Inhibition of Apoptosis Reduces Diploidization of Haploid Mouse Embryonic Stem Cells during Differentiation

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SUMMARY  
Phenotypes of haploid embryonic stem cells (haESCs) are dominant for recessive traits in mice. However, one major obstacle to their use is self-diploidization in daily culture. Although haESCs maintain haploidy well by deleting p53, whether they can sustain haploidy in differentiated status and the mechanism behind it remain unknown. To address this, we induced p53-deficient haESCs into multiple differentiated lineages maintain haploid status in vitro. Haploid cells also remained in chimeric embryos and teratomas arising from p53-null haESCs. Transcriptome analysis revealed that apoptosis genes were downregulated in p53-null haESCs compared with that in wild-type haESCs. Finally, we knocked out p73, another apoptosis-related gene, and observed stabilization of haploidy in haESCs. These results indicated that the main mechanism of diploidization was apoptosis-related gene-triggered cell death in haploid cell cultures. Thus, we can derive haploid somatic cells by manipulating the apoptosis gene, facilitating genetic screens of lineage-specific development.  
INTRODUCTION  
Sexually reproductive diploid animals exhibit evolutionary advantages over asexual organisms due to meiosis and the combination of two haploid gametes from different genetic backgrounds (Kondrashov and Crow, 1991; Perrot et al., 1991). Diploid genomes have allelic backups, but this hinders the elucidation of the gene function of recessive traits. Recently developed haploid cell lines, including near-haploid tumor cells (Carette et al., 2009; Gibbons et al., 1991) and haploid embryonic stem cells (haESCs) (Leeb and Wutz, 2011; Sagi et al., 2016), greatly facilitate forward and reverse genetic screening in mammalian species (Li and Shuai, 2017). It is easy to identify not only toxicant-targeting genes (Elling et al., 2011) but also crucial genes regulating critical biological processes (such as differentiation [Leeb et al., 2014] or X chromosome inactivation [Monfort et al., 2015]) through high-throughput screening in haESCs. However, daily cultured haESCs tend to revert to diploid status, and periodic sorting for haploids is necessary, which hampers the application of these cell lines in genetic screening. In addition, haESCs undergo more severe diploidization during differentiation both in vitro and in vivo, making the derivation of haploid lineage-specific somatic cells very difficult (Shuai and Zhou, 2014). Therefore, spontaneous diploidization of haESCs jeopardizes the exploration of the application of haploid technology in mammalian organ development or disease-related genetic screening despite their original single-genome status.  
Many efforts have been made to clarify the mechanism underlying the diploidization of mammalian haESCs; however, no precise regulatory pathway has yet been found. A previous report showed that the reason for the diploidization of mouse haESCs was a mistake in their cell cycle rather than the fusion of two single haploid cells, as indicated by the coculturing of haESCs with different marker genes (Leeb et al., 2012). Thereafter, chemical compounds inhibiting cell-cycle-related proteins were introduced into the culture medium to reduce diploidization (He et al., 2017; Takahashi et al., 2014), which still did not solve the problem completely. Single-cell dynamic analysis of mitosis in haESCs demonstrated that the metaphase of these cells was significantly longer than that of diploid ESCs (Guo et al., 2017). Thus, delayed mitosis might be the main cause of self-diploidization in haESCs. In this case, time-consuming and complicated fluorescence-activated cell sorting (FACS) was necessary to maintain the haploidy of haESCs. Recent studies showed that knockout of p53 (Olbrich et al., 2017) or overexpression of Dmtnt3b (He et al., 2018) could stabilize the haploid genome and slow down self-diploidization. In particular, p53-null haESCs could sustain haploid status well both under daily culture and in cells converting to an extraembryonic cell fate (Peng et al., 2019). p53 is an essential gene related to the cell cycle, DNA damage, and other crucial biological
processes (Aladjem et al., 1998; Cross et al., 1995; Fujiwara et al., 2005). Nevertheless, whether the p53-null mutation is beneficial for the maintenance of a haploid status during differentiation is still unknown. The exact mechanism by which p53-null reduces self-diploidization is also not clear.

In this study, we deleted p53 in mouse parthenogenetic haESCs with the CRISPR/Cas9 system and performed differentiation both in vitro and in vivo to assess whether p53-null stabilized haploidy in the somatic stage. The results revealed that by deleting p53, haploid somatic cells could be easily obtained from differentiated haESCs. We further analyzed global transcriptome data and found that genes regulating apoptosis were the main reason for diploidization during the differentiation of haESCs.

RESULTS

p53-Null HaESCs Maintain Haploidy Much More Stably Compared with Wild-Type HaESCs under Long-Term Culture

We generated two mouse parthenogenetic haESC lines from chemically activated oocytes according to a previous protocol (Shuai et al., 2014). These haESCs showed a standard mouse ESC morphology (Figure S1A) and grew well with the haploid genome assisted by FACS (Figure S1B). To introduce p53 mutations in haESCs, we designed two Cas9-mediated p53-single guide RNA (sgRNA) vectors to perform electroporation (Figure S1C). Two days after transfection, approximately 58.6% of the analyzed cells were Cas9-GFP-positive (Figure S1D), and these cells were harvested for further culturing. We randomly picked three subclones from the sorted cultures and determined whether they were gene edited. All three subclones (p53 KO-1, p53 KO-2, and p53 KO-3) carried different mutations in the p53 gene according to DNA sequencing (Figure 1A). The T7EN1 cleavage assay results also confirmed this (Figure 1B). To assess whether the P53 protein was lost, we performed western blotting in p53 KO-1, p53 KO-2, p53 KO-3, wild-type (WT) haESCs, and WT diploid ESCs (WT-diESCs). The results demonstrated that P53 was absent in all three p53-KO haESC lines (Figure 1C).

To determine whether p53 mutation affects the pluripotency of haESCs, we examined specific markers and alkaline phosphatase activity in p53-KO haESCs. Positivity was observed not only for pluripotency markers (OCT4, NANOG, and SSEA-1) but also for alkaline phosphatase in p53-KO haESCs (Figures S1E and S1F). Karyotype analysis showed that most chromosome spreads of p53-KO haESCs contained 20 chromosomes (Figure S1G). Next, we analyzed pluripotent genes and p53-related genes in p53-KO haESCs by real-time PCR with WT-haESCs as a control. The expression levels of pluripotent markers (Oct4 and Nanog) and Cyclin B did not change significantly in p53-KO haESCs compared with WT-haESCs. However, expression of p53 and p21 decreased, meanwhile Cdk2 and MAPK11 increased significantly in all three p53-KO haESC lines (Figure 1D). This result indicated that p53 mutations did not affect pluripotency but do affect p53-related genes, including some cell-cycle genes. Since p53 deletion increased haploidy maintenance in the ESC stage, as previously described, we tested whether p53-KO haESCs exhibited advantages in haploidy maintenance in daily culture without sorting for 3 weeks. There was still a high percentage of haploid cells remaining in the 1n peak (G0/G1 phase) of p53-KO haESCs (p53 KO-1, 21.4%; p53 KO-2, 20.9%; p53 KO-3, 20.2%), whereas the percentage of the 1n peak (G0/G1 phase of haploid cells) in WT-haESCs decreased to 2.4% (Figure 1E). Here, we chose p53 KO-1 (the most stable) for subsequent experiments. We designed p53 overexpression (p53 OE) vectors (Figure S1H) and transferred them into p53 KO-1 cells having a high proportion of haploids, with an empty vector as control. After transfection, transfected cells expressing exogenous GFP (a GFP cassette was included in the p53 OE vector or empty vector) were enriched for GFP and haploidy double-positive cells by FACS (Figures 1F and 1G). Real-time PCR result revealed that p53 OE-haESCs (p53 KO-1+p53 OE) had re-expressed p53 gene compared with WT-haESCs, p53 KO-1, and p53 KO-1-vector (Figure 1H). Western blotting analysis further confirmed this result (Figure S1I). Thereafter, we checked the haploidy-maintaining ability among p53 KO-1, p53 KO-1-vector, WT-haESCs, and p53 KO-1-p53 OE cells without sorting for 10 days (all the samples started as 100% of haploid cells). DNA-content analysis demonstrated that only p53 KO-1 and p53 KO-1-vector were able to maintain haploidy stably in this period, whereas p53 KO-1-p53 OE and WT-haESCs diploidized to some degree (Figure 1I). The rescue of p53 expression in p53-KO haESCs made them unable to sustain the phenotype of stable haploidy any longer, which proved that p53 was the main trigger of the diploidization phenomenon. As Cdk2 and MAPK11 (two important cell-cycle genes) were upregulated in p53-KO haESCs according to the above qPCR analysis, we conducted overexpression (OE) of Cdk2 and MAPK11 separately in WT-haESCs to address whether either of the two genes would affect the process of diploidization. Although the OE-haESCs showed upregulation of Cdk2 and MAPK11 independently (Figures S1I and S1K), neither of them obtained an advantage in maintaining haploidy like p53-KO in long-term cell culture (Figure S1L).

p53-Null HaESCs Differentiate Reliably with a Haploid Genome In Vitro

To examine the haploidy-maintaining ability of p53-KO haESCs during differentiation in vitro, we performed
Figure 1. *p53* Deletion in haESC and DNA Analysis during Proliferation
(A). *p53*-deleted genotypes in subclones *p53* KO-1, *p53* KO-2, and *p53* KO-3.
(B) T7EN1 cleavage analysis of the *p53*-KO lines *p53* KO-1, *p53* KO-2, and *p53* KO-3. Cleaved products (red arrowheads) indicate the presence of mutations.
(C) Western blotting to detect P53 in WT-diESCs, WT-haESCs, *p53* KO-1, *p53* KO-2, and *p53* KO-3 cells. GAPDH was used as a loading control.
(D) Expression levels of pluripotent, *p53*-related, and cell-cycle-related genes (*Oct4*, *Nanog*, *p53*, *p21*, *CyclinB*, *Cdk2*, and *MAPK11*) in WT-haESCs, *p53* KO-1, *p53* KO-2, and *p53* KO-3 cells determined by qPCR (n = 3 independent experiments). Data presented as mean ± SEM. t test: *p < 0.05, **p < 0.01, ***p < 0.001.
(E) DNA content analysis of *p53*-KO haESCs during culture on day 0, day 14, and day 21 without sorting, with WT-haESCs as a control. The percentages of the 1n (G0/G1) peak in WT-haESCs were 27.4%, 9.33%, and 2.4%, respectively; in *p53* KO-1 cells, 23.5%, 22.8%, and 21.4%, respectively; in *p53* KO-2 cells, 23.6%, 21.1%, and 20.9%, respectively; in *p53* KO-3 cells, 23.7%, 21.5%, and 20.2%, respectively.
(F) Percentage of GFP+ cells in *p53*-KO *p53* OE cells after transfection. WT-haESCs without transfection were used as a negative control.
(G) Bright-field (BF) and FITC images of *p53* KO-1-*p53* OE cells, Scale bar, 100 μm.
(H) Expression levels of *p53* in WT-haESCs, *p53* KO-1-Vector, *p53* KO-1, and *p53* KO-1-*p53* OE cells determined by qPCR (n = 3 independent experiments). Data presented as mean ± SEM. t test: ***p < 0.001.
(I) Percentages of haploids in *p53* KO-1, *p53* KO-1-Vector, WT-haESCs, and *p53* KO-1-*p53* OE cells 10 days after sorting. Nearly 44% of sorted cells remained in haploidy in *p53* KO-1 and *p53* KO-1-Vector group, whereas WT-haESCs and *p53* KO-1-*p53* OE exhibited only 29% staying as haploid cells (n = 3 independent experiments). Data presented as mean ± SEM. t test: ***p < 0.001.
See also Figure S1 and Table S4.
Figure 2. In Vitro Differentiation of p53-KO haESCs

(A) Morphology of 7-day haEBs derived from p53 KO-1 cells. Scale bar, 100 μm.

(B) DNA-content analysis of 7-day haEBs derived from p53 KO-1 cells. The percentage of the 1n (G0/G1) peak in haEBs was 21.7%.

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differentiation of p53-KO haESCs at different stages. Standard embryoid bodies (EBs) formed (Figure 2A), and 29.7% of haploid cells (at G0/G1 phase) were observed in 7-day EBs from p53-KO haESCs (Figure 2B). The expression levels of pluripotency genes and specific genes of the three germ layers showed that the haploid EBs had differentiated beyond the ESC stage (Figure 2C). Although WT parthenogenetic haESCs were not able to remain in the epiblast stem cell-like cell (EpiLC) stage with a haploid genome (Leeb et al., 2012), p53-KO haESCs differentiated into haploid EpiLCs (haEpiLCs) quite easily and stably sustained haploidy (Figures 2D and 2E). The primed state haEpiLCs expressed pluripotent markers such as OCT4 and NANOG (Figure 2F). Besides, naive-primed distinguishing marker TFE3 (Betschinger et al., 2013) was located in the cytoplasm of haEpiLCs (Figure 2G), demonstrating that p53-KO haEpiLCs could remain in the epiblast stage with a single genome in the presence of basic fibroblast growth factor (bFGF) and activin A. The expression levels of specific genes further proved that these haploid cultures expressed primed stage genes (FGF5 and Otx2), rather than naive genes (Rex1 and Klf4), with WT-haESCs and WT epiblast stem cells (WT-EpiSCs) as controls (Figures 2H and 2I). To assess differentiation potentials of haEpiLCs, we induced neural differentiation according to a previous protocol (Ying et al., 2016). Around 7–10 days post differentiation, NESTIN- and TUJ1-positive cells were observed in the adherent haNSCLCs (haNSCLCs) by immunostaining (Figures 2J and 2K), presenting the ability to aggregate into neural spheres (Figure S2C). DNA analysis revealed that the haNSCLCs could stably maintain haploidy during proliferation (Figure 2L). Next, we examined pluripotent and neural stem cell-specific markers in haNSCLCs by immunostaining. Pluripotent markers (OCT4 and NANOG) were absent in haNSCLCs (Figure 2M). Positivity for Pax6, Sox1, and Nestin was observed in adherent haNSCLCs (Figure 2N) and their neural spheres (Figure S2D). To assess the differentiation potential of haNSCLCs, we performed neural lineage specification. The results showed that glial cells (O4 and glial fibrillary acidic protein [GFAP] positive) and multiple neurons (MAP2, NEUN, and TUJ1 positive) could be generated from haNSCLCs (Figures 2O and 2P). In another parallel experiment, we analyzed DNA contents of glial mixture and neuronal mixture independently. There was 2.0% of glial mixture and 4.5% of neuronal differentiation.

(C) Expression levels of pluripotency and differentiation genes (Rex1, Oct4, Cdx2, Fgf5, Nestin, and T) in haEBS and haESCs determined by qPCR (n = 3 independent experiments). Data presented as mean ± SEM. t test: ***p < 0.001.

(D) Morphology of haEpiLCs derived from p53 KO-1 cells. Scale bar, 100 μm.

(E) DNA-content analysis of haEpiLCs derived from p53 KO-1 cells. The percentage of the 1n (G0/G1) peak in haEpiLCs was 21.8%.

(F) Immunofluorescence of OCT4 and NANOG in haEpiLCs derived from p53 KO-1 cells. DNA was stained with DAPI. Scale bar, 50 μm.

(G) Immunofluorescence of TFE3 in haEpiLCs derived from p53 KO-1 cells and haESCs. DNA was stained with DAPI. Scale bar, 50 μm.

(H) Expression levels of pluripotency genes (OCT4, Nanog, Rex1, and Klf4) in WT-haESCs, WT-EpiSCs, and haEpiLCs determined by qPCR (n = 3 independent experiments). Data presented as mean ± SEM. t test: ***p < 0.001.

(I) Expression levels of EpiSC-specific marker genes (Fgf5 and Otx2) in WT-haESCs, WT-EpiSCs and haEpiLCs determined by qPCR (n = 3 independent experiments). Data presented as mean ± SEM. t test: ***p < 0.001.

(J) Immunostaining of NESTIN and TUJ1 in 10-day differentiation cell cultures of haEpiLCs. Scale bar, 50 μm.

(K) Morphology of haNSCLCs derived from p53 KO-1 cells. Scale bar, 100 μm.

(L) DNA-content analysis of haNSCLCs derived from p53 KO-1 cells. The percentage of the 1n (G0/G1) peak in haNSCLCs was 28.5%.

(M) Immunofluorescence of OCT4 and NANOG in haNSCLCs. DNA was stained with DAPI. Scale bar, 50 μm.

(N) Immunofluorescence of neural stem cell-specific markers in haNSCLCs, Pax6, Sox1, and NR2F2. DNA was stained with DAPI. Scale bar, 50 μm.

(O) Immunostaining of oligodendrocytes (O4) and astrocytes (GFAP) derived from p53 KO-1. Scale bar, 50 μm.

(P) Immunostaining of neuronal specific markers (MAP2, NEUN, and TUJ1) in neurons derived from p53 KO-1. Scale bar, 50 μm.

See also Figure 2 and Table S4.
Figure 3. In Vivo Analysis of p53-KO haESCs
(A) Morphology of chimeric embryos at E6.5 derived from GFP-labeling WT-haESCs and p53 KO-1 cells. Scale bar, 100 μm. WT-E6.5 embryo was used as a control.
(B) DNA-content analysis of GFP+ cells and GFP- cells in chimeric E6.5 embryos derived from GFP-labeling WT-haESCs and p53 KO-1 cells. The percentages of the 1n (G0/G1) peak in GFP+ cells from p53 KO-1 E6.5 and WT-haESCs E6.5 were 28.5% and 4.7%, respectively.
(C) Morphology of chimeric embryo at E8.5 derived from GFP-labeling p53 KO-1 cells. WT-E8.5 embryo was used as a control.
(D) DNA-content analysis of GFP+ cells and GFP- cells in chimeric E8.5 embryo derived from GFP-labeling p53 KO-1 cells. The percentage of the 1n (G0/G1) peak from p53 KO-1 chimeric E8.5 GFP+ cells was 25.3%.
(E) Expression levels of pluripotency genes (Oct4, Nanog, Stella, Rex1, and Klf4) in chimeric E8.5 haploid cells from GFP+ cells, with WT-haESCs as a loading control (n = 3 independent experiments). Data presented as mean ± SEM. t test: **p < 0.01, ***p < 0.001.
(F) Morphology of chimeric embryos at E8.5 derived from GFP-labeling p53 KO-1 cells.
(G) Morphology of chimeric embryos at E10.5 derived from GFP-labeling p53 KO-1 cells. WT E10.5 was used as a control.
(H) DNA-content analysis of GFP+ cells and GFP- cells in chimeric E10.5 embryo derived from GFP-labeling p53 KO-1 cells. The percentage of the 1n (G0/G1) peak from p53 KO-1 chimeric E10.5 GFP+ cells was 21.4%.
(I) Teratoma formed from p53 KO haESCs was identified by H&E staining. Scale bar, 100 μm. The tissues shown were gut epithelium (endoderm), muscle (mesoderm), and neural tissue (ectoderm).
(J) DNA-content analysis of teratoma. The percentage of the 1n (G0/G1) peak from all the tissue was 3.1%.

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Haploid Cells Can Contribute to Chimeric Fetuses and Teratomas upon p53 Knockout

The finding that p53-KO haESCs showed excellent haploidy maintenance during differentiation in vitro led to the further investigation of whether they presented the same advantages during differentiation in vivo. We microinjected GFP-labeling p53-KO haESCs into blastocysts to construct chimeric embryos. The reconstructed embryos were transferred to the oviducts of 0.5 days post coitum (dpc) pseudopregnant mice. Chimeric embryonic day 6.5 (E6.5) embryos from GFP-labeling p53-KO haESCs and GFP-labeling WT-haESCs were obtained (Figures 3A and S3A). Around 17.2% cells of chimeric E6.5 1# embryo and 55.5% cells of chimeric E6.5 2# embryo generated from p53-KO haESCs microinjection were GFP-positive cells (Table S1). The haploid cells in the 1n peak of p53-KO haESCs-chimeric E6.5 embryos (p53 KO-1, chimeric 1#, 28.5%; p53 KO-2, chimeric 2#, 24.5%) were much higher than that in WT-haESCs-chimeric E6.5 embryo (WT-haESCs-chimeric, 4.7%) (Figures 3B and S3B; Table S1), demonstrating that p53-KO haESCs could contribute to an epiblast stage with a high proportion of haploid status. For chimeric E8.5 embryos, 12.4% cells of 1# embryo and 37.6% cells of 2# embryo from p53-KO haESCs microinjection were GFP-positive cells (Table S1). However, chimeric E8.5 embryos from p53-KO haESCs were abnormal (Figures 3C and S3C), possibly due to the high contribution of haploid cells (a haploid genome might not support full development) or deficiency of p53, with approximately 25% of these cells (p53 KO-1, chimeric 1#, 25.3%; p53 KO-2, chimeric 2#, 24.3%) remaining in the 1n peak (Figures 3D and S3D). To address the identity of these haploid cells from E8.5 embryos, we harvested them by FACS and performed qPCR analysis. Compared with WT-haESCs, the sorted haploid cells barely expressed any of the pluripotent markers (Oct4, Nanog, Stella, Rex1, and Klf4), demonstrating that they were indeed differentiated cells (Figure 3E). Thus, p53-KO haESCs could differentiate into a somite stage without diploidization. To investigate the effect of p53 deletion on development, we microinjected GFP-labeling p53-KO diploid ESCs into blastocysts as a parallel experiment. Interestingly, chimeric E8.5 embryos from p53-KO diploid ESCs were normal (Figure 3F), with GFP cells (24.9% in 1#) and 40.3% in 2#) contributing to the chimeric E8.5 embryos (Table S1). Furthermore, abnormal chimeric E10.5 embryos were also able to be obtained (Figures 3G and S3E). The percentages of GFP cells in chimeric E10.5 1# and 2# embryos from p53-KO haESCs microinjection were 1.6% and 3.3%, respectively (Table S1). According to DNA analysis, there were still around 22% of haploid cells in 1n peak (p53 KO-1, chimeric 1#, 21.4%; p53 KO-2, chimeric 2#, 22.3%) from E10.5 GFP-positive cells (Figures 3H and S3F), indicating that p53-KO haESCs could further differentiate in a haploid genome with limited development potentials.

To check the pluripotency of p53-KO haESCs to form the three germ layers, we injected approximately $1 \times 10^7$ p53 KO-1 cells under the skin of severe combined immunodeficiency (SCID) mice. Three weeks later, a standard teratoma formed (Figures S3G and S3H) and was divided into two parts. Histological analysis was performed on one part, and DNA-content analysis was performed on the other. Gut epithelium, muscle, and neural tube tissues, representing the three germ layers, were observed by H&E staining (Figure 3I). According to the DNA-content analysis, 3.1% haploid cells (1n peak) still existed in the 3-week teratoma, demonstrating that the p53-KO haESCs show a powerful ability to maintain haploidy in vivo (Figure 3J). The haploid cells from teratoma were also sorted by FACS and checked by qPCR. The result showed that the haploid cells in teratoma did not express the pluripotent marker genes (Oct4, Nanog, Stella, Rex1, and Klf4), indicating that they had entered a differentiated state instead of a pluripotent state (Figure 3K).

Transcriptome Analysis of p53-KO HaESCs

To determine why p53-KO haESCs show great advantages in haploidy maintenance, we compared the transcriptomes between p53-KO haESCs and WT-haESCs by RNA-sequencing (RNA-seq) analysis. The results revealed a very high correlation ($R^2 = 0.9947$) between p53-KO haESCs (p53 KO-1, p53 KO-2, and p53 KO-3) and WT-haESCs (three independent WT-haESC lines) (Figure 4A). According to comprehensive summaries with all expression data (Tables S2 and S3), there were 2,785 differentially expressed genes (p < 0.01, fold change >2) between p53-KO haESCs and WT-haESCs, which were further analyzed to elucidate the reason of haploidy stabilization. Hierarchical clustering (heatmap) showed the top 50 differentially expressed genes between p53-KO haESCs and WT-haESCs, considering a fold change of 2.0 and p < 0.05 (Figure S4A and Table S2). These differentially expressed genes were mainly related to the extracellular matrix and basic development according to gene ontology (GO) analysis (Figures S4B and S4C).

(K) Expression levels of pluripotent genes (Oct4, Nanog, Stella, Rex1, and Klf4) in the 1n cells sorted from teratoma determined by qPCR, with WT-haESCs as a loading control (n = 3 independent experiments). Data presented as mean ± SEM. t test: ***p < 0.001. See also Figure S2; Tables S1 and S4.
GO analysis data further indicated that most of the upregulated genes were correlated with male meiotic nuclear division, Piwi-interacting RNA metabolic process, and other biological processes, whereas the downregulated genes were related to vasculature development and extracellular matrix organization (Figure 4B). Since p53 is a very important gene involved in multiple biological processes, including DNA-damage repair, cell-cycle arrest, and apoptosis (Hafner et al., 2019), we analyzed the expression of specific genes involved in these processes between p53-KO haESCs and WT-haESCs in volcano plots of p values (−log10 scale) versus fold changes (log2 scale). Interestingly, there were several differentially expressed genes related to apoptosis identified between the two groups (Figure 4C). However, we did not observe distinctly expressed genes related to the cell cycle or DNA damage in the RNA-seq data (Figure 4C). As we also found that the p53-KO haESCs showed better survival ability during differentiation...
compared with WT-haESCs in vitro (data not shown), we as-
sessed carefully the differentially expressed genes corre-
lated with apoptosis. TP73 (p73, also known as Tri73),
Phlda3, Cdkn1a, and Bbc3 (downstream target genes of
p53) were significantly downregulated in p53-KO haESCs
(Figure 4D) and were listed as the top four downregulated
genes in p53-KO haESCs related to apoptosis. We further
detected the expression levels of some target genes of p53
related to apoptosis by qPCR and found that only p73
and Bbc3 were significantly downregulated (Figure 4E).

To address whether apoptosis was the main reason for
the diploidization of haESCs, we assessed the survival
rate of p53-KO haESCs 24 h after sorting for haploids
compared with WT-haESCs. Nearly 70.5% of the sorted
cells survived and expanded in the p53-KO group, whereas
in the control group this percentage was only 52.9% (Fig-
ure S4D). As caspase-3 is a key molecule in the process
of apoptosis (Song et al., 2015), we measured the caspase-3
activity between p53-KO haESCs and WT-haESCs after
sorting. The caspase-3 activities in p53 KO-1, p53 KO-2,
and p53 KO-3 were significantly decreased compared
with that in WT-haESCs (Figure 4F). In summary,
apoptosis of p53-KO haESCs was reduced in daily culture
and FACS processes.

p73 Is a Major Target of p53-Regulating Diploidization
To find out more about apoptosis-related gene-regulating
diploidization, we designed p73 and Bbc3 knockout vectors
(Figures S5A and S5B) and transfected them separately into
WT-haESCs to introduce gene editing. Among the 60
randomly picked subclones of the p73-edited group, 25
subclones maintained haploidy (Figure S5C). We
sequenced 11 subclones with high percentages of haploid
cells and found that four of them were p73-KO cells

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**Figure 5. p73 Is a Major Target of p53-Regulating Diploidization**

(A) p73-deleted genotypes in haESCs by DNA sequencing.
(B) Western blot to detect P73 in p73 KO-1 cells, p73 KO-2 cells, p73 KO-1 cells, WT-haESCs, and WT-diESCs. GAPDH was used as a loading
control.
(C) Progression of the percentage of haploid cells in the p73-KO haESCs and WT-haESCs for 14 days, 28 days, and 35 days after sorting (n = 3
independent experiments). Data presented as mean ± SEM. t test: ***p < 0.001.
(D) Caspase-3 activity of p73 KO-1, p73 KO-2, and p73 KO-3 cells, with WT-haESCs as a control (n = 3 independent experiments). Data
presented as mean ± SEM. t test: ***p < 0.001.
(E) Expression levels of apoptosis-related genes (p53, Bbc3, and Bax) and proliferation-related gene MAPK11 in p73 KO-1, p73 KO-2, and
p73 KO-3 haESCs determined by qPCR, with WT-haESCs as a control (n = 3 independent experiments). Data presented as mean ± SEM. t test:
*p < 0.05, **p < 0.01.
See also Figure S5 and Table S4.
(Figure 5A). To assess whether the P73 protein was lost, we performed western blotting in p73 KO-1, p73 KO-2, p73 KO-3, WT-haESCs, and diploid ESCs. The results demonstrated that P73 was absent in three p73-KO haESC lines (Figure 5B). However, in the Bbc3-edited group, 18 out of 60 randomly selected subclones exhibited haploid cells, 9 of which showed a high percentage of haploids, which were sequenced and did not contain a Bbc3 mutation (Figure S5C). We randomly checked two diploidized subclones by sequencing and found Bbc3 mutations (Figure S5D). DNA-content analysis again confirmed that the Bbc3 mutant subclones were diploid, without any haploid cells (Figure S5E). Notably, this result indicated that p73 alone was the key regulator of diploidization, rather than Bbc3.

We further assessed whether p73-KO haESCs also showed advantages in haploidy maintenance similar to those of p53-KO haESCs. The results demonstrated that p73-KO haESCs exhibited better proliferation ability after sorting (Figure S5F) and a lower rate of diploidization during long-term culture (Figure 5C) compared with WT-haESCs. The caspase-3 activity in p73 KO-1, p73 KO-2, and p73 KO-3 was much lower than that in WT-haESCs, suggesting that apoptosis could be inhibited by p73-KO (Figure 5D). Next, we examined the expression of p53-targeted genes related to apoptosis in p73-KO haESCs (p73 KO-1, p73 KO-2 and p73 KO-3) and WT-haESCs and did not find any significant differences in the downstream gene (Figure 5E). This result indicated that p73 played a crucial role in affecting diploidization without interacting with its upstream genes, including p53.

**DISCUSSION**

Haploidy in lower organisms, including yeast, is fairly stable, which makes haploid individuals a powerful tool for genetic engineering (de Godoy et al., 2008). Medaka fish haESCs also show durable haploidy in both undifferentiated and differentiated statuses, without being vertebrate species (Yi et al., 2009). However, mammalian haESCs tend to revert to diploidy under daily culture or differentiation, making complicated and frequent sorting of haploids necessary. Although haNSCLCs have been obtained by an optimized epiblast-based method (Shuai et al., 2015) or by introducing a ROCK inhibitor (Y27632) into the culture medium (He et al., 2017), these cells are not easy to handle, and the underlying mechanisms require further investigation. Our findings provide a simple strategy for deriving multiple haploid somatic cells by knocking out a single gene (p53-KO or p73-KO). We find that reduced apoptosis may be a major reason why p53-KO haESCs stably maintain haploidy. Additionally, knockout of p73, a target of p53, can also stabilize haploidy in haESCs.

Although p53 or p73 deficiency can promote haploidy maintenance in mouse haESCs, whether this phenomenon is applicable to other species remains unknown. Previous studies reported that primate haESCs maintain haploidy more stably than rodent haESCs (Sagi et al., 2016; Yang et al., 2013), and whether the mechanisms involved correlate with p53 or p73 warrants further investigation. Another study showed that although rhesus monkey haESCs maintained haploidy well in the ESC state, they underwent severe diploidization during differentiation for reasons related to apoptosis (Wang et al., 2018). Therefore, apoptosis may be the major cause of the diploidization of mammalian haploid cells. Generally, both haESCs and haploid somatic cells do not exhibit growth properties equivalent to those of their diploid counterparts (Olbrich et al., 2017). Our data revealed that p53-KO or p73-KO haESCs survived better after FACS (Figure S4D) and regained their proliferative ability faster (Figure S5F) than WT-haESCs. Previous studies have indicated that some haESCs experience cell-cycle errors such as mitosis escape, resulting in diploidization (Guo et al., 2017; He et al., 2017). According to our RNA-seq data, however, p53-KO haESCs did not show significant changes in the expression of cell-cycle-related genes (Figure 4C). We propose that the stabilization of haploidy in p53-KO haESCs depended on a reduction in apoptosis instead of regulation of the cell cycle. Nevertheless, both p53 and p73 are essential genes involved in many pathways, including key cell-cycle checkpoints (Melino et al., 2002; Stiewe, 2007). The exact mechanism by which p53-KO or p73-KO haESCs avoid mitosis escape has not yet been clarified.

Due to diploidization, complicated and time-consuming FACS purification is necessary for haploidy maintenance. Non-staining sorting methods have been introduced to enrich haploids (Freimann and Wutz, 2017; Qu et al., 2018), but their efficiency cannot be guaranteed. In our study, p53-KO or p73-KO haESCs could maintain haploidy without sorting for at least 3 weeks, which facilitates the expansion of large-scale haploid cell cultures rapidly for high-throughput genetic screening. However, previous reports demonstrated that p53-deficiency diploid ESCs showed limited pluripotency (Li et al., 2018a; Lin et al., 2005). In our study, p53-KO haESCs also showed nearly 2,800 differentially expressed genes from WT-haESCs at global transcriptome level. This indicated that there might be potential restriction of p53-KO haESCs in differentiation, which needs further investigation. Nevertheless, p53-KO haESCs could contribute to chimeras (up to E10.5) and teratomas in haploid status, which would mean that haploid somatic cells could be derived in a rigorous way in vivo, thus expanding the application of such a haploid system for lineage-specific genetic screening.
EXPERIMENTAL PROCEDURES

Transfection
To obtain p53-KO haESCs lines, we electroporated approximately 4 × 10⁶ WT-haESCs with PX461-sgRNA1 (3 μg) and PX461-sgRNA2 (3 μg), using a Neon (Invitrogen) electroporator at 1,400 V for 10 ms with three pulses. GFP-positive cells were sorted by FACS 2 days post electroporation. For the overexpression of p53, a combination of 2 μg of the PBase plasmid (SBI, PB210PA-1) and 6 μg of the overexpressing (or empty piggyBac control) plasmid were electroporated into 4 × 10⁶ p53-KO haESCs using the same electroporating conditions described above. After transfection for 4–5 days, GFP and haploidy double-positive cells were sorted for subsequent experiments. Overexpression of Cre and MAPK11 plasmids and PBase plasmid were transfection into WT-haESCs, respectively. After transfection for 4–5 days, GFP and haploidy double-positive cells were sorted for subsequent experiments. To obtain GFP-labeling rating conditions described above. After transfection for 4–5 days, GFP-positive cells were sorted by FACS and were sacrificed for subsequent experiments. Overexpression of p53-KO diploid ESCs, or WT-haESCs cells into CD-1 background blastocysts. Reconstructed embryos were transferred to the oviducts of pseudopregnant CD-1 mice at 0.5 dpc. At E6.5, E8.5, and E10.5, pseudopregnant mice were sacrificed to dissect chimeric fetuses. Chimeric embryos were trypsinized and GFP-positive and DNA-content analysis performed by FACS.

For teratoma analysis, approximately 1 × 10⁷ p53 KO-1 cells were injected subcutaneously into the limbs of 8-week-old male SCID mice. Fully formed teratomas were dissected 3 weeks later, then fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with H&E for further analysis. Alternatively, fully formed teratomas were dissected to analyze the DNA contents.

Data and Code Availability
The Gene Expression Omnibus accession number for the RNA-seq data reported in this paper is GEO: GSE148118.

A complete description of the methods can be found in Supplemental Information.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2020.05.004.

AUTHOR CONTRIBUTIONS
L.S. conceived and designed the study. W.Z., Y.T., and Q.G. performed most of the experiments. X.L., J.Z., C.Y., Y.W., H.W., Y.Z., and Q.Z. participated in some of the cell culture and molecular experiments. Y.L. analyzed the bioinformatics data. L.L., Y.Y., Y.F., and L.S. wrote the paper.

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Supplemental Information

Inhibition of Apoptosis Reduces Diploidization of Haploid Mouse Embryonic Stem Cells during Differentiation

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Figure S1. Gene editing in haESCs and characterization. Related to Figure 1

(A) Colonies of WT-haESCs in bright field. Scale bar, 100 μm.

(B) DNA content analysis of WT-haESCs for p53-deletion. The percentage of the 1n peak (G0/G1 phase) in WT-haESCs was 7.0%.

(C) DNA information for p53 knocking out using the CRISPR/Cas9 system.

(D) Percentage of Cas9-GFP positive cells in transfected WT-haESCs 2 days after transfection by FACS indicating. WT-haESCs without transfection were used as control.

(E) Immunofluorescence staining of pluripotent markers, including OCT4, NANOG and SSEA1 in p53-KO haESCs. DNA was stained with DAPI. Scale bar, 50 μm.

(F) Alkaline phosphatase (AP) staining of p53-KO haESCs.

(G) Chromosome spread analysis of p53-KO haESCs. Scale bar, 10 μm.

(H) Schematic overview of the vectors used for overexpression of p53.

(I) Western blotting analysis of P53 in p53 KO-1, p53 KO-1-Vector, WT-haESCs and p53 KO-1-p53 OE cells. GAPDH was used as a loading control.

(J) Expression levels of Cdk2 in WT-haESCs, WT-haESCs-Vector and WT-haESCs-Cdk2 OE cells determined by qPCR. (n=3 independent experiments). t test, ***p < 0.001. Data was represented as the mean ± SEM.

(K) Expression levels of MAPK11 in WT-haESCs, WT-haESCs-Vector and WT-haESCs-MAPK11 OE cells determined by qPCR. (n=3 independent experiments). t test, ***p < 0.001. Data was represented as the mean ± SEM.

(L) Percentage of haploid cells in WT-haESCs, WT-haESCs-Vector, WT-haESCs-MAPK11 OE, WT-haESCs-Cdk2 OE and p53-KO haESCs at day 10 after sorting. Nearly 29% of sorted cells remained in haploidy in WT-haESCs-Cdk2 OE, WT-haESCs-MAPK11 OE, WT-haESCs and WT-haESCs-Vector, whereas p53 KO-1 still had 44% of cells stayed in haploidy. (n=3 independent experiments). t test, ***p < 0.001. Data was represented as the mean ± SEM.
Figure S2. *In vitro* differentiation potentials of *p53*-KO haESCs. Related to Figure 2

(A) FACS analysis of *Pax6*-GFP-positive cells on 9-day of neural differentiation. *p53*-KO haESCs was used as the *Pax6*-GFP-negative control.

(B) DNA content analysis of *Pax6*-GFP-positive and -negative cells derived from *p53*-KO haESCs. The percentage of the 1n (G0/G1) peak in *Pax6*-GFP-positive cells was 6.2%, and that in *Pax6*-GFP-negative cells was 27.8%.

(C) The haNSCLCs spontaneously aggregate to form compact neural sphere structures in suspension culture. Scale bar, 100 μm.

(D) Immunostaining of PAX6, NESTIN and SOX1 in the neural spheres derived from *p53*-KO haESCs. Scale bar, 50 μm.

(E) DNA content analysis of glial mixture differentiated from haNSCLCs. 2.0% of cells remained in the 1n peak.

(F) DNA content analysis of neuronal mixture differentiated from haNSCLCs. 4.5% of cells remained in the 1n peak.
Figure S3. *In vivo* differentiation potentials of p53-KO haESCs. Related to Figure 3

(A) Morphology of chimeric embryo at E6.5 derived from GFP labelling p53 KO-2 cells. Scale bar, 100 μm.

(B) DNA content analysis of GFP+ cells and GFP- cells in chimeric E6.5 embryo derived from p53 KO-2 cells. The percentage of the 1n (G0/G1) peak in GFP+ cells was 24.5%.

(C) Morphology of chimeric embryo at E8.5 derived from GFP labelling p53 KO-2 cells. Scale bar, 100 μm.

(D) DNA content analysis of GFP+ cells and GFP- cells in chimeric E8.5 embryo derived from p53 KO-2 cells. The percentage of the 1n (G0/G1) peak in GFP+ cells was 24.3%.

(E) Morphology of chimeric embryo at E10.5 derived from GFP labelling p53 KO-2 cells. Scale bar, 100 μm.

(F) DNA content analysis of GFP+ cells and GFP- cells in chimeric E10.5 with p53 KO-2 cells. The percentage of the 1n (G0/G1) peak in chimeric GFP+ cells was 22.3%.

(G-H) Teratoma derived from p53-KO haESCs.
Figure S4. RNA-seq and survival analysis of p53-KO haESCs. Related to Figure 4

(A) Hierarchical clustering (heat map) showed the top 100 differentially expressed genes among WT-ESCs and p53-KO haESCs, considering a fold change of 2.0 and p < 0.05. To observe the differences between samples, we selected the top 50 variant genes.

(B) Enriched terms of differentially expressed genes in p53-KO haESCs (more than 2 folds) compared to WT-haESCs, statistical significance was represented by a discrete color scale.

(C) Enrichment network visualization for differential expressed genes between WT-haESCs and p53-KO haESCs. Each node represents an enriched term and cluster annotations are shown in color.

(D) Cell survival rates of WT-haESCs and p53-KO haESCs for 24 h after sorting. Nearly 70.5% of sorted cells survived in p53-KO haESCs, whereas that in WT-haESCs exhibited only 52.9%. (n=3 independent experiments). t test, **p < 0.01. Data was represented as the mean ± SEM.
Figure S5. Gene function validation of p73 and Bbc3 in haESCs. Related to Figure 5

(A-B) DNA information for knocking out of p73 and Bbc3 in WT-haESCs using the CRISPR/Cas9 system.

(C) Summary of haploidy and gene mutations in p73-KO and Bbc3-KO ESCs. 25/60 subclones of p73-KO and 18/60 subclones of Bbc3-KO were haploid. The subclones whose 1n peak >10% were sequenced, and there were 4 p73-KO subclones but no Bbc3-KO subclone.

(D) Bbc3-KO genotypes in the mixture Bbc3-KO ESCs.

(E) DNA content analysis of Bbc3-KO ESCs. The percentage of the 1n peak (G0/G1 phase) was 0%.

(F) Cell counts of p53 KO-1, p73 KO-1, p73 KO-2 and p73 KO-3 for 24 h, 48 h, 72 h and 96 h after sorting, with WT-haESCs as a control. (n=3 independent experiments). t test, **p < 0.01, ***p < 0.001. Data are represented as the mean ± SEM.
Table S1. Contribution of p53-KO ESCs in chimeric embryos. Related to Figure 3.

|       | Percentage of GFP+ cells (%)<sup>a</sup> | Percentage of cells in the 1n peak (%)<sup>b</sup> |
|-------|----------------------------------------|-----------------------------------------------|
|       | p53-KO haploid | p53-KO diploid | p53-KO haploid |
| E6.5  | 17.2          | ND<sup>c</sup> | 28.5          |
|       | 55.5          | ND             | 24.5          |
| E8.5  | 12.4          | 24.9           | 25.3          |
|       | 37.6          | 40.3           | 24.3          |
| E10.5 | 1.6           | ND             | 21.4          |
|       | 3.3           | ND             | 22.3          |

<sup>a</sup>The percentage of GFP+ cells were calculated based on all cells dissected from respective chimeric embryo by FACS analysis.

<sup>b</sup>The percentage of cells in the 1n peak were calculated based on GFP+ cells from respective chimeric embryo by FACS analysis.

<sup>c</sup>ND, not determined.
Table S4. Summary of used primers. Related to Experimental Procedures and Figures 1-5, S1 and S5

| Type                        | Target Name | Sequence 5’-3’ |
|-----------------------------|-------------|----------------|
| **p53 knockout sgRNA**     | sgRNA1-1    | CACCGAGGAGCTCCTGACACCTCGGA |
|                             | sgRNA1-2    | AAACCTCGAGTGTCAGGACCTCT   |
|                             | sgRNA2-1    | CACCGACCCTGTCAGGAGACCCCT |
|                             | sgRNA2-2    | AAACAGGGGTGTCGCTAGGACCTGC |
| **Bbe3 knockout sgRNA**    | sgRNA1-1    | CACCGGTTGGCTCTATGCCGCCGTCTC |
|                             | sgRNA1-2    | AAACGGGTAGGGGAGGCTAGGGAGC |
|                             | sgRNA2-1    | CACCGGCAGGCGTGTCGCTGACCTGC |
|                             | sgRNA2-2    | AAACCATTGCGAGGAGGCTAGGGAGC |
| **p73 knockout sgRNA**     | sgRNA1-1    | CACCGCCTGCAATACCGACTTCC |
|                             | sgRNA1-2    | AAACGGGTAGGGGAGGCTAGGGAGC |
|                             | sgRNA2-1    | CACCGGCGGCGTGTCGCTGACCTGC |
|                             | sgRNA2-2    | AAACCATTGCGAGGAGGCTAGGGAGC |
| **p53 overexpression**     | p53         | F: GCTCTAGAATGACTGCCATGAGGAGTC |
|                             |             | R: CGGAATTCTCTAATCCGCGGTCACCC |
| **Cdk2 overexpression**    | Cdk2        | F: GCTCTAGAATGTCGCTCTGAGAAGC |
|                             |             | R: CGGAATTCTCTATCCTGCTGAGGAGC |
| **MAPK11 overexpression**  | MAPK11      | F: CTTACTCCAGAATGCTGCAAGC |
|                             |             | R: CGGAATTCTCTGCTGAGGAGC |
| **qPCR**                   | Gapdh       | F: AGGTCGGTGTGGAACCCAGATGTGTAC |
|                             |             | R: TGTAGACCATGATGTTAGGGTGTA |
|                             | Oct4        | F: GGAATGGCATCTGCTGAGGACCC |
|                             |             | R: TTTCACTGGCTGAGGACTTCA |
|                             | Nanog       | F: CCAAGGGTATCTGCTAGGAGACCC |
|                             |             | R: CCCGAAGTATGAGGAGGAGC |
|                             | p53         | F: CATGAAACCGCGACCTATCC |
|                             |             | R: GCAGTTCAGAGGCAAGGACTC |
|                             | p21         | F: CCCGAGAACGTTGGAACCTT |
|                             |             | R: AGAAGTAGGAGACGGCAGAAGC |
|                             | Cyclin B    | F: GACGCGATGATGAGGAGGTG |
|                             |             | R: GCAAAACATGGGCGACACCT |
|                             | Cdk2        | F: CCTCTGACAGACTGAGGGAGC |
|                             |             | R: TGGCTGACACTTCAAGG |
|                             | Rex1        | F: CCCTCGACAGACTGAGGGAGC |
|                             |             | R: TCAGGCGACTTCAAGG |
|                             | Hand1       | F: GCAGCGTCTAGGAGCAGAAGC |
|                             |             | R: GCCAGTCTAGGAGCAGAAGC |
|                             | Cdx2        | F: GTCCCTAGGAGGAGAGTGAAGC |
|                             |             | R: TGGCTGACACTTCAAGG |
|                             | FGF5        | F: GAAGCGTCTGACACTTCAAGG |
|                             |             | R: GAAGAAAACGTCGCGCTACT |
| Gene  | Forward Primer | Reverse Primer |
|-------|----------------|----------------|
| Nestin| F: CCCTGAAGTCGAGGAGCTG | R: CTGCTGCACCTCTAAGCGA |
| T     | F: GCTTCAAGGAGCTAACTAACGAG | R: CGTCACGAAGTCCAGCAAGA |
| Klf4  | F: TCGCAGTCATGTGTGTGCTC | R: GCCTCCGGTCCTCTCCTAGGG |
| Otx2  | F: ACACACGAGGTAGTGACGC | R: TCTGAGTCGCCGTGGAAAAG |
| p73   | F: ATGCTTTACGTCGGTGACCC | R: GCACTGCTGAGCAAATTTGGAAC |
| MAPK11| F: GCCGGATTCTACCGCGCAAG | R: GAGCAGACTGAGCGCTAGGG |
| Phlda3| F: CCGTGGAGTGCGTAGAGAG | R: TCTGGATGGCCTGTTGATTCT |
| Bbc3  | F: AGCAGCACTTAGAGTCGCC | R: CCTGGGTAAGGGGAGGAGT |
| Bax   | F: TGAAGACAGGGGCCTTTTTG | R: AAATCGCCGAGACACTCG |
Supplemental Experimental Procedures

Mice
All the specific pathogen-free (SPF)-grade mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed at the Nankai University Animal Center. All experiments were guided by the Institutional Animal Care and Use Committee of Nankai University, and all experiments conformed to the relevant regulatory standards.

Cell Culture
HaESCs were derived from chemically activated oocytes with a 129Sv/Jae genetic background as described previously (Gao et al., 2018). The culture medium of the haESCs referred to a previous protocol (Shuai et al., 2014). For the purification of haploids, haESCs were incubated with 5 μg/ml Hoechst 33342 (Invitrogen, H3570) for 20 min at 37°C. Subsequently, the haploid (1n) peak was gated to sort cells with a diploid control on a Moflo XDP sorter (Beckman). For DNA content analysis, cell samples were fixed with 75% ethanol overnight and then incubated with 50 μg/ml propidium iodide (Sigma, P4170) for 30 min at 37°C. Subsequently, the haploid (1n) peak was analyzed with a diploid control on a BD LSR Fortessa flow cytometer.

Vector Construction
For the p53-, Bbc3- or p73- knockout vector, sgRNAs were designed with reference to the CRISPR design tool (www.crispr.mit.edu), then annealed and inserted into SpCas9n (BB)-2A-GFP (PX461, Addgene, #48140) vectors. For the p53-, Cdk2- and MAPK11- overexpression vector, the cDNA of p53, Cdk2 and MAPK11 were amplified from the total cDNA of mouse ESCs by using Phanta Max Super-Fidelity DNA Polymerase (Vazyme, P505-d2). Then, p53, Cdk2 and MAPK11 cDNA was linked in the reconstructive PiggyBac Dual promoter (SBI, PB513B-1) separately by using the BamHI and EcoRI sites (p53), AgeI and EcoRI sites (Cdk2) and XbaI and EcoRI sites (MAPK11). All the primers used are listed in Table S2.

Quantitative Real-time PCR
Following the manufacturer’s protocol to purify total RNAs from cells using TRIzol Reagent (Invitrogen, 15596018), cDNA was obtained by reverse transcription using a Prime Script™ RT reagent Kit with a gDNA Eraser (Takara, RR047A). Quantitative real-time PCR was performed on an ABI QuantStudio™ 6 Flex machine with FS Universal SYBR Green Master (Roche, 4913914001). Relative expression levels were normalized to Gapdh. Averages and SD values were calculated based on three independent experiments. All primers used are listed in Table S2.

Western Blotting
For western blotting experiments, protein samples were extracted from cells by using RIPA lysis solution (Solarbio, R0020) following the manufacturer’s protocol. Equal amounts of the samples were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (GE, RPN303F). The membranes were blocked in 5% nonfat dry milk for 2 hours, washed three times with PBST, and incubated with primary antibodies overnight at 4°C. Then, the membranes were washed and incubated with appropriate secondary antibodies for 1 hour at room temperature. The signal was detected with Enlight™ (Engreen, 29050) and imaged. The primary antibodies used in western blotting included anti-P53 (Santa, sc-126), anti-P73 (Abcam, ab40658) and anti-GAPDH (Santa, sc-365062).

T7EN1 Assay
Following the manufacturer’s recommended protocol for T7 Endonuclease I (NEB, E3321), the DNA fragments containing sgRNA target sites were amplified from transfected cells and wild-type cell genomic DNA by using Phanta Max Super-Fidelity DNA Polymerase (Vazyme). These two kinds of
DNA fragments were hybridized in Buffer 2.0 (NEB). After the hybridization reaction, T7 Endonuclease I was added to the mixture, and digestion was performed for 2 hours at 37 °C. The products were analyzed by agarose gel electrophoresis.

**Immunostaining, AP staining, and Karyotype Analysis**

For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde at 4°C overnight. The samples were washed three times with PBS. Then, the cells were permeabilized with 0.3% Triton X-100 (Sigma, T9284) for 40 minutes and incubated with 1% BSA (Sigma, B2064) for 40 minutes at room temperature. Next, the samples were incubated with primary antibodies against OCT4 (Abcam, ab18976), SSEA1 (Santa, sc-21702), NANOG (Abcam, ab80892), TFE3 (Sigma, HPA023881), PAX6 (Abcam, ab5790), SOX1 (Abcam, ab87775), NESTIN (Abcam, ab6320), O4 (R&D Systems, MAB1326), NEUN (Abcam, ab104225), GFAP (Abcam, ab7260), MAP2 (Abcam, ab32454) and TUJ1 (Abcam, ab18207) at 4°C overnight. The primary antibodies were diluted in blocking buffer (1% BSA). After three wash steps, the cells were stained with secondary antibodies at room temperature for 1 hour. After another three wash steps, the nuclei were stained with DAPI (YEASEN, 40727ES10) for 10 minutes at room temperature. Immunofluorescence images were taken with a TCS SP8 confocal laser scanning microscope (Leica). AP staining was performed according to the manufacturer’s instructions for the alkaline phosphatase kit (Beyotime, P0321). For karyotype analysis, cells were incubated with 0.2 μg/mL nocodazole (MCE, HY-13520) overnight. The samples treated with hypotonic solution were fixed in methanol: acetic acid (3:1) for 20 min twice and dropped onto precooled slides. Then, the cells were stained with Giemsa for 15 min before observation.

**Analysis of RNA-seq Data**

All the RNA-seq data were sequenced by a local company (Novogene). For data analysis, quality control was performed for the raw sequencing reads using the FastQC application. Raw data were trimmed using Trimmomatic to remove low-quality bases (quality score < 5) or reads (low-quality bases > 50% of the read size) (Bolger et al., 2014). Then, the abundance of each transcript was quantified using Salmon (Patro et al., 2017). Differential gene expression analysis was performed using the DEseq2 package, and gene expression was normalized using the relative-log-expression (RLE) in DESeq2 (Love et al., 2014). Genes with summed reads greater than 10 across all samples were kept. The R function of heatmap was used to generate the hierarchical clustering of the top 50 variant genes. Scatterplots were visualized with ggplot2 and ggrepel.

For Gene Ontology analysis, metascape (http://metascape.org) was employed to perform gene enrichment and functional annotation analyses in both WT ESCs and p53 KO-1 cells with the following ontology sources: KEGG Pathway, GO Biological Processes, and Reactome Gene Sets (Zhou et al., 2019). Genes with p-values < 0.001, a minimum count of 3, and an enrichment factor > 1.5 were identified and grouped into clusters based on their membership similarities.

**Cell Survival Test, Cell Growth Curve and Caspase-3 Activity**

For the cell survival test, 50,000 haploid cells were sorted and plated in a 24-well plate under feeder-free conditions. Each group included three repetitions. After 24 hours, the cells were digested into single cells and counted with a cell counter (Logos Biosystems, LUNAII). To describe the cell growth curve, the number of living cells was recorded at 24, 48, 72 and 96 hours. Averages and SD values were calculated based on three independent experiments.

To analyze the rate of diploidization, purified p53-KO, p73-KO and WT-haESCs were passaged every 2-3 days. Then, the haploid proportions were analyzed at 10 days, 14 days, 28 days and 35 days after sorting and calculated according to a previous report (Xu et al., 2017). Averages and SD values were
calculated based on three independent experiments. For the Caspase-3 activity, $5 \times 10^5$ haploid cells were sorted and plated in a 35 mm petri-dish under feeder-free condition. After 24 hours, the Caspase-3 activity was detected by the Caspase 3 Activity Assay Kit (Beyotime, C1116) follow the instructions. Averages and SD values were calculated based on three independent experiments.

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