The teratomas from one $\Delta 133p53$

Figure 4 Overexpression of $\Delta 133p53$ enhances reprogramming from human fibroblasts to iPSC. (**a** and **b**) BJ human fibroblasts with overexpressed $\Delta 133p53$ and the control vector (Vec) (those examined in Figure 3) were used for generating iPSC by retrovirally expressing three reprogramming factors (Oct-4, Sox-2 and Klf-4) (**a**) or four reprogramming factors (the former three plus c-Myc) (**b**). BJ human fibroblasts with shRNA knockdown of p53 (also examined in Figure 3) were included as a positive control. The numbers of iPSC colonies generated from $\Delta 133p53$ -overexpressing and p53-knockdown cells are shown in relative to those from control (Vec defined as 1.0). Means $\pm$ S.D. are shown, $n=3$. (**c**) CRL-2097 human fibroblasts with overexpressed $\Delta 133p53$ and the control vector (Vec) (those examined in Supplementary Figure S2) were used for generating iPSC by transfection of synthetic mRNAs encoding the four reprogramming factors. The graphs show the numbers of iPSC colonies generated per 1×10^4 cells. Means $\pm$ S.D. are shown, $n=3$. A representative image is shown in Supplementary Figure S4a. (**d**) BJ human fibroblasts were transduced with a Tet repressor vector and then with a lentiviral vector for inducible expression of $\Delta 133p53$. The inducible expression by treatment with doxycycline was confirmed by western blot (as shown in Supplementary Figure S4b). The cells in the presence (+) or absence (–) of doxycycline (Doxy) were used in synthetic mRNA-mediated iPSC reprogramming as performed in **c**. Data are also presented as in **c**. Means $\pm$ S.D. are shown, $n=3$. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, as determined by unpaired two-tailed Student's *t*-test. (**e**) Teratoma formation assay showing *in vivo* differentiation of iPSC generated from $\Delta 133p53$ -overexpressing cells. Two iPSC lines generated from CRL-2097 fibroblasts overexpressing $\Delta 133p53$ (Ci133-3 and Ci133-5; isolated and expanded from the experiment shown in **c**) were subcutaneously injected into NOD/SCID mice. Teratomas were resected after 7 weeks (Ci133-3) or 21 weeks (Ci133-5), followed by formalin fixation and paraffin embedding, and then hematoxylin-eosin staining of tissue sections. Differentiated tissues derived from all three germ layers are shown

overexpression-derived iPSC (Ci133-5) were more maturely differentiated (Figure 4e, lower panels) than those from the other four lines for currently unknown reason.

Six iPSC lines generated from $\Delta 133p53$ -overexpressing fibroblasts (Bi133-1, Bi133-3, Ci133-2, Ci133-3, Ci133-4 and Ci133-5), two lines from control or untransduced fibroblasts (CiC1 and CiV2) and an iPSC line from p53-knockdown fibroblasts (Bi53KD) were examined by G-banding chromosome analysis, and all of them were found to have normal karyotype (46, XY) with no gross chromosomal aberrations (Supplementary Table S1 and Supplementary Figure S4c). When five highly variable microsatellite loci (ACTC, BAT26, D5S107, D5S406 and D13S153)³⁴ were examined, all iPSC lines had the identical allelic profiles to their original fibroblasts BJ or CRL-2097 at the all five loci (Supplementary Table

S2 and Supplementary Figure S5), suggesting that these microsatellite repeats were stably maintained and confirming that all the iPSC lines established in this study were indeed derived from their original fibroblasts. The copy numbers of mitochondrial DNA (mtDNA) were different from cell line to cell line, ranging from 0.9- to 3.3-fold of the original fibroblasts, and there was not a consistent change associated with iPSC lines from $\Delta 133p53$ -overexpressing fibroblasts (Supplementary Figure S6). A mtDNA D-loop region, which was previously reported to contain homoplasmic and heteroplasmic mutations in iPSC,³⁵ was sequenced and there was no mutation in any of the iPSC lines established in this study, although the BJ- and CRL-2097-specific polymorphisms again confirmed the origin of each iPSC line (Supplementary Table S2).

iPSC lines generated from $\Delta 133p53$ -overexpressing fibroblasts, as well as control iPSC lines, have lower rates of somatic mutations than an iPSC line generated from p53-knocked-down fibroblasts. For precise and genome-wide examination of genetic changes in iPSC, we performed whole exome sequencing (Supplementary Table S3) and analyzed the data for somatic changes between each iPSC line examined and its original fibroblasts. As predicted from the total inhibition of p53 activities, an iPSC from p53-knocked-down fibroblasts (Bi53KD) had the highest rate of somatic mutations (5.1 mutations per Mb), including synonymous and nonsynonymous single-nucleotide variants (SNV) and small insertions and deletions (InDel) (Figure 5a). Four iPSC lines from $\Delta 133p53$ -overexpressing fibroblasts had 1.2 (Bi133-1), 0.5 (Bi133-3), 0.8 (Ci133-2) and 0.2 (Ci133-3) somatic mutations per Mb, while the mutation rates in four control iPSC lines were 0.4 (BiC1), 1.8 [Bi133(-)], 0.1 (CiV2) and 0.9 (CiV4), indicating that these two groups of iPSC lines have similar rates of somatic mutations (Figure 5a). When somatic mutations affecting amino acid sequences in protein-coding genes (i.e., missense, premature stop and frameshift mutations) were compared, Bi53KD again had the most mutations (Figure 5b and Supplementary Table S4). Although the numbers of such coding mutations in the other iPSC lines widely varied from 4 to 20, there was no association with whether or not they were generated from $\Delta 133p53$ -overexpressing fibroblasts (Figure 5b). These results using a mutant allele frequency cutoff of 0.30 are roughly consistent with the previous studies that reported an average of 6 or 12 somatic coding mutations in homozygous or heterozygous state in iPSC lines.^{36,37} Out of 116 genes we identified as carrying somatic coding mutations, 114 genes were unique to a single iPSC line (Supplementary Table S4), strongly supporting that each iPSC line is an independent clone.

iPSC lines generated from $\Delta 133p53$ -overexpressing fibroblasts and control iPSC lines both express similar levels of $\Delta 133p53$ protein and p53 target genes. As above, although $\Delta 133p53$ overexpression enhances iPSC generation, the iPSC lines we established have similar biological and genetic characteristics whether or not their original fibroblasts had $\Delta 133p53$ overexpression. We thus examined whether they are also similar in expression of $\Delta 133p53$ protein and p53 target genes. Both $\Delta 133p53$ overexpression-enhanced (Ci133-1, Ci133-2, Ci133-3 and Ci133-4) and control iPSC lines (CiV1, CiV2 and CiV4) were found to express similar ranges of $\Delta 133p53$ protein, whether exogenous or endogenous (Supplementary Figure S7a), which are also similar to those in previously established iPSC and ESC lines (Figure 1). The expression levels of the p53 target genes in these iPSC lines (Supplementary Figure S7b–h) also reproduced the results in previously established iPSC and ESC lines (Figure 2) and there was no consistent difference between $\Delta 133p53$ overexpression-enhanced and control iPSC lines in any of the genes examined. These results suggest that both exogenous overexpression and endogenous upregulation of $\Delta 133p53$ similarly contribute to iPSC reprogramming, although at an increased efficiency with the former.

Figure 5 Summary of whole-exome sequencing data in iPSC lines. (a) Numbers of somatic mutations per megabase (Mb), including synonymous and nonsynonymous single-nucleotide changes (SNV) and small insertions and deletions (InDel). The iPSC lines derived from $\Delta 133p53$ -overexpressing fibroblasts ($\Delta 133p53$) and those from fibroblasts without $\Delta 133p53$ overexpression (control) are indicated. (b) Numbers of protein-coding genes that have a missense, premature stop or frameshift mutation with mutant allele frequency of 0.30 or greater. The genes mutated in each iPSC line are listed in Supplementary Table S4

Overexpressed $\Delta 133p53$ displaces full-length p53 from the promoter regions of the p53-inducible genes. Consistent with the retention of the C-terminal oligomerization domain (Figure 6a), $\Delta 133p53$ was shown to physically interact with FL-p53 (Figure 6b) as previously reported.²¹ To examine how $\Delta 133p53$ affects the transactivation function of FL-p53 and to gain mechanistic insight into its role in enhancing iPSC reprogramming, we performed a chromatin immunoprecipitation (ChIP) assay for binding of FL-p53 to the p53 response elements within the regulatory regions of the *miR-34a*, *p21^{WAF1}*, *BAX* and *PUMA* genes (Figure 6a). Since the DO-1 antibody used in this assay recognizes the N-terminal amino acid residues 20–25 of FL-p53 (Figure 6a), any N-terminally truncated p53 isoforms (including $\Delta 133p53$) by themselves could not be immunoprecipitated. Moreover, the major pattern of C-terminal alternative mRNA splicing in proliferating human fibroblasts was found to be α -splicing encoding FL-p53, with no or minor fractions of β - and γ -splicing patterns leading to C-terminal truncations.^{18,38} Thus, this ChIP assay was designed to exclusively or at least mainly detect DNA binding complexes involving FL-p53. We also confirmed that overexpressed $\Delta 133p53$ did not interfere with the ability of DO-1 to immunoprecipitate FL-p53 (Figure 6b). While no binding to the negative control region (*HBB*) was observed in immortalized human fibroblasts with or without $\Delta 133p53$ overexpression, the *miR-34a*, *p21^{WAF1}*, *BAX* and *PUMA* promoter regions showed significant binding

Figure 6 Overexpressed $\Delta 133p53$ displaces full-length p53 from the p53-inducible gene promoters. (a) Chromatin immunoprecipitation (ChIP)-qPCR assay for p53 binding to the regulatory regions of the *miR-34a*, *p21^{WAF1}*, *BAX* and *PUMA* genes. Domain structures of full-length p53 (FL-p53) and $\Delta 133p53$ are shown (TAD, transactivation domain; Pro, proline-rich region; DBD, DNA-binding domain; NLS, nuclear localization signal; and OD, oligomerization domain). Since the anti-p53 antibody used, DO-1, recognizes the N-terminal amino acids 20–25, this assay detects DNA binding of protein complexes containing FL-p53, but not that of any N-terminally truncated p53 isoforms by themselves, including $\Delta 133p53$. Also shown are the positions and DNA sequences of p53 response elements (p53RE) that transcriptionally activate *miR-34a*, *p21^{WAF1}*, *BAX* and *PUMA*. Upper and lowercase letters show matched and mismatched bases, respectively, to the canonical p53RE sequence (RRRCWWGYYY), with numbers of intervening bases in the parentheses. Arrows indicate the transcription start sites. Paired arrowheads indicate the regions amplified by qPCR. (b) Overexpression of $\Delta 133p53$ does not affect the efficiency of immunoprecipitation by the DO-1 antibody. Protein samples from immortalized BJ fibroblasts overexpressing $\Delta 133p53$ or with control vector (Vec), either after immunoprecipitation (IP) with DO-1 or directly (No IP), were used in western blot (IB) with PAb421, an antibody recognizing the C-terminus of p53, and with anti-GAPDH antibody (a loading control and a negative control for IP with DO-1). FL-p53 was immunoprecipitated by DO-1 at similar efficiencies in both cells. The interaction between FL-p53 and $\Delta 133p53$ was also detected. (c) Quantitative data of FL-p53 binding to the p53RE. Crosslinked protein-DNA complexes from immortalized BJ fibroblasts overexpressing $\Delta 133p53$ or with control vector (Vec) were analyzed by ChIP-qPCR as outlined in (a). *HBB* (hemoglobin subunit β) was used as a negative control. Normalized binding values are shown as % input. The relative binding in the presence of overexpressed $\Delta 133p53$ compared with its absence is shown in the parenthesis for each gene. Means $\pm$ S.D. are shown, $n = 3$. $**P < 0.01$, as determined by unpaired two-tailed Student's *t*-test

in the absence of overexpressed $\Delta 133p53$ (Figure 6c, Vec). The overexpression of $\Delta 133p53$ significantly reduced the FL-p53 binding to these four promoter regions (Figure 6c, $\Delta 133$), suggesting that $\Delta 133p53$ is able to displace FL-p53 from the p53 response elements through its hetero-oligomerization with FL-p53. The residual fraction of FL-p53 binding in the presence of overexpressed $\Delta 133p53$ to the *BAX* and *PUMA* promoters (approximately 20% of the binding in its absence) was larger than that to the *miR-34a* and *p21^{WAF1}* promoters (9% and undetectable, respectively). It is currently unknown whether the residual binding of FL-p53 is due to remaining

FL-p53 homooligomers or newly formed heterooligomers of $\Delta 133p53$ and FL-p53 in a promoter context-specific manner.

Discussion

This study for the first time establishes the role of $\Delta 133p53$ in human pluripotent stem cells by showing that: (i) endogenous $\Delta 133p53$ is upregulated in human iPSC and ESC lines (Figure 1); (ii) a subset of p53-inducible genes that mediate cellular senescence are downregulated in human pluripotent stem cells (Figure 2); (iii) the same subset of p53-inducible

genes are repressed by $\Delta 133p53$ overexpression in human fibroblasts (Figure 3); and (iv) $\Delta 133p53$ enhances reprogramming from human fibroblasts to iPSC (Figure 4). Our whole-exome sequencing data suggest that $\Delta 133p53$ overexpression, unlike p53 knockdown, does not increase mutation rates during iPSC reprogramming (Figure 5). This study also provides a mechanistic insight into the dominant-negative inhibition of p53-inducible senescence genes by $\Delta 133p53$ (Figures 6 and 7). Our findings represent significant progress in understanding the p53 isoform-mediated regulation of the p53 signalling network in human development and physiology, although our $\Delta 133p53$ knockdown method established for normal fibroblasts, CD8⁺ T lymphocytes and astrocytes^{18–20} failed to successfully knock down the abundant levels of endogenous $\Delta 133p53$ in iPSC and ESC (data not shown).

We previously showed that downregulation of $\Delta 133p53$ during replicative senescence in normal human cells occurs through protein degradation via chaperone-assisted selective autophagy,²¹ while others reported that upregulation of $\Delta 133p53$ in some cancer cell lines and *Helicobacter pylori*-infected cells may involve transcriptional activation.^{39,40} Our findings (Figure 1 and Supplementary Figures S1b, c and d) suggest that both reduced autophagic degradation and increased mRNA expression during reprogramming events contribute to the elevated levels of $\Delta 133p53$ in human pluripotent stem cells, although the exact regulators of these processes remain to be identified.

The ability of $\Delta 133p53$ to repress the p53-inducible senescence-mediating genes (Figure 3 and Supplementary Figure S2) and to enhance iPSC reprogramming (Figure 4) is well consistent with the upregulation of endogenous $\Delta 133p53$ (Figure 1) and the repression of the same subset of genes in human pluripotent stem cells (Figure 2). Our data are also consistent with the previous reports that p21^{WAF1} and miR-34a function to restrict reprogramming to iPSC.^{7,41–43} This study suggests that overexpressed $\Delta 133p53$, and likely endogenously upregulated $\Delta 133p53$ as well, dominant-negatively inhibits the transactivation function of FL-p53 on the p53-inducible senescence genes (such as *miR-34a* and *p21^{WAF1}*), leading to the suppression of p53-mediated cellular senescence, to induce and maintain the self-renewing potential in human pluripotent stem cells (Figure 7). Our findings that $\Delta 133p53$ did not, or did to a lesser degree, affect the expression of BAX, PUMA and p53R2 (Figure 3 and Supplementary Figure S2) are reminiscent of a previous report by Rasmussen *et al.*⁴³ that transient suppression of p53 enhances iPSC reprogramming without affecting apoptosis and DNA damage response. The maintained or elevated expression of these genes in human iPSC and ESC (Figure 2) likely contributes to high sensitivity to apoptosis for eliminating damaged cells¹⁶ and maintenance of genomic stability⁴⁴ in these human pluripotent stem cells.

There are a number of precedents for the transcriptional regulation of a select subset of p53 target genes, including those inducing either apoptosis or cell cycle arrest and senescence.^{45–47} In this study, the preferential repression of p21^{WAF1} and miR-34a, but not BAX and PUMA, by $\Delta 133p53$ may not fully be explained by the FL-p53 binding to their promoter regions in the ChIP-qPCR assay. However, since the expression of BAX and PUMA in the presence of

Figure 7 Schematic representation of the mechanism by which endogenously upregulated or exogenously overexpressed $\Delta 133p53$ contributes to the generation and maintenance of human pluripotent stem cells. $\Delta 133p53$ was previously shown to physically interact with FL-p53 (ref. 21), although the exact stoichiometry of the interaction is still unknown (a heterotetramer consisting of two each is shown in this scheme)

overexpressed $\Delta 133p53$ was still dependent on FL-p53 (Supplementary Figure S3) and the BAX and PUMA promoters showed the more fractions of residual binding of FL-p53 in the presence of overexpressed $\Delta 133p53$ (Figure 6c), we speculate that $\Delta 133p53$ /FL-p53 heterooligomers may bind to and activate these genes in a promoter context-dependent manner in human fibroblasts. Since $\Delta 133p53$ loses one amino acid making direct contact with DNA (Lys120) but retains all the other responsible amino acids,⁴⁸ it is likely that such heterooligomers can still function as a DNA-binding transcriptional regulator but in a more context-dependent manner and/or at a more limited set of genes. Our future studies will be aimed at determining the DNA binding profiles of $\Delta 133p53$ at a larger and genome-wide scale.

The tumorigenicity of iPSC is a serious safety concern^{14,49} and iPSC from p53-deficient or knocked-down cells were reported to become malignant and form teratocarcinoma *in vivo*.^{8,11} Our iPSC lines from $\Delta 133p53$ -overexpressing fibroblasts, as well as control iPSC lines from fibroblasts without $\Delta 133p53$ overexpression, formed well-differentiated, benign teratomas with no malignant pathology (Figure 4e and Supplementary Table S1), rather than teratocarcinoma, indicating that $\Delta 133p53$ does not principally cooperate with the Yamanaka reprogramming factors towards malignant transformation. The whole exome sequencing analysis also revealed that the iPSC lines from $\Delta 133p53$ -overexpressing fibroblasts, similar to control iPSC lines, showed lower somatic mutation rates and had fewer numbers of protein-coding mutations than an iPSC line from p53-knocked-down fibroblasts (Figure 5 and Supplementary Table S4). In addition, $\Delta 133p53$ overexpression-induced iPSC lines and control iPSC lines were found to be similar in eventual levels of $\Delta 133p53$ protein, p53 target gene expression and genomic stability markers (Supplementary Figures S5, S6 and S7 and Supplementary Table S2). Our data suggest that the upregulation of $\Delta 133p53$ facilitates normal human somatic cells to be reprogrammed to a pluripotent stem cell state, and that overexpression of $\Delta 133p53$, unlike knockdown of p53, could be a non- or less oncogenic and mutagenic method to enhance the reprogramming process.

While this study highlights the role of $\Delta 133p53$ in human pluripotent stem cells through the regulation of p53-mediated cellular senescence, a more efficient iPSC reprogramming by

p53 knockdown than by Δ 133p53 overexpression (Figure 4a and b) may suggest other p53-regulated processes (e.g., transient induction of apoptosis during reprogramming^{9,42} and regulation of energy metabolism^{1,50}) as another barrier to reprogramming. Since this study and our previous publications^{18–21} place Δ 133p53 at the crossroads of the regulation of autophagy, cellular senescence and pluripotent stem cells, it is interesting to investigate whether Δ 133p53 also contributes to the activation of autophagy, which is associated with self-renewal of various types of stem cells and is required for efficient iPSC reprogramming.⁵¹ Our initial experiment to address this possibility showed that overexpression of Δ 133p53 upregulates autophagy-mediating factors composing the core autophagy machinery, such as Beclin-1, ATG7 and ATG5-ATG12 conjugate,²² not only in normal human fibroblasts (Supplementary Figure S8a) but also in a p53-null fibroblast cell line (Supplementary Figure S8b). Further studies will elucidate whether Δ 133p53-induced upregulation of these factors plays a complementary role in enhancing iPSC reprogramming via autophagy activation. These autophagy-mediating factors are also to be examined as possible targets of the trans-activation function of Δ 133p53 in a manner independent of FL-p53 (ref. 52).

Materials and Methods

Cells and cell culture. Normal human fibroblast strains derived from newborn foreskin (CRL-2097 and BJ) and fetal lung (MRC-5) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Immortalized BJ fibroblasts were generated by retroviral transduction of human telomerase reverse transcriptase (hTERT).⁵³ MDAH041 $-/-$, a p53-null fibroblast cell line with a homozygous frameshift mutation at codon 184 (refs 18,21), was provided by M. Tainsky (Case Western Reserve University). These fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Human iPSC lines (i14 through i25) were derived from CRL-2097 by lentiviral transduction of the four Yamanaka factors at NIH Stem Cell Unit (<http://stemcells.nih.gov>).⁵⁴ Human ESC lines (WA01, WA07 and WA09) were also obtained from NIH Stem Cell Unit. These previously established iPSC and ESC lines were maintained feeder-free in mTeSR1 medium (STEMCELL Technologies, Vancouver, BC, Canada) on cell culture wells and plates coated with BD Matrigel hESC-qualified Matrix (BD Biosciences, San Jose, CA, USA). Harvesting of cells was carried out using ReLeSR (STEMCELL Technologies). Newly generated iPSC clones were initially expanded on mitomycin-C-treated SNL feeder cells (Cell Biolabs, San Diego, CA, USA) in Primate ES Cell Culture Medium supplemented with 4 ng/ml bFGF (ReproCELL, Yokohama, Japan) or in DMEM/F12 medium containing KnockOut Serum Replacement (KSR, 20%; Thermo Fisher Scientific, Waltham, MA, USA), L-glutamine (2 mM), non-essential amino acids (1×10^{-4} M), 2-mercaptoethanol (1×10^{-4} M) and bFGF (4 ng/ml),³² and then transferred to feeder-free culture as above. Treatment of iPSC with bafilomycin A1 (Sigma-Aldrich, St. Louis, MO, USA) was at 100 nM for 4 h.

Protein lysate preparation and western blot analysis. Cells were lysed in 10 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.1% SDS/1% NP-40/1 mM EDTA containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein lysates (20–40 μ g per sample) were separated on 10% or 4–20% gradient SDS-polyacrylamide gels (Thermo Fisher Scientific) and transferred to Hybond-P PVDF membranes (GE HealthCare Life Sciences, Pittsburgh, PA, USA). Incubations with primary and secondary antibodies were carried out in 10 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.05% Tween 20 containing 5% non-fat dry milk (LabScientific, Highlands, NJ, USA) or SuperBlock (Thermo Fisher Scientific). Signal detection was performed using ECL western blotting detection system (GE HealthCare Life Sciences) or SuperSignal West Dura chemiluminescence substrate (Thermo Fisher Scientific). Quantitative image analysis was performed using the ImageJ 1.40 g software (<http://rsb.info.nih.gov/ij/>). The anti- Δ 133p53 antibody (MAP4, rabbit polyclonal; 1:5000) was used by Fujita et al.,¹⁸ Mondal et al.,¹⁹

Horikawa et al.²¹ and Turnquist et al.,²⁰ and its specificity to Δ 133p53 has been established in these previous publications. Commercially available primary antibodies used were as follows: anti-Oct-4 (sc-5279, Santa Cruz Biotechnology, Dallas, TX, USA; 1:1000); anti-p53 (DO-1, Santa Cruz Biotechnology; 1:2000); anti-p53 (PAb421, EMD Millipore, Billerica, MA, USA; 1:1000); anti-p62/SQSTM1 (PM045, MBL International, Woburn, MA, USA; 1:5000); anti-LC3B (#2775, Cell Signaling Technology, Danvers, MA, USA; 1:2000); anti-Becn1-1 (#3788, Cell Signaling Technology; 1:2000); anti-ATG7 (PM039, MBL International; 1:5000); anti-ATG5 (#2630, Cell Signaling Technology; 1:5000); anti-GAPDH (EMD Millipore; 1:5000) and anti- β -actin (AC-15, Sigma-Aldrich; 1:10 000).

RNA isolation and qRT-PCR. Total RNA samples were prepared using TRIzol (Thermo Fisher Scientific). Reverse transcription was carried out using SuperScript III 1st strand cDNA synthesis system (Thermo Fisher Scientific). Quantitative real-time RT-PCR (qRT-PCR) assays were performed using Taqman Universal PCR Master Mix (Thermo Fisher Scientific) and the following primers/probe sets (all from Thermo Fisher Scientific): p21^{WAF1} (CDKN1A, Hs99999142_m1); PAI-1 (SERPINE1, Hs01126606_m1); IGF1BP7 (Hs00944483_m1); BAX (Hs00180269_m1); PUMA (BBC3, Hs00248075_m1); p53R2 (RRM2B, Hs00968432_m1); and Nanog (Hs02387400_g1). GAPDH (Hs03929097_g1) was used as an internal control. For miR-34a, reverse transcription was performed using Taqman MicroRNA Reverse Transcription Kit and a miR-34a-specific primer, followed by quantitative real-time PCR reaction using Taqman MicroRNA Assay for miR-34a. RNU66 was used as a control for miR-34a (all reagents were from Thermo Fisher Scientific). Quantitative data analysis was performed using the $\Delta\Delta$ Ct method according to the supplier's protocol (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). All qRT-PCR data were means $\pm$ S.D. from technical triplicate ($n=3$). The qRT-PCR experiments for the effect of Δ 133p53 on p53-inducible genes were replicated in two independent fibroblast strains BJ (Figure 3) and CRL-2097 (Supplementary Figure S2). The conventional RT-PCR analysis shown in Supplementary Figure S1b used the following primers: 5'-TGG GTT GCA GGA GGT GCT TAC-3' and 5'-CCA CTC GGA TAA GAT GCT GAG G-3' for Δ 133p53 (32 cycles); and 5'-CCA TCT TCC AGG AGC GAG A-3' and 5'-TGT CAT ACC AGG AAA TGA GC-3' for GAPDH (17 cycles). Quantitative image analysis was performed using the ImageJ 1.40 g software (<http://rsb.info.nih.gov/ij/>).

Vectors for Δ 133p53 overexpression and p53 knockdown and their transduction. A retroviral shRNA construct for p53 knockdown, which targets amino acid positions 259–264, was derived from pSUPER.retro.puro (OligoEngine, Seattle, WA, USA).¹⁸ A control construct containing the scrambled sequence was also generated from this vector. A retroviral construct for Δ 133p53 overexpression was generated by inserting the Δ 133p53 cDNA into pQCXIN vector (BD Biosciences).¹⁸ The Δ 133p53 cDNA was also cloned into pLenti6.3/TO/V5-DEST vector (Thermo Fisher Scientific) by using Spe I and Mlu I sites. This lentiviral Δ 133p53 expression vector was used for either constitutive overexpression by itself (in Figures 4c and 6 and Supplementary Figure S2) or inducible overexpression with a Tet repressor vector pLenti3.3/TR (Thermo Fisher Scientific) (in Figure 4d and Supplementary Figure S4b). These retroviral or lentiviral vectors were transiently transfected into 293T/17 cells (ATCC) with the ViraPort retroviral expression system (Agilent Technologies, Santa Clara, CA, USA) or the ViraPower lentiviral expression system (Thermo Fisher Scientific), respectively. Vector supernatants were collected 48 h after transfection and then used to transduce human fibroblasts for 16–20 h. Two days after transduction, the cells were selected with puromycin (2 μ g/ml; Sigma-Aldrich), G418 (500 μ g/ml; Sigma-Aldrich) or blasticidin (5 μ g/ml; Thermo Fisher Scientific). Knockdown of full-length p53 without affecting Δ 133p53 was performed by Lipofectamine RNAiMAX (Thermo Fisher Scientific)-mediated transfection of a siRNA oligonucleotide targeting amino acid positions 49–54 (Thermo Fisher Scientific, ID# 115182), along with a negative control oligonucleotide (Thermo Fisher Scientific, cat# AM4611).

iPSC reprogramming via retroviral vector transduction. Retroviral vector-mediated iPSC reprogramming was carried out as previously described.³² Briefly, vector supernatants were prepared from four retroviral pMXs constructs driving each of the Yamanaka reprogramming factors (i.e., Oct-4, Sox-2, Klf-4 and c-Myc),⁵⁵ as described above. Human fibroblasts, which had been retrovirally or lentivirally transduced and selected with appropriate antibiotics as above, were plated in six-well plates (2×10^5 cells per well), and then infected with either all of the four vector supernatants or three of them (Oct-4, Sox-2 and Klf-4) every 12 h for 2 days. Three days after the completion of vector infection, the cells were harvested

and replated on SNL feeder cells, followed by medium replacement with the above DMEM/F12–20% KSR medium. Culture was maintained for 3–4 weeks in the absence of drug selection with daily medium changes. After several well-separated iPSC colonies were picked up for expansion, all the plates were stained for alkaline phosphatase (AP) using AP Staining Kit II (Stemgent, Lexington, MA, USA). For quantitation of iPSC generation efficiency, each reprogramming transduction had a parallel transduction containing the same set of reprogramming vectors plus a GFP-expressing vector (pBabe-Puro-GFP).³² The transduction efficiency was measured by flow cytometry detection of GFP and used to normalize data from the total number of AP staining-positive colonies. Data were means $\pm$ S.D. from biological triplicate ($n=3$; three independent vector transductions of the Yamanaka reprogramming factors).

iPSC reprogramming via synthetic mRNA transfection. Reprogramming by means of transfection of synthetic modified mRNAs³³ was carried out using the mRNA Reprogramming Factors Set (Stemgent) according to the supplier's protocol (http://assets.stemgent.com/files/1310/original/Stemgent_mRNAUserManual_2012.pdf). Human fibroblasts were plated on NuFF-1 MITC feeder cells (GlobalStem, Rockville, MD) in 6-well plates at 2.5×10^4 cells per well (for picking up colonies) or at 5×10^4 cells per well (for AP staining). Starting from the following day, mRNA transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) daily for 18 consecutive days, the first 6 days of which used fresh Pluriton mRNA Reprogramming Medium (Stemgent) and the remaining 12 days with the same medium pre-incubated with NuFF-1 MITC feeder. Each mRNA transfection consisted of recommended amounts of mRNAs encoding the four Yamanaka reprogramming factors (Oct-4, Sox-2, Klf-4 and c-Myc) and an mRNA encoding nuclear GFP, which allowed the confirmation of similar transfection efficiencies between control and Δ133p53-overexpressing cells. After 3–5 more days of daily medium changes with NuFF-1 MITC-conditioned Pluriton medium, visible colonies were counted on AP-stained and unstained wells. Data were means $\pm$ S.D. from biological triplicate ($n=3$; three independent transfections of the synthetic mRNA cocktail). After colony counting, well-separated colonies were isolated from unstained wells for expansion.

Teratoma formation assay. Immunodeficient NOD/SCID mice were purchased from Charles River Laboratory (Frederick, MD, USA). iPSC in culture were treated with Y-27632 (Sigma-Aldrich) at $10 \mu\text{M}$ for 3 h and harvested into Primate ES Cell Culture Medium (ReproCELL) with $10 \mu\text{M}$ of Y-27632. The iPSC colonies were broken into small clumps by pipetting up and down several times. Per injection site, approximately 4×10^6 cells were suspended in $200 \mu\text{l}$ of the above medium containing 50% Matrigel (BD Biosciences),⁵⁶ followed by subcutaneous injection into the dorsal flank of two NOD/SCID mice per iPSC line. When cystic masses grew rapidly, cystic fluid was drained with a sterile syringe so that solid parts would grow for several days⁵⁶ before mice were euthanized at 7 weeks after injection. When solid masses grew slowly, mice were maintained through 21 weeks after injection. All animal experiments and maintenance conformed to the guidelines of the Animal Care and Use Committee and of the American Association of Laboratory Animal Care. Resected teratoma tissues were fixed in 10% formalin, embedded in paraffin blocks, sectioned at a thickness of $5 \mu\text{m}$, and mounted on glass slides. Staining with hematoxylin and eosin was performed using standard procedures.

Chromosome analysis. High-quality G-band karyotyping analysis was performed at Cell Line Genetics (Madison, WI, USA). Twenty metaphases were analyzed per cell line and the results were verified by clinically certified cytogeneticists. When all 20 metaphases examined were normal or when at least 18 metaphases were normal with one or two demonstrating a non-clonal, simple chromosome loss (which is most likely a technical artifact), the cell line was concluded to have normal karyotype.

Isolation of genomic DNA. Genomic DNA samples were purified from cultured fibroblasts and iPSC lines using PureLink Genomic DNA Mini kit (Thermo Fisher Scientific), which was previously used to quantitate mitochondrial DNA copy numbers.⁵⁷ The quantity and quality of DNA samples were verified by 1.0% agarose gel electrophoresis.

Whole-exome sequencing. Whole-exome sequencing was performed at Novogene Corporation (Chula Vista, CA, USA). One μg of genomic DNA per sample was used as input material for DNA sample preparation. Sequencing

libraries were generated using Agilent SureSelect Human All Exon kit (Agilent Technologies) following manufacturer's recommendations and index codes were added to each sample. Briefly, fragmentation was carried out by the hydrodynamic shearing system Covaris S2 (Covaris, MA, USA) to generate 180- to 280-bp fragments. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and then the enzymes were removed. After adenylation of 3'-ends of DNA fragments, adapter oligonucleotides were ligated. DNA fragments with ligated adapter molecules on both ends were selectively enriched in a PCR reaction. After PCR reaction, they were hybridized with biotinylated probes, then streptavidin-coated magnetic beads were used to capture 334 378 exons in 20 965 genes. Captured libraries were PCR-amplified to add multiplex index tags. The products were purified using AMPure XP system (Beckman Coulter, Beverly, MA, USA) and quantified using the Agilent high-sensitivity DNA assay on Bioanalyzer 2100 system (Agilent Biotechnologies). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina sequencing platform. Sequencing statistics of each sample are summarized in Supplementary Table S3. The somatic mutation calling algorithms Mutect and Strelka were used to detect somatic SNPs and InDels, respectively, in each iPSC line versus its original fibroblasts.

Microsatellite DNA analysis. Five unstable microsatellite loci (D5S107, D5S406, ACTC, D13S153 and BAT26) were PCR-amplified in a single multiplex reaction using the previously reported primer pairs (one primer of each pair was 5'-labeled with FAM)³⁴ and Type-it Microsatellite PCR kit (Qiagen, Valencia, CA, USA) following the supplier's protocol. The PCR products, along with GeneScan 500 ROX Size Standard (Applied Biosystems, Foster City, CA, USA), were analyzed on a fluorescence-based capillary electrophoresis (3130xl Genetic Analyzer, Applied Biosystems).

Assay of mitochondrial DNA (mtDNA) copy number. The qRT-PCR-based quantitation of mtDNA copy numbers, in parallel to nuclear DNA copy numbers, was performed using 1.0 ng of DNA samples and Human Mitochondrial DNA Copy Number Assay kit (Detroit R&D, Detroit, MI, USA) following the supplier's protocol. The mtDNA copy numbers normalized to nuclear DNA copy numbers were calculated using the $\Delta\Delta\text{Ct}$ method.

DNA sequencing of mtDNA. To examine whether the iPSC lines established in this study harbor homoplasmic or heteroplasmic mtDNA mutations,⁵⁵ DNA samples from the iPSC lines and the original fibroblasts were PCR-amplified for a mtDNA D-loop region with frequent mutations (nt. 128–599) using the primers 5'-CTG TCT TTG ATT CCT GCC TC-3' and 5'-TTG AGG TAA GCT ACA T-3', as previously reported,⁵⁸ and Platinum *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific). The PCR products were purified through Performa DTR Gel Filtration Cartridges (EdgeBio, San Jose, CA, USA), followed by direct sequencing in both directions using BigDye Terminator v1.1 Cycle Sequencing kit (Thermo Fisher Scientific).

Chromatin immunoprecipitation (ChIP). Immortalized BJ fibroblasts with Δ133p53 overexpression or control vector were cultured in 10-cm plates to ~90% confluence, and one plate was used for each immunoprecipitation. Cells were treated with $50 \mu\text{M}$ etoposide (Sigma-Aldrich) during the last 24 h of culture, followed by fixation with 1% formaldehyde (Sigma-Aldrich) for 15 min with gentle agitation and then treatment with 125 mM glycine (Sigma-Aldrich) for 10 min. Fixed cells were rinsed with phosphate-buffered saline (PBS) twice, collected into microtubes using cell scraper, and lysed with ChIP lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, Complete Protease Inhibitor Cocktail (Roche Diagnostics)) on ice for 10 min. To fragment chromatin, samples were sonicated using a Bioruptor (Diagenode, Denville, NJ) for 3 cycles of 30 s (output level High) with an interval of 30 s. Then, samples were centrifuged at $14\,000 \times g$ at 4°C for 10 min and supernatants were collected. After taking an aliquot for input control (5% of total volume), the remaining samples were diluted 10-fold in ChIP dilution buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, Complete Protease Inhibitor Cocktail), and then incubated overnight at 4°C with $40 \mu\text{l}$ of Dynabeads M-280 sheep anti-mouse IgG (Thermo Fisher Scientific), which had been pre-incubated for 3 h with $5 \mu\text{g}$ of DO-1 antibody (sc-126X, Santa Cruz Biotechnology). After washed with ChIP wash buffer (50 mM HEPES-KOH (pH 7.0), 0.5 M LiCl, 1 mM EDTA, 0.7% deoxycholate, 1% NP-40) five times and with TE

(10 mM Tris-HCl (pH 7.5), 1 mM EDTA) once, immunoprecipitated materials were eluted from the beads by heating overnight at 65 °C in elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS). From these immunoprecipitated materials and the above aliquot taken before immunoprecipitation, DNA was purified using MinElute PCR purification kit (Qiagen). Quantitative PCR (qPCR) was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and the following primers: HBB (hemoglobin subunit β as a negative control locus), 5'-AAC GTG CTC GCC TTT CTC-3' (forward) and 5'-GAA GCA GAACTC TGC ACT TC-3' (reverse); p21^{WAF1}, 5'-AGC CTT CCT CAC ATC CTC CT-3' (forward) and 5'-GGA ATG GTG AAA GGT GGA AA-3' (reverse); miR-34a, 5'-GGC ACG AGC AGG AAG GAG-3' (forward) and 5'-AAT CTC CAA ATG CCC CCG AT-3' (reverse); BAX, 5'-AGG CTG AGA CGG GGT TAT CT-3' (forward) and 5'-AAA GCT CAG AGG CCC AAA AT-3' (reverse); and PUMA, 5'-GTC GGTC TG TGTACG CAT CG-3' (forward) and 5'-AGA CAC CGG GAC AGT CGG ACA C-3' (reverse). DNA from the aliquot before immunoprecipitation was serially diluted and used to generate a standard curve for quantitative determination of DNA binding data calculated as % input. Data were means $\pm$ S.D. from biological triplicate ($n=3$; three independent 10-cm plates for immunoprecipitation).

Statistical analysis. Data are presented as means $\pm$ S.D. from triplicate experiments ($n=3$) unless otherwise stated. Statistical comparisons were made using unpaired two-tailed Student's t test. Differences were considered significant at a value of $*P<0.05$, $**P<0.01$, $***P<0.001$ or $****P<0.0001$.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

IH, KYP, KI, MS and CCH conceived and designed the project. IH, KYP, KI, HL, YH, KA, AIR, AMM and KF performed the experiments. IH, KYP, KI, HL, YH, KA, AIR, MS and CCH analyzed the data. All authors contributed to writing the manuscript.

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