

Erk signaling is indispensable for genomic stability and self-renewal of mouse embryonic stem cells

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Inhibition of Mek/Erk signaling by pharmacological Mek inhibitors promotes self-renewal and pluripotency of mouse embryonic stem cells (ESCs). Intriguingly, Erk signaling is essential for human ESC self-renewal. Here we demonstrate that Erk signaling is critical for mouse ESC self-renewal and genomic stability. Erk-depleted ESCs cannot be maintained. Lack of Erk leads to rapid telomere shortening and genomic instability, in association with misregulated expression of pluripotency genes, reduced cell proliferation, G1 cell-cycle arrest, and increased apoptosis. Erk signaling is also required for the activation of differentiation genes but not for the repression of pluripotency genes during ESC differentiation. Furthermore, we find an Erk-independent function of Mek, which may explain the diverse effects of Mek inhibition and Erk knockout on ESC self-renewal. Together, in contrast to the prevailing view, Erk signaling is required for telomere maintenance, genomic stability, and self-renewal of mouse ESCs.

Erk | Mek | embryonic stem cells | self-renewal | genomic stability

Embryonic stem cells (ESCs) are pluripotent and, hence, promising donor cell sources for regenerative medicine. Transcriptional regulation plays an essential role in pluripotency maintenance of ESCs, and a transcriptional regulation network for pluripotency has been characterized (1, 2). The core component of the pluripotency transcriptional regulation network is a feed-forward self-regulating circuitry formed by transcription factors Oct4, Sox2, and Nanog (3, 4). ESCs are cultured in media supplemented with growth factors. Through signaling pathways, growth factors affect the pluripotency transcriptional regulation network and regulate the self-renewal and differentiation of ESCs. For example, LIF and BMP signaling controls the transcriptional activities of the downstream effectors Stat3 and Smad1 to promote the self-renewal of mouse ESCs (5–7).

The extracellular signal-regulated kinase (Erk)/mitogen-activated protein kinase (MAPK) signal-transduction cascade mediates the effect of growth factors by sequential activation of Ras-like GTPase, Raf kinase, serine/threonine protein kinase Mek, and Erk to regulate cell-cycle progression, proliferation, differentiation, and carcinogenesis (8, 9). Erk signaling also plays a pivotal role in pluripotency maintenance. Inhibition of Mek/Erk signaling constrains the differentiation of mouse ESCs (10). Mouse ESCs can be derived and maintained in medium supplemented with inhibitors of Mek and Gsk3 signaling (2i) (11). Moreover, inhibition of Mek facilitates the conversion of mouse epiblast stem cells (epiSCs) to ESC-like cells (12). Similarly, the Mek inhibitor PD0325901 is used in establishing and maintaining human ground-state pluripotent stem cells (13–16). Conversely, activation of Mek/Erk signaling promotes the differentiation of ESCs. Ectopic expression of an activated H-RAS mutant leads to mouse ESC differentiation toward the trophectodermal lineage. Mek/Erk signaling is the downstream effector of Ras mediating the cell-fate change (17). Attenuating Erk signaling by knocking out the upstream activator Fgf4 or by Mek inhibition impairs the neural differentiation of ESCs (18, 19). Erk signaling may control ESC self-renewal by phosphorylating

pluripotency factors. For example, Erk1 and Erk2 phosphorylate Klf4 at Ser123, leading to recruitment of ubiquitin E3 ligase mediated by β TrCP1 and β TrCP2 and to Klf4 ubiquitination and degradation (20). Nanog is also demonstrated to be a substrate of Erk kinases (21, 22). Nanog phosphorylation by Erk reduces Nanog transactivation activity and protein stability (22). Recently, it has been demonstrated that in mouse ESCs, Erk2, in cooperation with PRC2, binds to developmental genes and phosphorylates RNA polymerase II at Ser5 to maintain a poised transcription status (23).

The above data are consistent with the prevailing view that Erk signaling is dispensable for ESC self-renewal but required for the differentiation of ESCs. However, FGF2, an upstream activator of ERK signaling, is a necessary growth factor for conventional human ESCs (24). Knockdown of ERK2 indeed compromises the pluripotency of conventional human ESCs. ERK2 binds to and activates genes involved in pluripotency as well as metabolism, the cell cycle, and translation in conventional human ESCs (25). The conflicting roles of Erk signaling in human and mouse ESCs might be due to species divergence. Human ESCs resemble mouse epiSCs in a primed pluripotency state, whereas mouse ESCs are maintained in a naïve pluripotency state (26, 27). Nevertheless, due to the redundant role of Erk1 and Erk2, many experiments studying Erk signaling rely on Mek inhibitors. Even

Significance

Signaling pathways regulate the self-renewal and differentiation of embryonic stem cells (ESCs). Suppression of Mek/Erk signaling by pharmacological inhibitors promotes self-renewal and pluripotency maintenance of mouse ESCs, supporting the prevailing view that Erk signaling is dispensable for ESC self-renewal. However, using inducible Erk knockout ESCs, we demonstrate that Erk signaling is critical for ESC self-renewal. ESCs cannot be maintained for more than four passages after Erk depletion, associated with misregulated expression of pluripotency genes, reduced proliferation rate, G1 cell-cycle arrest, increased apoptosis, rapid shortening of telomeres, and impaired genomic stability. We further demonstrate an Erk-independent function of Mek, which may explain the diverse effects of Mek inhibition and Erk knockout on ESC self-renewal.

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though these Mek inhibitors are claimed to be of high specificity, we cannot rule out the nonspecific effect of Mek inhibitors. Moreover, Erk1 and Erk2 have long been conceived as the only physiological substrates of Mek (8, 9). However, it remains possible that Mek has other unidentified downstream targets. Indeed, HSF1 has been identified as a novel MEK substrate (28). Therefore, to clarify the function of Erk signaling in ESCs, simultaneous knockout (KO) of *Erk1* and *Erk2* genes is required. *Erk1* and *Erk2* have been knocked out individually in mice or ESCs (29–32). However, no *Erk1* and *Erk2* double KO ESCs have been generated.

In this study, we find that constitutively active Mek1 (caMek1), but not constitutively active Erk1 or Erk2 (caErk1 or caErk2), suppresses the expression of *Nanog* in ESCs, implying that Mek1 might repress *Nanog* expression through downstream factors other than Erk1/2. To clarify the function of Erk1/2 in ESCs, endogenous *Erk1* and *Erk2* genes were disrupted in ESCs harboring an exogenous doxycycline (Dox)-inducible *Erk1* gene, resulting in *iErk1*; *Erk* KO ESCs. *iErk1*; *Erk* KO ESCs grows like wild-type (WT) ESCs when Dox is added to the medium. However, upon Dox withdrawal, Erk1 expression diminishes, leading to rapid telomere shortening, genomic instability, and compromised self-renewal of ESCs such as reduced proliferation rate, altered cell-cycle structure, and apoptosis. Consistent with previous data, ESC differentiation is blocked in the absence of Erk signaling. Moreover, with the inducible *Erk* KO ESCs, we demonstrate an Erk-independent function of Mek. Altogether, our data show that Erk signaling is indispensable for genomic stability that is critical for self-renewal and differentiation of ESCs, and also suggest that Mek regulates self-renewal and differentiation of mouse ESCs by both Erk-dependent and Erk-independent pathways.

Results

caMek1, but Not caErk1/2, Suppresses the Expression of *Nanog*. The Mek inhibitor PD0325901 (PD) has been routinely used to inhibit Mek/Erk signaling in 2i medium for mouse ESCs, and Mek inhibition enhances the expression of *Nanog* in mouse ESCs cultured in a serum/LIF condition (11, 33). We analyzed Erk phosphorylation (p-Erk) at Thr202 and Tyr204 upon Mek inhibition by PD (1 μ M), and surprisingly found that the level of p-Erk is only reduced at early time points (6 and 12 h). Nevertheless, p-Erk recovers at late time points (24 and 48 h) to a level similar to or even slightly higher than that of the starting time point (0 h). Interestingly, *Nanog* expression increases at 24 and 48 h after PD treatment, regardless of the increased level of p-Erk (Fig. 1A). N2B27 medium without serum is used for mouse ESC 2i culture (11). To rule out the possibility of medium effects on Mek inhibition, ESCs cultured in N2B27 medium supplemented with LIF also were treated with PD. The dynamic change of p-Erk is similar to that in a serum/LIF condition. Moreover, both p-Erk and *Nanog* are higher in ESCs cultured in 2i medium than in ESCs cultured in serum/LIF (Fig. 1B). These data demonstrate that Mek inhibition increases the expression of *Nanog* and is associated with an elevated p-Erk level, arguing against Mek suppressing *Nanog* expression through activating Erk signaling.

To clarify the effect of Mek and Erk signaling on *Nanog* expression, we overexpressed caMek1, caErk1, and caErk2 in mouse ESCs. Surprisingly, only caMek1, but not caErk1 or caErk2, suppresses the expression of pluripotency genes *Nanog*, *Oct4*, and *Sox2*, with the most eminent repression effect on the *Nanog* gene (Fig. 1C). At the protein level, *Nanog* expression is also reduced by caMek1 overexpression but not by caErk1 or caErk2 (Fig. 1D). These data imply that Mek1 might repress *Nanog* expression via an Erk-independent pathway.

Construction of *Erk* KO ESCs. The above data seem to be inconsistent with the prevailing view of Erk signaling in mouse ESCs. It is important to note that previous studies of Erk signaling in mouse ESCs mainly relied on Mek inhibitors (11, 12, 19). However, the possibility of the nonspecific effect of a Mek inhibitor

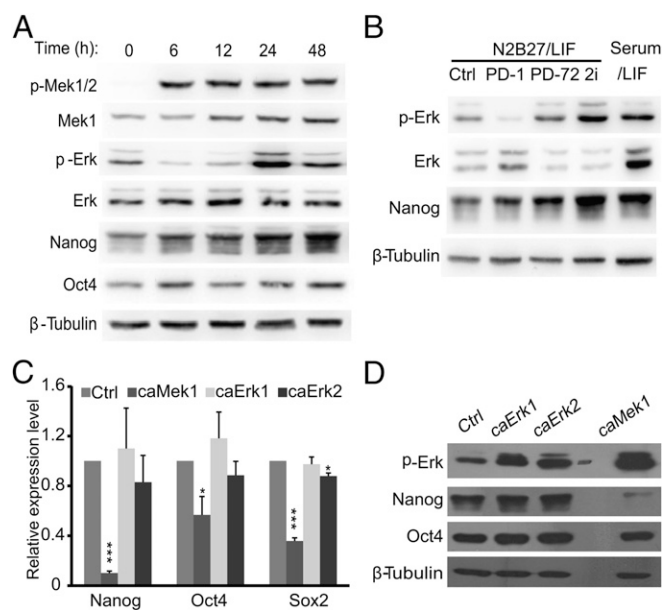


Fig. 1. *Nanog* expression is not negatively correlated with Erk phosphorylation. (A) Short-term PD treatment, but not prolonged PD treatment, reduces the level of p-Erk. V6.5 ESCs were cultured in a serum/LIF condition, and PD0325901 (1 μ M) was added to the medium at the indicated time points before harvesting. Cells were harvested and subjected to Western blot analysis. (B) Expression of p-Erk of ESCs in N2B27 medium. V6.5 ESCs were switched from the serum/LIF condition to N2B27/LIF medium for 72 h (Ctrl). PD0325901 was added to the medium at 1 h (PD-1) or 72 h (PD-72) before harvesting cells. V6.5 ESCs cultured in serum/LIF or adapted to the 2i condition (2i) for more than five passages were also included. (C and D) caMek1, but not caErk1 or caErk2, suppresses *Nanog*. Plasmids overexpressing caMek1, caErk1, and caErk2 were transfected into V6.5 ESCs. Cells were harvested 48 h after transfection. The expression of pluripotency genes *Nanog*, *Oct4*, and *Sox2* was determined by quantitative RT-PCR (C) and Western blot (D). * $P < 0.05$; *** $P < 0.001$. Error bars are standard deviations (SDs).

or an additional downstream effector(s) of Mek cannot be ruled out. To elucidate the roles of Erk signaling in ESCs, we applied a genetic approach to delete the *Erk1* and *Erk2* genes. *Erk1*^{−/−} or *Erk2*^{−/−} ESCs were established from WT ESCs (V6.5, KH2, iMek1, and iErk1) by transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) technology (Fig. S1A and B). The CRISPR/Cas system seems to be more efficient than the TALENs (Table S1). Due to the redundant role of Erk1 and Erk2 (34), it is necessary to generate *Erk1* and *Erk2* double KO ESCs to study the function of Erk signaling. Thus, we attempted to knock out *Erk1* and *Erk2* in *Erk2*^{−/−} and *Erk1*^{−/−} ESCs, respectively. However, no *Erk1*^{−/−}; *Erk2*^{−/−} ESC colonies can be identified, even though the cutting efficiency of TALENs and CRISPR/Cas remains unchanged in *Erk2*^{−/−} and *Erk1*^{−/−} ESCs. These data imply that Erk signaling is required for mouse ESCs.

We then used another strategy to generate *Erk* KO ESCs (Fig. S1C). First, both alleles of *Erk1* were disrupted in KH2 cells by TALENs (*Erk1*^{−/−} ESCs) (35). An exogenous *Erk1* gene driven by a tetracycline-inducible promoter was integrated into the engineered *ColA1* locus through FLPe recombinase-mediated recombination, resulting in a Dox-inducible *Erk1* transgene (*iErk1*; *Erk1*^{−/−} ESCs). Then, *iErk1*; *Erk1*^{−/−} cells were transfected with the Cas9 vector targeting *Erk2* and cultured in the presence or absence of Dox. A week later, ESC colonies from single cells were picked, and the genotypes of *Erk2* in these ESC clones were analyzed. Without Dox, no *Erk2*^{−/−} ESCs were isolated from *iErk1*; *Erk1*^{−/−} ESCs. In contrast, when *Erk1* was induced by Dox, both *Erk2* alleles were modified in 11 out of 45 picked colonies (Table S1). Sequencing results confirmed that both *Erk2* alleles are disrupted in two ESC

lines (*iErk1*; *Erk KO* ESCs) out of three sequenced clones (Fig. S1D). With the same strategy, *iErk2*; *Erk KO* ESCs were also established (Fig. S2A). We selected two *iErk1*; *Erk KO* ESC lines and two *iErk2*; *Erk KO* ESC lines for subsequent analysis. All data are consistent among these four cell lines. Only one set of data from one *iErk1*; *Erk KO* ESC line is shown in the main text, and one set of data from one *iErk2*; *Erk KO* ESC line is shown in Supporting Information (Figs. S2–S4).

Erk Depletion Compromises ESC Self-Renewal. We demonstrated that Erk1 expression diminishes in *iErk1*; *Erk KO* ESCs 48 h after Dox withdrawal (Fig. 2A). Thus, we are able to induce Erk depletion by withdrawing Dox, and *iErk1*; *Erk KO* ESCs cultured in the absence of Dox for longer than 48 h are considered to be *Erk KO* ESCs. When *iErk1*; *Erk KO* ESCs were continuously cultured in serum/LIF ESC medium without Dox, the colonies became flattened and less compacted (Fig. 2B). After three or four passages in the absence of Dox, cell proliferation rate is significantly reduced and the cell culture crashes (Fig. 2C). The expression of pluripotency genes *Nanog*, *Oct4*, and *Sox2* gradually decreases after Dox withdrawal, except for elevated expression of *Nanog* at passage 1 (Fig. 2D and E). *Erk KO* leads to the suppression of endodermal, mesodermal, and trophodermal markers, whereas ectodermal markers, *Fgf5* and *Nestin*, are transiently activated after *Erk KO* (Fig. 2F). These data suggest that Erk signaling is indispensable for ESC self-renewal.

To address whether the kinase activity of Erk is required for ESC self-renewal, WT Erk2 and kinase-dead mutant (K52R) and phosphorylation-site mutant (T183A, Y185A) of Erk were introduced into *iErk1*; *Erk KO* ESCs. Only WT Erk2, but not the two Erk2 mutants, is able to rescue the slow proliferation rate induced by Erk depletion (Fig. S5B), suggesting that the kinase activity of Erk indeed is essential for ESC self-renewal.

Erk Signaling Is Essential for ESC Differentiation. Next, we asked whether Erk signaling is essential for the differentiation of mouse ESCs. *iErk1*; *Erk KO* ESCs were cultured in ESC medium with or without Dox for 48 h and allowed to form embryoid bodies (EBs) with the hanging-drop method. EBs formed by *Erk KO* cells have irregular edges, rather than smooth and round edges, and are smaller than EBs formed by cells with Erk (Fig. 3A). Moreover, without Erk signaling, lineage-specific genes, including ectodermal, endodermal, mesodermal, and trophodermal genes, are not activated in EBs, whereas the down-regulation of pluripotency markers *Nanog*, *Oct4*, and *Sox2* is not affected by *Erk KO* (Fig. 3B). In a neural differentiation system (36), *Erk KO* ESCs are defective in activating neural markers *NeuroD3*, *Ngn2*, and *Nestin*. Again, pluripotency markers *Nanog*, *Oct4*, and *Sox2* are repressed after differentiation, regardless of the absence or presence of Erk signaling (Fig. 3C). These data indicate that during ESC differentiation, Erk signaling is essential for the activation of differentiation genes but is not required for the suppression of pluripotency genes.

Lack of Erk Perturbs the Cell Cycle and Induces Cell Death. To understand the underlying mechanism for the reduced proliferation rate of *Erk KO* ESCs, we analyzed the cell-cycle profile of *iErk1*; *Erk KO* ESCs after Dox withdrawal by propidium iodide (PI) staining. It is notable that the sub-G1 population (apoptotic cells) and G1 cells increase over time after Dox withdrawal (Fig. 4A), indicating that *Erk KO* leads to cell arrest at the G1 phase, as well as apoptosis. Consistently, increased cell apoptosis after *Erk KO* was detected by Annexin V/PI staining (Fig. S5F).

Three major MAPK pathways, Mek/Erk, JNK, and p38, cross-talk with each other (37). Inhibition of p38 and JNK supports naive pluripotency in human ESCs (13). Moreover, Erk and p38 signaling have been implicated in nitric oxide-induced apoptosis of mouse ESCs (38). We hypothesized that *Erk KO* might activate p38, consequently triggering apoptosis of ESCs. To test this hypothesis, we first examined the expression dynamics of p38 and

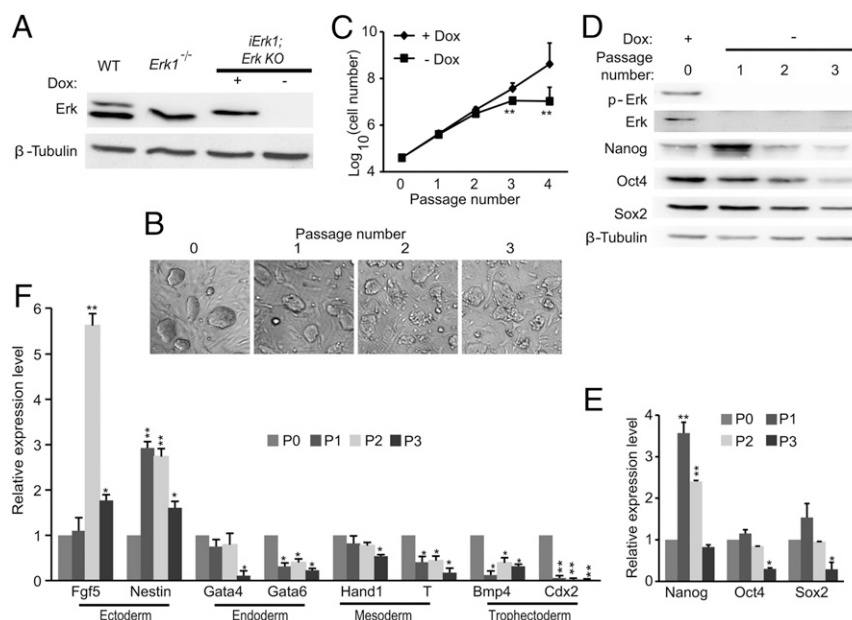


Fig. 2. Erk signaling is essential for the self-renewal of ESCs. (A) Western blots demonstrate the diminishment of Erk expression in *iErk1*; *Erk KO* ESCs at 48 h after Dox withdrawal. (B) Colony morphology change upon *Erk KO*. *iErk1*; *Erk KO* ESCs were continuously cultured in the absence of Dox for three passages. Phase-contrast images of ESC colonies at each passage are shown. (C) Reduced proliferation of ESCs after *Erk KO*. *iErk1*; *Erk KO* ESCs were cultured in serum/LIF medium with or without Dox. Cell numbers were counted every passage, and equal amounts of ESCs were plated onto tissue-culture dishes. (D–F) Expression dynamics of pluripotency and differentiation markers after *Erk KO*. Cells were cultured as described in C. The protein (D) and mRNA (E) levels of pluripotency markers *Nanog*, *Oct4*, and *Sox2* were measured by Western blot and quantitative RT-PCR. (F) Expression of differentiation genes after *Erk KO*. * $P < 0.05$; ** $P < 0.01$. Error bars are SDs.

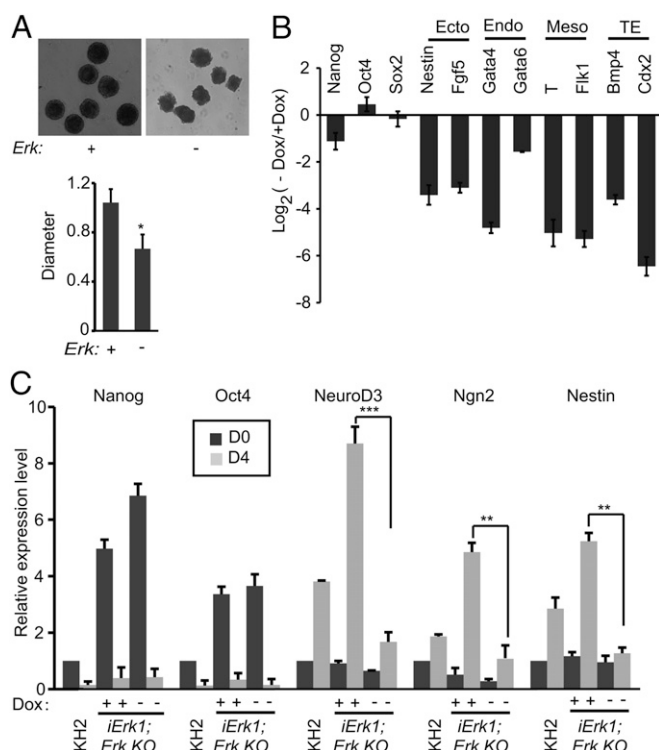


Fig. 3. Erk signaling is required for ESC differentiation. (A and B) *iErk1*; *Erk* KO ESCs were cultured with or without Dox for 48 h. The cells were then used for EB differentiation, again with or without Dox. (A) Images of day 4 EBs with or without Dox. The relative diameters of day 4 EBs with or without Dox ($n > 10$) are plotted. (B) RNA was isolated from day 4 EBs and analyzed by quantitative RT-PCR. TE, trophectoderm. (C) *iErk1*; *Erk* KO ESCs were cultured with or without Dox for 48 h (D0, day 0). These cells, as well as KH2 ESCs, were cultured in N2B27 with or without Dox to induce neural differentiation for 4 d. The expression of pluripotency genes and neural genes in the resulting cells was analyzed by quantitative RT-PCR. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars are SDs.

phosphorylated p38 (p-p38) at Thr180 and Tyr182 after *Erk* KO. Indeed, the level of p-p38 increases upon *Erk* KO, whereas total p38 remains stable (Fig. 4B). We then treated *iErk1*; *Erk* KO ESCs with a p38 inhibitor, SB203580, upon Dox withdrawal. The number of apoptotic cells induced by *Erk* KO is significantly reduced by p38 inhibition, demonstrated by both PI staining and Annexin V/PI staining (Fig. 4C and D and Fig. S5G). Knockdown of p38 by shRNA also suppresses *Erk* KO-induced apoptosis (Fig. S5H and I). Therefore, *Erk* KO triggers ESC apoptosis through activation of p38. Nevertheless, inhibition of p38 does not affect G1 cell-cycle arrest caused by *Erk* KO (Fig. 4D).

Erk Depletion Leads to Rapid Telomere Shortening and Genomic Instability. Genomic stability is essential for the self-renewal and potentiality of ESCs (39). Telomere damage and dysfunction can contribute to genomic instability and impair cell proliferation (40, 41). Impaired genomic stability resulting from lack of Erk might also reduce cell proliferation and contribute to increased apoptosis in ESCs. To test this hypothesis, we analyzed chromosome integrity and the length of telomeres in *Erk* KO ESCs by metaphase spread and telomere quantitative fluorescence in situ hybridization (Q-FISH). Telomere Q-FISH revealed that telomeres of ESCs shorten within only two passages after Erk depletion (Fig. 5A). Rapid telomere shortening in *Erk* KO ESCs was further validated by quantitative PCR (Fig. 5C). Meanwhile, the genomic stability of ESCs is compromised. Chromosome fusion and breakage events are more frequent in *Erk* KO ESCs in com-

parison with those of WT ESCs (Fig. 5A and B). Telomeres are primarily elongated by telomerase (42). However, the expression levels of key telomerase genes, *Tert* and *Terc*, are only reduced by less than twofold in *Erk* KO ESCs (Fig. S5D). Even in the absence of telomerase activity, around 50 d of culture and 15 passages are required to detect comparable telomere shortening in *Terc*^{-/-} ESCs and induced pluripotent stem cells (iPSCs), respectively (43, 44). Therefore, telomere shortening induced by *Erk* KO is unlikely caused by lack of telomerase activity. Instead, rapid telomere shortening might be due to DNA damage. Indeed, we noticed that DNA damage at telomeres, as indicated by γ H2AX and Trf1 colocalized foci, gradually increases after *Erk* KO (Fig. 5D–F). Reduced expression of Trf1 and an unchanged level of γ H2AX (Fig. 5G) imply that Erk signaling is involved in safeguarding telomeric DNA and telomere structure but not overall genomic DNA.

We have shown that activation of p38 induces apoptosis of *Erk* KO ESCs. Telomere shortening and genome instability might trigger apoptosis. We then asked whether p38 activation is upstream of telomere shortening in order to cause apoptosis. p38 inhibition cannot prevent rapid telomere shortening in *Erk* KO ESCs (Fig. S5J), suggesting that the impaired genome stability and rapid telomere shortening are not caused by p38 activation.

Erk-Dependent and -Independent Effect of Mek Inhibition in ESCs. To gain insights into the role of Erk signaling in Mek inhibition of ESCs, six samples, WT KH2 ESCs treated with or without PD for 48 h (KH2+PD and KH2, respectively), *iErk1*; *Erk* KO ESCs

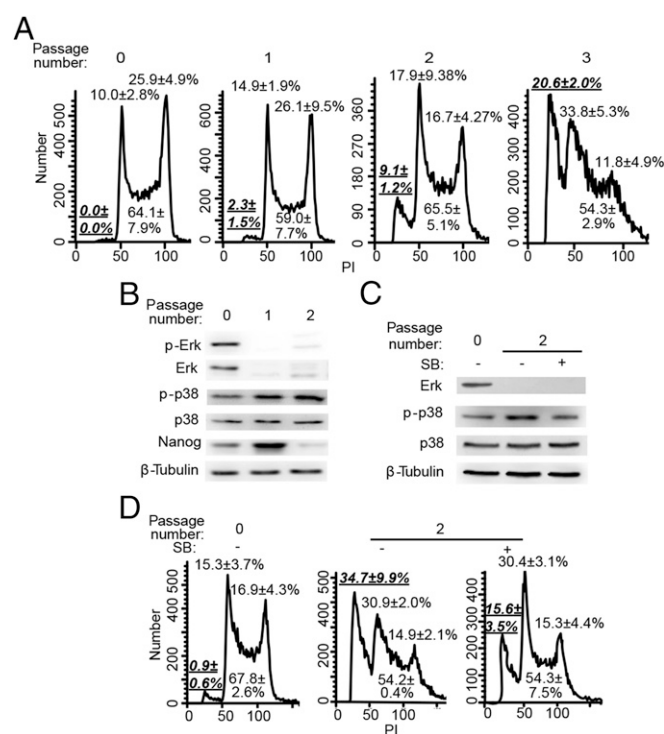


Fig. 4. Loss of Erk signaling leads to G1 cell-cycle arrest and apoptosis of ESCs. (A) Cell-cycle analysis of *iErk1*; *Erk* KO ESCs at different passages after Dox withdrawal. (B) p38 is activated upon *Erk* KO. The expression of p38 and p-p38 in *iErk1*; *Erk* KO ESCs at different passages after Dox withdrawal was determined by Western blot. (C and D) Inhibition of p38 suppresses ESC apoptosis induced by *Erk* KO. *iErk1*; *Erk* KO ESCs were cultured in the absence of Dox for two passages, with or without the p38 inhibitor SB203580. (C) Expression of p38 and p-p38 in *Erk* KO ESCs treated with or without SB203580 (SB). (D) Cell-cycle analysis was performed with these cells. (A and D) Percentages of sub-G1 cells in total cells (in bold and underlined) and percentages of G1, S, and G2/M cells in nonapoptotic cells are shown.

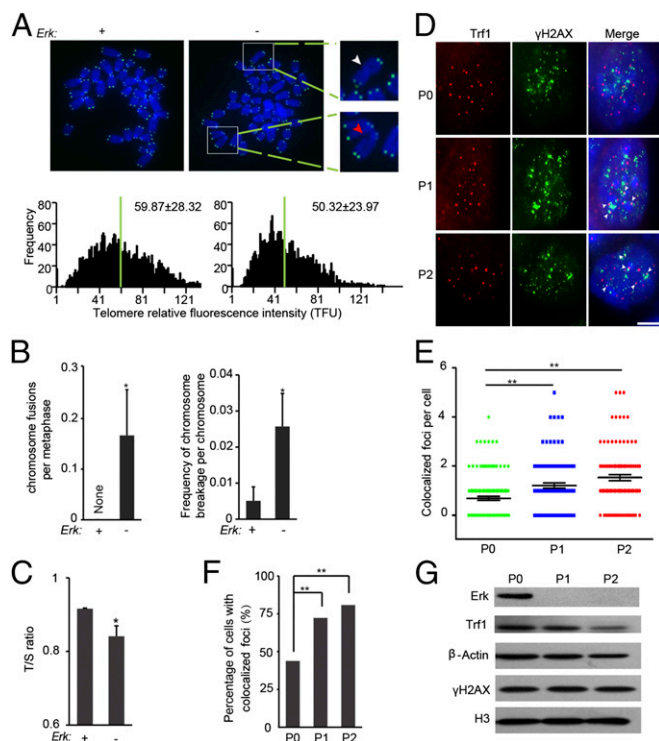


Fig. 5. Rapid telomere shortening occurs in *Erk KO* ESCs. *iErk1*; *Erk KO* ESCs with Dox or two passages after Dox withdrawal were subjected to telomere Q-FISH analysis (A) and telomere/single copy gene 36B4 (T/S) ratio analysis (C). (A, Upper) Telomere Q-FISH images of chromosome spreads. Blue, DAPI-stained chromosomes; green dots, telomeres. Blown-up images are examples of chromosome fusion (marked with a white arrowhead) and chromosome breakage (indicated by a red arrowhead). (A, Lower) Histograms showing the distribution of relative telomere length displayed as TFU by Q-FISH. Green bars mark the average telomere length. Mean \pm SD of telomere length is shown in each panel. Heavy black bars on the y axis show the frequency of telomere signal-free ends. Fifteen chromosome spreads were quantified for each group. (B) Quantification of chromosome fusion and chromosome breakage frequencies from the Q-FISH data in A. Mean \pm SE are plotted ($n \geq 15$). (C) Relative telomere length shown as T/S ratio measured by quantitative PCR. (D–G) *iErk1*; *Erk KO* ESCs cultured with Dox, 48 and 96 h after Dox withdrawal, were subjected to immunofluorescence and Western blot analyses. (D) Immunofluorescence images of TRF1 (red) and γ H2AX (green). Colocalized foci are indicated by arrowheads. (Scale bar, 5 μ m.) Approximately 100 nuclei from images captured in D were quantified for colocalized TRF1 and γ H2AX foci per cell (E) and percentage of cells with TRF1 and γ H2AX colocalized foci (F). Mean \pm SE are shown in E. (G) Protein levels of TRF1 and γ H2AX as measured by Western blot. * $P < 0.05$; ** $P < 0.01$.

cultured with Dox (P0) and 48 and 96 h after Dox withdrawal (P1 and P2, respectively), and *iErk1*; *Erk KO* ESCs cultured without Dox for 96 h and treated with PD in the last 48 h (P2+PD), were subjected to RNA-sequencing (RNA-seq) analysis. By comparing KH2+PD with KH2 [denoted Meki (WT)], 570 up-regulated genes and 196 down-regulated genes were identified; comparison between P1 and P0 (named *Erk KO*) identified 362 up-regulated genes and 290 down-regulated genes; and 186 up-regulated genes and 67 down-regulated genes were identified by comparing P2+PD with P2 [called Meki (*Erk KO*)] (Dataset S1). Consistent with the role of Erk signaling in ESC differentiation, the differentially expressed genes in the three comparisons are enriched for differentiation- and development-related genes (Dataset S2). Clustering analysis revealed that Meki (WT) is more closely related to Meki (*Erk KO*) than to *Erk KO* (Fig. 6A). Venn diagrams also show that only a small proportion of genes, 57 up-regulated genes and 66 down-regulated genes, are shared by Meki (WT) and *Erk KO*

(Fig. 6B). The RNA-seq results were confirmed by quantitative RT-PCR for selected genes, including Meki-regulated genes (*Thbs1*, *Postn*, and *Fgf5*), *Erk KO*-regulated genes (*Gata6*, *Egr1*, and *Dusp14*), and both Meki- and *Erk KO*-regulated genes (*Myc*, *Bmp4*, and *Prdm14*) (Fig. S6A–C). These data suggest that Mek inhibition and *Erk KO* have quite distinct effects on the transcriptional profile of ESCs.

When comparing Meki (WT) with Meki (*Erk KO*), fewer genes are affected by PD treatment in *Erk KO* ESCs than in WT ESCs, consistent with the current view that PD inhibits Mek and subsequently Erk signaling to regulate gene expression. Nevertheless, a significant fraction of genes, 156 up-regulated genes and 44 down-regulated genes, are regulated by PD treatment in the same way, regardless of the presence or absence of Erk signaling (Fig. 6B). The RNA-seq results of selected genes, including *Dusp6*, *Dusp9*, *Bmp4*, and *Postn*, were validated by quantitative RT-PCR (Fig. S6D). These data demonstrate that PD regulates a group of genes through an Erk-independent mechanism, whereas Erk signaling mediates the PD effect for the majority of genes.

The Erk-independent effect of PD might be due to the off-target effect of PD or the Erk-independent function of Mek. To demonstrate that Mek might have a biological function independent of Erk signaling, caMek1 was overexpressed in *iErk1*; *Erk KO* ESCs with or without Dox, and the expression of several known Mek downstream genes was analyzed by quantitative RT-PCR. caMek1 fails to suppress *Prdm14* in *Erk KO* ESCs. Thus, Mek1 regulates *Prdm14* through Erk signaling. In contrast, *Klf4* and *Tbx3* are

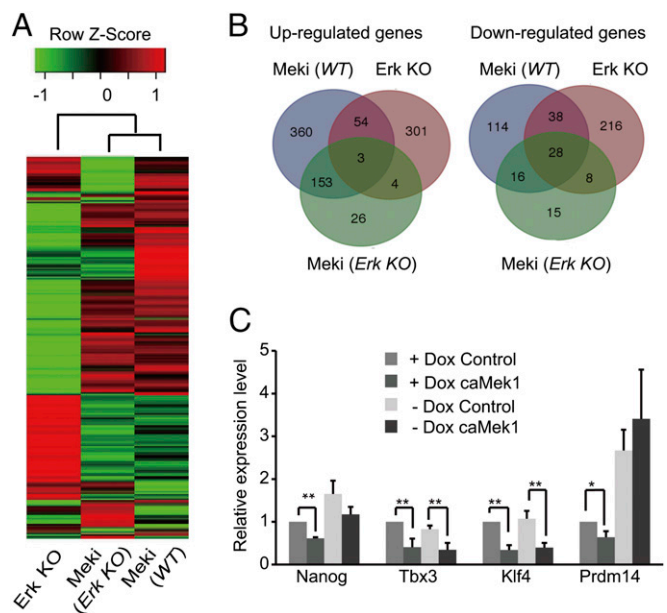


Fig. 6. Erk-dependent and -independent functions of Mek. (A and B) RNA-seq analysis of six samples: WT KH2 ESCs treated with or without PD for 48 h (KH2+PD and KH2, respectively), *iErk1*; *Erk KO* ESCs cultured in the presence of Dox (P0) and 48 and 96 h after Dox withdrawal (P1 and P2, respectively), and *iErk1*; *Erk KO* ESCs cultured without Dox for 96 h and treated with PD in the last 48 h (P2+PD). Meki (WT), Meki (*Erk KO*), and *Erk KO* are paired comparisons of KH2+PD and KH2, P2+PD and P2, and P1 and P0, respectively. Differentially expressed genes were identified in these three comparisons with the criteria of more than twofold change and false discovery rate (FDR) < 0.001 (Dataset S1). (A) Clustering analysis of differentially regulated genes. (B) Venn diagrams of up- and down-regulated genes from three conditions. (C) caMek1 represses the expression of *Klf4*, *Tbx3*, and *Nanog* in the absence of Erk signaling. *iErk1*; *Erk KO* ESCs with Dox or 48 h after Dox withdrawal were transfected with empty vector or caMek1 overexpression vector. Forty-eight hours after transfection, cells were harvested for quantitative RT-PCR analysis. * $P < 0.05$; ** $P < 0.01$. Error bars are SDs.

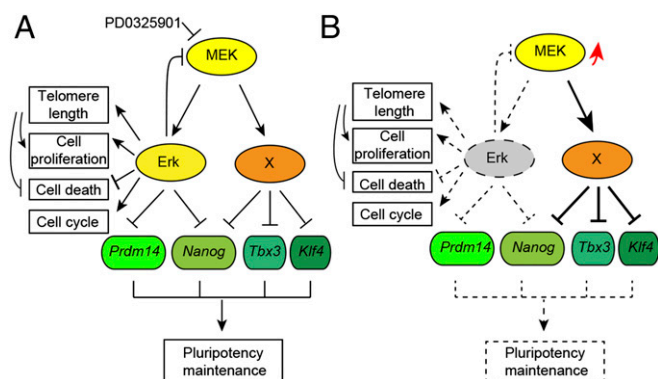


Fig. 7. Working model for Mek/Erk signaling in mouse ESCs. (A) Mek might suppress the expression of pluripotency genes through Erk-dependent and -independent pathways. In addition to its role in repressing *Prdm14* and *Nanog*, Erk signaling is required for cell proliferation, cell-cycle progression, and telomere-length maintenance as well as suppression of apoptosis. (B) Upon Erk KO, Mek and its Erk-independent pathway are activated (marked by a red arrow) to perturb the pluripotency regulation network.

suppressed by caMek1 in both WT and *Erk KO* cells. caMek1 also down-regulates the expression of *Nanog* in *Erk KO* ESCs, even though the difference is not statistically significant (Fig. 6C). These data indicate that Mek may regulate gene expression independent of Erk signaling.

Discussion

To clarify the role of Erk signaling in mouse ESCs, we constructed inducible *Erk KO* ESCs and demonstrated that Erk signaling is indispensable for both self-renewal and differentiation of ESCs. Erk negatively regulates the expression of pluripotency genes, especially for *Nanog*, in mouse ESCs. At 48 h after Dox withdrawal, *Nanog* expression is increased in *iErk1*; *Erk KO* ESCs (Fig. 2 D and E), likely due to the suppression of the heterogeneous expression of *Nanog* in ESCs (Fig. S5A). Consistently, enhanced expression of pluripotency genes, including *Nanog*, *Tbx3*, *Prdm14*, and *Klf4*, has been observed in *Erk2 KO* ESCs and *Erk2 KO/Erk1* knockdown ESCs (23, 30). In addition to transcriptional regulation, Erk may regulate the protein stability of pluripotency factors, such as *Nanog* and *Klf4*, by phosphorylation modification (20–22). The down-regulation of pluripotency genes after prolonged culture of *Erk KO* ESCs might be an indirect effect of loss of Erk signaling. For example, loss of Erk signaling activates Mek (Fig. S5E), which in turn may function through an Erk-independent mechanism to suppress pluripotency genes. However, Erk signaling is not required for suppressing pluripotency genes during ESC differentiation. In EB and neural differentiation experiments, pluripotency genes *Oct4*, *Sox2*, and *Nanog* are repressed in *Erk KO* cells (Fig. 3 B and C). In contrast, the activation of differentiation genes requires Erk signaling. *Erk KO* leads to down-regulation of differentiation genes in ESCs (Fig. 2F), and failure to activate differentiation genes, when ESCs differentiate into EBs or neural cells (Fig. 3 B and C). It is consistent with the role of Erk in phosphorylating RNA polymerase II and maintaining poised transcription status of differentiation genes (23). It has been reported that *Erk2* is dispensable for multilineage commitment (30). In that study, only *Erk2* is knocked out, and the redundant function of *Erk1* ensures normal differentiation of ESCs.

The pluripotency status of ESCs is associated with rapid progression through the cell cycle and a short G1 phase (45). Even though Erk signaling promotes the G1-to-S transition by activating cyclin D1 expression in somatic cells (46), Erk phosphorylation has been shown to be dispensable for the progression from G1 to S phase in ESCs (47). However, we observed that loss of Erk signaling induces G1 cell-cycle arrest and cell

death of ESCs (Fig. 4A). ESCs express low levels of D-type cyclins and undetectable cyclin D-Cdk4-associated kinase activity (48). Rather, increased cyclin E and extended cyclin E-Cdk2 activity lead to hyperphosphorylation of Rb and facilitate the G1-to-S phase transition (49, 50). When the expression of cell-cycle regulators for the G1-to-S transition was examined, we found that *cyclins D1, D3, E2, Cdk2, Cdk4, Cdk6, Cdc25A*, and *c-Myc* are down-regulated at passage 2 after *Erk KO* (Fig. S5C). Given the importance of cyclin E-Cdk2 activity in cell-cycle progression of ESCs, the reduced expression of *Cdk2* and *cyclin E2* might contribute to G1 cell-cycle arrest in *Erk KO* ESCs. In addition, down-regulation of *c-Myc*, which activates the transcription of *cyclin E* and *Cdc25A* (51), might be another reason for *Erk KO*-induced G1 arrest.

Erk KO triggers apoptosis of ESCs through the activation of p38. It has been shown that dual-specificity phosphatases (Dusps) mediate the cross-talk among three major MAPK cascades, Erk, JNK, and p38 (37). RNA-seq detected down-regulation of multiple *Dusp* genes, including *Dusp4*, *Dusp5*, *Dusp6*, and *Dusp9*, upon *Erk KO*, which might lead to the activation of p38 and apoptosis subsequently. In addition, genes regulating cell death, which are differentially expressed upon *Erk KO* but not by PD treatment, were also identified by RNA-seq. These genes, including up-regulated positive regulators for cell death *Fgd4*, *Smpd2*, and *Tiam2* and down-regulated negative regulators for cell death *Cntfr*, *Nrbp2*, and *Nrg1*, might contribute to the apoptosis in *Erk KO* ESCs. Moreover, *Erk KO*-induced rapid shortening of telomeres might initiate apoptosis as well. A component of the shelterin complex, *Trf1*, deletion of which leads to apoptosis of iPSCs (52), is down-regulated upon *Erk KO* (Fig. 5G). This might be another reason for *Erk KO*-induced apoptosis.

Notably, telomere damage and rapid shortening occur in *Erk KO* ESCs. Within two passages, the average length of telomeres is reduced by about 9 kb (Fig. 5A). This is unlikely due to lack of telomerase activity. The rate of telomere loss has been measured as 50–100 bp per cell division in somatic cells, in which telomerase activity is low or absent (53). Around six or seven cell doublings occur during two passages of mouse ESC culture. If lacking telomerase is the only reason for telomere shortening, telomeres should become shorter by less than 1 kb. Moreover, much slower telomere shortening was observed in telomerase-deficient mouse ESCs and iPSCs than in *Erk KO* ESCs (43, 44). In fact, increased DNA damage was detected at telomeres in *Erk KO* ESCs, whereas the overall DNA damage signal remains stable (Fig. 5 D–G). Therefore, Erk signaling might be involved in maintaining telomere structure and preventing DNA damage. *Erk KO* is associated with down-regulation of *Trf1* (Fig. 5G). It has been shown that deletion of *Trf1* leads to telomere fusion and increased telomere fragility but not telomere shortening (54, 55). Thus, reduced expression of *Trf1* might account for the increased chromosome fusion and breakage in *Erk KO* ESCs. However, rapid telomere shortening in *Erk KO* ESCs could be caused by other mechanisms. Targeted telomere insertion (TTI) might contribute to telomere shortening (56). Whether TTI is activated in *Erk KO* ESCs needs to be validated experimentally. Additional causes for genetic and epigenetic instability following the depletion of Erk cannot be excluded, and remain to be explored.

Our results do not support the prevailing view that Erk signaling is dispensable for ESC self-renewal. Almost all previous data are from experiments using pharmacological inhibitors of Mek. Thus, the discrepancy might be due to the difference between pharmacological inhibition of Mek and genetic KO of *Erk*. Obviously, the off-target effect of Mek inhibitors might account for the difference between Mek inhibition and *Erk KO*. Nevertheless, the off-target effect of Mek inhibitors is unlikely to be the reason, because multiple Mek inhibitors, including U0126, PD98059, PD184352, and PD0325901, have been shown to have similar effects in promoting pluripotency maintenance (10, 11, 57). Instead, Mek inhibition and

Erk KO have distinct effects on Erk signaling. Upon *Erk* KO, Erk signaling is completely blocked. However, during Mek inhibition, Erk signaling is only attenuated, because the Mek-Erk cascade has properties of a negative-feedback amplifier, stabilizing outputs in response to drug-induced perturbations (58, 59). Furthermore, we found that Mek inhibition only reduces p-Erk levels at an early time point, and the level of p-Erk comes back to a level similar to or even higher than that before Mek inhibition (Fig. 1*A* and *B*). Activation of Mek and down-regulation of Erk phosphatase might contribute to the elevated p-Erk level after prolonged Mek inhibition. The level of phosphorylated Mek1/2 at Ser217/221 is enhanced after Mek inhibition (Fig. 1*A*). Our RNA-seq and quantitative RT-PCR results demonstrate the down-regulation of several dual-specificity phosphatase genes, including *Dusp6* and *Dusp9*, upon Mek inhibition (Fig. S6*D*). The latter has been shown to be a key downstream target gene of BMP4 signaling to modulate Erk activity in mouse ESCs (60). Moreover, Mek might function through another immediate downstream target(s), in addition to Erk1/2. Even though Erk1/2 have been conceived as the only substrates for Mek1/2 (8, 9), a recent study identified HSF1 as a novel MEK substrate (28). Our data also provide evidence that Mek might have a function(s) independent of Erk1/2. First, Mek inhibition regulates a large group of genes (643 genes), which are not affected by *Erk* KO (Fig. 6*B*). Second, with or without Erk signaling, a significant portion of genes (200 genes) responds to PD treatment in the same way (Fig. 6*B* and Fig. S6*D*). This group of genes is likely regulated by Mek through an Erk-independent mechanism. Third, in *Erk* KO cells, overexpression of caMek1 represses the expression of pluripotency genes *Nanog*, *Klf4*, and *Tbx3*, further confirming the Erk-independent function of Mek (Fig. 6*C*). More investigation is required to identify a novel Mek substrate(s) involved in pluripotency maintenance.

In summary, our work has demonstrated that Erk signaling is indispensable not only for ESC differentiation but also for the self-renewal of mouse ESCs. Erk signaling has dual roles in pluripotency regulation. On one hand, it represses the expression of pluripotency genes such as *Prdm14* and *Nanog*. On the other hand, Erk is required for cell proliferation, cell-cycle progression, and telomere-length maintenance, as well as suppression of apoptosis in ESCs (Fig. 7*A*). Thus, *Erk* KO leads to reduced proliferation, G1 arrest, rapid telomere shortening, and apoptosis of ESCs. In addition, upon *Erk* KO, Mek is activated, and may subsequently exert its Erk-independent function to suppress the expression of pluripotency genes, including *Tbx3* and *Klf4*, perturbing the pluripotency maintenance network (Fig. 7*B*). In contrast to the effect of *Erk* KO, Mek

inhibition by PD0325901 facilitates the maintenance of pluripotency, because both Erk-dependent and -independent functions of Mek are suppressed. Moreover, during PD0325901 treatment, residual Erk activity remains to facilitate the self-renewal of ESCs. Our data suggest an Erk-independent function of Mek. Characterization of this Erk-independent function of Mek is of great significance not only for understanding the role of Mek in pluripotency maintenance but also for cancer therapy targeting MEK.

Materials and Methods

Quantitative RT-PCR. Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche) with random primers according to the manufacturer's instructions. PCR was performed with FastStart Universal SYBR Green Master (Roche) in a Bio-Rad iQ5 System. PCR cycling conditions were 95 °C for 2 min and 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s, and then a melting curve of the amplified DNA was acquired. Quantification of target genes was normalized with β -actin. Primer information is listed in Table S2.

Telomere Q-FISH. Telomere length and function (telomere integrity and chromosome stability) were measured by telomere Q-FISH, as described previously (61). Telomeres were denatured at 80 °C and hybridized with telomere PNA probe (0.5 μ g/mL) (Panagene). Chromosomes were stained with 0.5 μ g/mL DAPI. Fluorescence from chromosomes and telomeres was digitally imaged on a Zeiss microscope with FITC/DAPI filters, using AxioCam and AxioVision software 4.6. Telomere length shown as telomere fluorescence unit (TFU) was integrated using the TFL-TELO program (a gift kindly provided by P. Lansdorp, Terry Fox Laboratory, Vancouver, Canada).

RNA Sequencing. Construction of an RNA-sequencing library, sequencing with Illumina HiSeq 2000, and bioinformatic analysis were performed by BGI Tech. The RNA-seq dataset (accession no. GSE70304) has been deposited in the Gene Expression Omnibus database.

Statistical Analysis. Data were analyzed by two-tailed Student's *t* test, except for Fig. 5*F*, in which χ^2 test was performed to calculate the *P* value. Statistically significant *P* values are indicated in the figures as follows: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Averages and SDs of at least three independent experiments are shown in the figures when applicable.

Detailed information is provided in *SI Materials and Methods*.

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