ERK2 Suppresses Self-Renewal Capacity of Embryonic Stem Cells, but Is Not Required for Multi-Lineage Commitment

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Abstract

Activation of the FGF-ERK pathway is necessary for naïve mouse embryonic stem (ES) cells to exit self-renewal and commit to early differentiated lineages. Here we show that genetic ablation of Erk2, the predominant ERK isozyme expressed in ES cells, results in hyper-phosphorylation of ERK1, but an overall decrease in total ERK activity as judged by substrate phosphorylation and immediate-early gene (IEG) induction. Normal induction of this subset of canonical ERK targets, as well as p90RSK phosphorylation, was rescued by transgenic expression of either ERK1 or ERK2 indicating a degree of functional redundancy. In contrast to previously published work, Erk2-null ES cells exhibited no detectable defect in lineage specification to any of the three germ layers when induced to differentiate in either embryoid bodies or in defined neural induction conditions. However, under self-renewing conditions Erk2-null ES cells express increased levels of the pluripotency-associated transcripts, Nanog and Tbx3, a decrease in Nanog-GFP heterogeneity, and exhibit enhanced self-renewal in colony forming assays. Transgenic add-back of ERK2 is capable of restoring normal pluripotent gene expression and self-renewal capacity. We show that ERK2 contributes to the destabilization of ES cell self-renewal by reducing expression of pluripotency genes, such as Nanog, but is not specifically required for the early stages of germ layer specification.

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Introduction

Pluripotent mouse embryonic stem (ES) cells are immortal, karyotypically stable, cell lines derived from the inner cell mass (ICM) or early epiblast of pre-implantation embryos [1–3]. They maintain many of the characteristics of their in vivo counterparts even after prolonged periods of culture, not least of which is the ability to generate differentiated cell types of all three germ layers as well as contribute to the germ line [4]. The efficient derivation and maintenance of ES cells is heavily dependent on genetic background [5,6], and supported by the combined activities of leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) signaling [7]. Both genetic studies [8,9] and whole transcriptome analysis [10] have provided a wealth of information as to the factors that maintain ES cells in an undifferentiated state, and the signaling networks that regulate their expression [11]. Our current understanding is that the combined activities of a core network consisting of the transcription factors OCT4, SOX2 and Nanog act to maintain ES cell identity by inhibiting the expression of lineage affiliated genes [12] and supporting a pluripotent epigenetic signature [10].

Although much is now known about the molecular requirements for efficient ES cell self-renewal, the means by which cells exit this state and acquire the competence to respond to differentiation cues is less well understood. Genetic studies have shown that fibroblast growth factor (FGF) signaling through GRB2-RAS is essential for peri-implantation mouse embryogenesis and the formation of the primitive endoderm lineage [13–16]. The ES cell, and presumably ICM/epiblast expression of Fgf4 is driven by a ternary complex consisting of OCT4, SOX2 and the Fgf4 distal enhancer [17], and acts as a major auto-inductive cue for ES cell differentiation, but is largely dispensable for proliferation [18,19]. Moreover, it has been shown that the combined pharmacological inhibition of FGF-ERK and glycogen synthase kinase 3 (GSK3) signaling pathways promotes long-term self-renewal of ES cells in the absence of both LIF and BMP4 [7]. This dual inhibitor (2i) culture condition permitted the derivation of ES cell lines from previously refractory genetic backgrounds such as CBA [5], and non-obese diabetic (NOD) mice [20], and importantly the establishment of authentic rat ES cells [21,22].

Because ERK2 is the predominant ERK enzyme expressed in ES cells, and because of its early embryonic phenotype [23,24], we sought to determine the effect of Erk2 genetic ablation on FGF signaling, self-renewal and lineage specification. Here we present evidence that ERK2 is necessary for maximal FGF-ERK signaling and its deletion results in a modest, but consistent increase in self-renewal capacity and reduction in heterogeneity of Nanog expression in self-renewing culture conditions. In contrast to our
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To test whether the targeted mutation generated the predicted null allele, Erk2 expression was assessed by qRT-PCR and ERK2 protein by western blotting. A 50% reduction of Erk2 expression was observed in heterozygous Erk2+/− ES cell clones, and an absence of mRNA from both homozygous null lines (Figure 2A). Moreover, there was no evidence of a compensatory increase in Erk1 mRNA expression. This was also confirmed on the protein level by immunoblotting with a polyclonal antibody that recognizes both ERK1 and ERK2 (Figure 2B).

ERK2 Depletion Attenuates Short Term, but not Long Term FGF-ERK Signaling

To test whether ERK signaling was perturbed in the absence of ERK2, lysates from WT and Erk2−/− ES cells, as well as Erk2+/− cells expressing FLAG-ERK2 from a randomly integrated transgene were examined for phosphorylation status of the ERK, MEK, and p90RSK enzymes. In steady-state self-renewing conditions ERK2 depletion resulted in a 3-fold increase in the ratio of phospho-ERK to total ERK, which was returned to a normal ratio in FLAG-ERK2 expressing cells (Figure 2C,D). However, the amount of total phospho-ERK, i.e. pERK1(−/−)pERK2)/GAPDH, was decreased by approximately 50% in ERK2-deficient ES cells (Figure 2D). Moreover the decrease in total phospho-ERK in Erk2 knockout-out cells translated into a similar decrease in phospho-p90RSK levels on the ERK-dependent sites T358 and S362 (Figure 2E) [26]. Interestingly, depletion of ERK2 resulted in an increase in the levels of phospho-MEK1/2 (upstream ERK kinases) at S211 and S217 (Figure 2E), suggesting that the observed increase in ERK1 phosphorylation in Erk2-null ES cells was due to decreased ERK-dependent negative feedback up-stream in the kinase cascade [27]. To examine FGF responsiveness in the absence of ERK2 we inhibited endogenous FGF signaling with a chemical inhibitor (PD173074) for 24 h followed by a wash-out and stimulation with recombinant FGF2 for 5–120 min. This acute stimulation with FGF2 exhibited similar time-to-peak (10–30 min) and decay kinetics in the presence or absence of ERK2 (Figure 2F). However, the ERK2-deficient ES cells exhibited a decrease in p90RSK phosphorylation indicating a reduction in the overall output of ERK signaling (Figure 2F).

As both ERK1/2 and p90RSK activities have been shown to be essential for the induction of immediate-early genes (IEGs) cFos, Egr1, and Egr2 in other systems [28,29], we next asked if this global reduction in ERK activity was reflected in the transcriptional response to FGF stimulation. qRT-PCR analysis of IEGs Egr1 and Egr2 expression following acute stimulation with FGF2 showed an approximately 70% reduction in their induction at 1 hour post-stimulation in ERK2-deficient ES cells consistent with a reduction in FGF pathway activity (Figure 3A). The induction of another IEG, cFos, was only reduced by approximately 35% in Erk2−/− ES cells (Figure 3A). However, its induction was similar to FGF stimulation of WT cells in the presence of the MEK inhibitor PD0325901. This indicated that only a fraction of cFos induction was MEK-dependent, with the MEK-independent cFos expression possibly being due to FGF-mediated phosphatidylinositol 3'-OH kinase (PI3K)-AKT induction of cFos [30].

Dusp6 expression and its protein stability are both regulated by ERK activity [31,32] where they act in a negative feedback loop that regulates the duration of ERK activity [29]. As the duration of ERK activity can determine the expression of late response genes [33], we asked if the expression of Dusp6 was attenuated in the absence of ERK2 and if this correlated with a difference in late response gene expression such as D-type cyclins. Similar to Egr1, Egr2, and cFos, the magnitude of Dusp6 expression was reduced in Erk2−/− ES cells (Figure 3B), however FGF2-induced, MEK-dependent, Cnd2 expression was not affected by loss of ERK2 (Figure 3C) indicating that the initial decrease in pathway activity can be compensated for in the long term, possibly by a decreased amount of induced negative feedback. Consistent with studies into the unique cell cycle architecture of mouse ES cells [34], Cnd3 expression was independent of both ERK and MEK activity (Figure 3D).

To test whether the attenuation of IEG induction in Erk2-null ES cells was isoyme specific, or a consequence of reduced total ERK activity, ERK2 depletion was rescued by transgenic expression of either 3xFLAG-ERK1 or 3xFLAG-ERK2. Western blot analysis showed that both exogenous ERK1 and ERK2 were expressed at comparable levels to endogenous ERK2 in WT cells, and were phosphorylated normally following FGF2 stimulation (Figure 4A). qRT-PCR analysis of IEG induction showed that either ERK isoyme was equally capable of reinstating normal levels of Egr1, Egr2 and cFos induction (Figure 4B) arguing that ERK1 and ERK2 overlap functionally with respect to induction of this subset of IEGs in mouse ES cells. We also examined the activity of other signaling pathways and found that ERK2 deletion does not perturb the stability of β-catenin nor the phosphorylation status of STAT3, AKT, and SMAD2 in self-renewing ES cells (Figure 4C).

ERK2 is Dispensable for Germ Layer Specification of ES Cells

We have previously reported a block in both neural and mesodermal differentiation in ERK2-deficient ES cells [18]. However, this block was not reversed upon transgenic rescue (Figure S2A,B), even though exogenous ERK2 was phosphorylated normally and rescued the defect in Egr1 induction (Figure S2C,D). Moreover, ERK2-deficient cells were capable of generating both endodermal and mesodermal cell types in embryoid
bodies (Figure S2E). Therefore it is likely that the differentiation defect exhibited by these ES cells is not related to the targeted mutation at the Erk2 locus, and possibly related to genetic background, since they were derived from a C57Bl/6–129 F1 cross [23], or to an unknown mutation during the derivation of these ES cell lines from blastocysts. We therefore examined the ability of new Erk2−/− ES cells generated on a pure 129 background to commit to various differentiated lineages. Embryoid body (EB) differentiation was employed as it produces descendants of all three germ layers in 3-D culture, and is therefore an unbiased reflection of the potency of mES cells in culture. EBs produced from Erk2−/− ES cells exhibited similar morphological features to parental E14Ju wild-type ES cells (Figure 5A), with cystic EBs visible from day 6, and rhythmically beating cardiomyocytes evident by day 8 (data not shown). A time course of gene expression analysis showed no major differences in the down-regulation of the pluripotency markers, Rex1 and Nanog, or up-regulation of endodermal (Gata4, Foxa2) and mesodermal markers (T, Acta2), indicating no general impairment in differentiation (Figure 5B). However, ERK2-deficient ES cells expressed...
higher levels of Nanog in self-renewing conditions (Day 0), and a more prominent induction of α-smooth muscle actin (Acta2) expression at Day 6 and Gata4 at Day 4 (Figure 5B).

Next we specifically investigated the requirement of ERK2 during neural induction using an established monolayer differentiation protocol in serum-free N2B27 medium [35], in which embryo-derived Erk2 knock-out ES cells failed to form neural tissue (Figure S2) [18]. Following 5 days of differentiation ES-derived Erk2-null cells generated βIII tubulin-positive neurons with similar efficiency to WT and heterozygous lines (Figure 6A). qRT-PCR analysis showed almost complete down-regulation of pluripotency markers (Oct4, Nanog, Rex1) in all cell lines by day 5 of neural induction, with a concomitant increase in neural markers, NeuroD3, Ngn2, and Mash1 (Figure 6B). As observed for the EB experiments, the expression of Nanog was higher in Erk2-null ES cells at Day 0 (Figure 6B), indicating that ES cells may self-renewal more efficiently in the absence of ERK2.

Figure 2. ERK2 ablation attenuates FGF-ERK signaling. (A) qRT-PCR analysis showing reduced or absent Erk2 transcript in the mutated lines, with no compensatory increase in Erk1 expression. (B) Western blot analysis of total ERK1/2 protein levels in WT, heterozygous, and homozygous Erk2 cell lines. ERK2 expression is reduced in the heterozygous line D2, and completely absent in the Erk2−/− lines D7N2 and D2N4. Tubulin is used as a loading control. The qRT-PCR and western results for the heterozygous line D7 were identical to clone D2 (not shown). (C) Western blot analysis of wild-type (E14Ju), Erk2−/−, FLAG-ERK2-rescued Erk2−/− ES cells. The levels of phospho-ERK1/2, phospho-RSK and phospho-MEK1/2 were examined in self-renewing culture conditions. (D) Quantification of western blots to examine the ratio of phosphorlated ERK1/2 to total ERK1/2 (left), and phospho-ERK1/2 to GAPDH (right) in E14Ju, Erk2−/−, and Erk2-rescued Erk2−/− lines. (E) Quantification of western blots to examine the ratio of phosphor-RSK to GAPDH (left), and phospo-MEK1/2 to total MEK1/2 (right) in E1Ju, Erk2−/−, and Erk2-rescued Erk2−/− lines. (F) Western blot analysis of ERK1/2 and RSK activation in wild-type (E14Ju) and Erk2−/− ES cells following acute FGF2 stimulation. Phosphorylation of p90RSK is reduced in the absence of ERK2. Membranes were stripped and sequentially blotted with the indicated antibodies.
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ERK2 Suppresses Expression of a Subset of Pluripotent Genes

To further investigate the elevated Nanog expression in ES cells lacking ERK2, two independent Erk2−/− ES cell clones were rescued with ERK2 expression constructs. Transgenic expression of ERK2 was capable of reinstating normal levels of Nanog to Erk2−/− ES cells (Figure 7A). A second elevated pluripotent marker, Tbx3, was similarly increased in Erk2−/− ES cells and rescued to wild-type levels by an ERK2 expression construct. A similar, but less dramatic effect was also observed for Klf4 (Figure 7A). No significant difference was observed for Oct4 levels across all cell lines tested. ERK2 deficiency had the most significant effect on Tbx3 levels (a one-tailed t-test giving p-values of 0.02 and 0.01 for D7N2 and D2N4, respectively). This is consistent with previous reports that Tbx3 is a negatively regulated direct target of ERK signaling in ES cells, and that Tbx3 can positively regulate Nanog promoter activity [11]. It is of note that Nanog, Tbx3, Klf4 are expressed heterogeneously in self-renewing ES cells [11] in contrast to the homogenous expression of Oct4, which did not exhibit significant differences in expression in the absence of ERK2. Therefore we hypothesized that ERK2 deficiency may reduce heterogeneity of self-renewing undifferentiated ES cells. To test this hypothesis, an EGFP cassette was knocked into the endogenous Nanog promoter in Erk2−/− ES cells (Figure S3) [36]. Two correctly targeted clones, one each from D7N2 and D2N4 Erk2-null lines, were stably transfected with an ERK2 expression cassette and the extent of EGFP expression was assessed by flow cytometry (Figure 7B). While the majority of Erk2-null ES cells exhibited high Nanog-GFP expression, a significant Nanog-GFP low subpopulation appeared when ERK2 was re-introduced (Figure 7B). This is consistent with other reports which show that Nanog heterogeneity is reduced in the presence of chemical inhibitors of MEK-ERK signaling [36,37]. To further examine this phenomenon for a different pluripotent gene, we generated a Tcfcp21i-Venus reporter construct and stably transfected it into Erk2−/− and ERK2-rescued Erk2−/− ES cells (Figure S4A). Tcfcp21i is a grainyhead transcription factor that is highly expressed in mouse ES cells and rapidly down-regulated upon differentiation [38], and it is a direct binding partner of OCT4 [39]. In self-renewing (+LIF) conditions 97% of Erk2−/− ES cells were positive for the Tcfcp21i-Venus reporter, but this was reduced to 86% when ERK2 was added back to the cells (Figure S4B). The kinetics of Tcfcp21i-Venus reporter expression during differentiation (removal of LIF) was examined for the Erk2−/− and ERK2-rescued ES cells and both cell lines efficiently down-regulated this reporter (Figure S4C). This result indicates that ERK2 plays a major role to generate heterogeneous gene expression in self-renewing ES cells, but has little impact on their down-regulation during differentiation.

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To test if this reduced heterogeneity was functionally significant the self-renewal capacity of ERK2-deficient ES cells was tested in a stringent clonal density assay in the presence of LIF. Self-renewing cells were identified by alkaline phosphatase activity after 7 days at clonal density [40]. Colonies were counted and scored based on the degree of differentiation observed. In WT and Erk2−/− ES cells, a distribution of 10% undifferentiated, 70% mixed, and 20% differentiated colonies was observed (Figure 7C). This was in contrast to Erk2-null ES cell lines were a undifferentiated colonies increased from 10% to 30% and differentiated colonies were reduced from 20% to 5%. These percentages returned to a WT distribution upon transgenic expression of ERK2, indicating that ERK2 depletion enhances self-renewal in the presence of LIF. However, in the absence of LIF all genotypes differentiated to a similar extent (data not shown). Taken together these data indicate that loss of ERK2 increases the self-renewal capacity via reducing heterogeneous expression of key pluripotency genes in the
presence of LIF, but is dispensable for lineage commitment of mouse ES cells. Our data supports a model where the total ERK activity, and not isozyme-specific activity, is critical for the balance between self-renewal and lineage commitment.

Discussion

Here we have determined the consequences of Erk2 deletion on FGF-ERK signal transduction, self-renewal capacity, and lineage specification in mouse ES cells. We have found that ES cells compensate for the loss of ERK2 by hyper-phosphorylation of the remaining ERK1 isozyme without an increase in Erk1 transcription or translation. The mechanism may be through attenuation of induced negative feedback mechanisms, such as MAPK phosphatases, or direct feedback within the MAPK core module. We have found that immediate-early gene (IEG) induction is reduced in the absence of ERK2, as well as the negative regulator Dusp6. However, expression of a late response gene, Cd2, was unaffected by the loss of ERK2, underscoring the robustness of the ERK pathway to various perturbations [41]. Taken together this data suggests that ERK activity is stabilized, and maintained within a certain range, by both short (MAPK core cascade) and long (induced phosphatases) feedback loops and indicates that targeting these feedback loops may prove effective in modulating ERK activity in mES cells. To address the issue of redundancy we have shown that the early transcriptional defects in response to FGF activation observed in Erk2-null ES cells is entirely rescued by expression of either ERK1 or ERK2 implying that in ES cells it is total ERK activity, rather than isozyme specificity, that is most important for IEG induction.

In contrast to blastocyst-derived Erk2-/- ES cells of mixed genetic background, Erk2 deletion by two rounds of homologous recombination in ES cells of a uniform genetic background (129/ Ola) has no detectable effect on lineage specification into either neural, endodermal, or mesodermal lineages, and in fact ERK2-deficient ES cells appeared to differentiate towards the mesoderm lineage more efficiently that either WT or heterozygous lines, as judged by increased induction of Gata4 and Acta2 expression (Figure 5B).

The lack of lineage commitment defects we observe is in agreement with recent embryological data indicating that ERK2 is not required for lineage specification in the embryo proper. Voisin and colleagues used tetraploid complementation, where the extra-embryonic tissues are wild-type, to produce grossly normal E13.5
Erk2^+/2 embryos [42]. As reported previously, the early phenotype of Erk2-null embryos was due to cell autonomous defects in the trophoblast lineage [23], and that the reported lack in mesoderm induction [24], is likely a secondary defect due to a lack of inductive signals from a defective extraembryonic ectoderm.

Elevated expression of key pluripotency-associated genes, Nanog, Tbx3, and Klf4 was detected in Erk2-null ES cells when compared to WT or ERK2-rescued cells. The Nanog-GFP reporter and Tcf21l-Venus reporter on the Erk2-null background suggests this increase in expression is probably due to reduced heterogeneity in ES cell cultures. Undifferentiated Nanog-GFP-low ES cells are more prone to differentiate than Nanog-GFP-high ES cells [36], and increased self-renewal activity of Erk2-null ES cells in stringent clonal assays is consistent with their reduced heterogeneity. Recent work has shown that ERK activity in ES cells increases the heterogeneous expression of the primitive endoderm marker, Hex, and epiblast marker, Tcf15 [43,44]. These results suggest that lowering the total active ERK pool in ES cells reduces fluctuation of lineage-priming genes and parts of the pluripotency transcriptional network, consistent with observations that chemical inhibition of the MEK-ERK pathway promotes self-renewal and inhibits lineage commitment [5,45]. The increase in Nanog and Tbx3 expression observed in Erk2^−/− ES cells may contribute to the enhanced performance in clonal assays, since over-expression of either gene strongly promotes the self-renewal capacity of ES cells [11,40]. In summary, this work reveals that mouse ES cells can cope with the removal of ERK2 partially by up-regulating the activity of ERK1. This apparent functional redundancy between the ERK enzymes has been clearly shown in embryonic fibroblasts, where Erk1−/−;Erk2−/− cells rapidly undergo senescence [42]. The complete genetic removal of both Erk1 and Erk2 from ES cells will be necessary to determine if naive mouse pluripotent stem cells require ERK enzymes for cell survival, self-renewal, and to efficiently exit self-renewal to generate committed lineages.

Materials and Methods

Targeting Vectors and Expression Constructs

Erk2^−/− targeting vectors were constructed with Erk2 homology arms kindly provided by S. Meloche and M. Saba-El-Lei (30). Homology arms were digested with BglII to remove a 2.5 kb fragment flanking exon 3. The vector backbone, containing 4.8 kb 5' homology and 2.8 kb 3' homology, was then ligated to a BamHI fragment containing the floxed CMV-Hph-TK cassette. To this a diphtheria toxin A cassette was added outside of the homology arms as a HindII fragment. The second targeting construct was generated by replacing the CMV-Hph-TK cassette with a BamHI fragment containing the floxed CMV-Hph-TK cassette. To this a diphtheria toxin A cassette was added outside of the homology arms as a HindII fragment. The second targeting construct was generated by replacing the CMV-Hph-TK cassette with a BamHI fragment containing the floxed CMV-Hph-TK cassette.

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Figure 5. Germ layer differentiation of ES cells is ERK2 independent. (A) Phase contrast images of embryoid bodies (EBs) at 12 days of differentiation, arrow indicate presumptive cysts. (B) Time-course analysis of the progression of EB differentiation, showing similar differentiation kinetics in WT (E14Ju), heterozygous (D2) and homozygous (D7N2 and D2N4) Erk2 as shown by markers of pluripotency (Oct4, Nanog), mesoderm (Acta2, T), and endoderm (Gata4, Foxa2). Error bars represent the standard deviation from the mean of technical replicates.

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epitope contained in a pCAG-driven expression vector with puromycin-resistance [46]. The Tcfcp2l1-Venus reporter construct was constructed as follows. An 8.5 kb SpeI fragment was taken from a BAC clone (bMQ332b16) which contained a putative promoter region, exon 1, and most of intron 1 of the Tcfcp2l1 gene. This was cloned upstream of an adenovirus splice acceptor (SA) and an internal ribosome entry site (IRES) from the gene-trap vector pGT1V3 which contained a Venus expression cassette [47]. A PEST sequence was added to the C-terminal of Venus to decrease its stability [48] and increase the sensitivity of the promoter. A second IRES from the encephalomyocarditis virus (ECMV) [49] was added to link expression of the puromycin N-acetyl-transferase (PAC) gene for selection of Venus expressing clones (Figure S4A). Vector sequence is available upon request.

ES Cell Culture and Gene Targeting

All ES cell lines were maintained in complete ES cell medium: GMEM (Sigma, G5154) supplemented with 10% FBS (Invitrogen), 100 μM 2-mercaptoethanol (BDH, 441413), 1×MEM non-essential amino acids (Invitrogen, 1140-036), 2 mM L-glutamine, 1 mM sodium pyruvate (both from Invitrogen), and 1000 units/ml LIF (made in-house) on gelatinised tissue culture flasks (Iwaki). The FGFR and Mek1 inhibitors, PD173074 and PD0325901, were used at 200 nM and 1 μM, respectively, and were both from Axon Medchem. All Erk2 gene targeting was performed in E14Ju ES cells, which is a subclone of E14Tg2a ES cells [50]. E14Ju ES cells (10⁶) were electroporated with linearized DNA (100 μg) using a BioRad GenePulser (0.8 kV and 10 μF). Stable transfectants were selected for with the appropriate antibiotic: Hygromycin (100 μg/ml); G418 (150 μg/ml); Puromycin (1 μg/ml). ES cell colonies were picked to 96-well plates, replica plated, and initial screening for targeting was by qPCR of genomic DNA for exon 3 of Erk2. Exon 3 primers are forward: 5’-CTAACCCTCTTGACCCCGTGAC-3’ and reverse: 5’-GATCACAAGTGGTGTTTCACCGAG-3’, used with Roche Universal Probe Library (UPL) probe #97 and control primers for diploidy were for Spry1 forward: 5’-CTAACCCTCTTGACCCCGTGAC-3’ and reverse: 5’-GATCACAAGTGGTGTTTCACCGAG-3’ used with UPL.

Figure 6. ERK2 is not required for ES cells to efficiently produce neurons under defined conditions. (A) Immunocytochemistry analysis of wild-type, Erk2+/− and Erk2−/− cultures after 5 days of neural induction for neuronal marker βIII Tubulin immunoreactivity (red). DNA is counterstained with DAPI in blue. (B) Gene expression analysis of cultures following 5 and 10 days of neural induction. Markers of pluripotency (Oct4, Nanog, and Rex1) and neural tissue (NeuroD3, Ngn2, and Mash1) are shown. Error bars represent the standard deviation from the mean of biological replicates. doi:10.1371/journal.pone.0060907.g006
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Panel A: Relative expression of Oct4, Nanog, Tbx3, and Klf4 in different cell lines.

Panel B: Graph showing the expression of GFP in clone D7N2 and clone D2N4, with control (CTRL) and Erk2-/- and Erk2-/- + Erk2 conditions.

Panel C: Colony Forming Assay showing the percentage of cells in different categories (Undifferentiated, Mixed, Differentiated) for different cell lines.

Images and graphs illustrate the suppression of self-renewal by ERK2 in ES cells.
probe #78. The EGFP-IRESPuroR cassette was targeted to the Nanog locus in Erk2−/− ES cells as previously described [36].

Southern Blotting

Southern blotting was performed by resolving 5 μg gDNA, digested with KpnI alone or KpnI-PstI, by agarose gel electrophoresis. gDNA was stained with ethidium bromide and nicked using a UV trans-illuminator, followed by denaturation in 500 mM NaOH, 1.5 M NaCl for 1 hour. The reaction was stopped by incubating the gel in 500 mM Tris, 1.5 M NaCl, (pH 5.5) and gDNA was transferred to a nitrocellulose membrane (Amersham) overnight by capillary action in the presence of 20X SSC. The DNA was then cross-linked to the membrane using a UV oven (Stratalink) and baked for 1 h at 120°C. The hybridization probe was labelled using the MegaPrime™ DNA Labeling System (Amersham) according to manufacturer’s instructions, using [α-32P] dCTP, 3000 Ci/mmol and incubated overnight at 68°C. Following extensive washes the membrane was exposed to X-ray film (Kodak) overnight at −80°C and subsequently developed in an automated autoradiographic film processor.

ES Cell Stimulation, Differentiation and Clonal Assays

For acute FGF2 simulation, ES cells were plated at 2 × 105 cells/cm2 in complete ES cell medium. The following day inhibitors were added (PD173074, 100 nM or PD0325901, 1 μM, both from Axon Medchem) and cultured for a further 24 hours. Inhibitors were washed out with PBS (2 × 2 ml) and cells were stimulated with complete ES cell medium supplemented with recombinant hFGF2 (10 ng/ml, NCI Frederick BRB Repository) for 5 min to 3 hours. Serum-free neural induction was performed as described by Ying et al. with N2B27 medium made in-house [35]. Embryoid body differentiation was performed in hanging drops of ES cell medium in the absence of LIF. Cells were diluted to a concentration of 3.2 × 104 cells/ml and aliquoted in 30 μl drops. After 2 days the aggregates were transferred to low adhesion bacterial grade culture dishes and cultured for the indicated times. Self-renewal capability was determined in a colony-forming assay by plating at low density (0.2 × 104 cells/cm2) in the presence or absence of LIF. Colonies were allowed to form over 7 days whereupon they were stained for alkaline phosphatase activity using the Leukocyte Alkaline Phosphatase Assay Kit (Sigma), and classified as either (i) undifferentiated, (ii) mixed, or (iii) differentiated.

Western Blotting

Western blotting and multi-stripe blotting were performed as described previously [18,31]. Membranes were probed with rabbit anti-total ERK1/2 #4370 (1:1000), rabbit anti-phospho-ERK1/2 #4695 (1:1000), rabbit anti-phospho-RSK T359/S363 #9344 (1:1000), rabbit anti-total RSK #9355 (1:1000), rabbit anti-phospho-SMAD2 #3108 (1:1000), rabbit anti-phospho-MEK1/2 #9134, mouse anti-MEK1/2 #4694 (1:1000), rabbit anti-phospho-STAT3 #9145, all from Cell Signaling, anti-Tubulin (1:2000, Abcam), mouse anti-GAPDH (1:35000, Sigma), mouse anti-α-Catenin (1:1000, Sigma) and detected with the appropriate HRP conjugated secondary (Pierce) and peroxidase activity was visualised with the SuperSignal West Pico Kit (Pierce). Membranes were stripped with 100 mM 2-mercaptoethanol in 72.5 mM Tris (pH 7.6) at 60°C for 15 minutes, and sequentially probed with the indicated antibodies. For quantitative western blotting, primary antibodies were detected with secondary antibodies conjugated to Alexa fluorophores (Molecular Probes, 1:5000) and visualized using the BioRad ChemiDoc MP system. Images were quantified based on pixel density using the ImageJ software package.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. First-strand synthesis was performed using M-MLV RT (Invitrogen) with 50 ng random hexamers (Thermo Fisher) in the presence of RNase inhibitor, RNaseOUT (Invitrogen), in a reaction volume of 20 μl. Following first-strand synthesis the reaction mixture was diluted to 80 μl, and 2 μl was used per qPCR reaction in Probes Master Mix (Roche) using Universal Probe Library (UPL) probes and the LightCycler 480 system (Roche). Concentrations were determined against a standard curve and values normalized to TBP expression, as a house-keeping gene. Primers sequences and UPL probe numbers are available in Supporting Table S1.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (w/v) at room temperature for 15 minutes followed by single step permeabilization and blocking in 0.1% Triton-X and 2% goat serum for 1 hour. Cells were incubated with anti-βIII Tubulin [TuJ1, 1:1000, R&D Systems] overnight. Following extensive washes, secondary antibodies conjugated to Alexa fluorophores (Molecular Probes) were diluted 1:1000 in blocking buffer and applied for 1 hour at room temperature. The cells were washed twice in PBS and a third time in PBS containing DAPI (10 μg/ml) before obtaining pictures on an Olympus IX51 inverted fluorescence microscope.

Flow Cytometry

Cells were collected by trypsinization and resuspended in PBS supplemented with 3% FBS. Samples were analyzed using a FACSCalibur flow cytometer (BD Biosystems) and data processed with FlowJo (Tree Star Inc.).

Supporting Information

Figure S1 Normal ploidy of Erk2-null ES cell lines. Metaphase spreads of four Erk2-null ES cell lines were stained with Giemsa and chromosome counts indicated largely normal ploidy. (TIF)

Figure S2 Embryo-derived Erk2−/− ES cell lines exhibit an ERK2-independent block in lineage specification. (A) Immunocytochemistry analysis of wild-type (E14Tg2a), embryo-derived Erk2−/− (B1) ES cells, and ERK2-rescued Erk2−/− ES cells following 7 days of neural induction for neuronal marker βIII Tubulin. DNA is counterstained with DAPI. (B) qRT-PCR
analysis of markers of pluripotency (Oct4), and neural tissue (Nestin, βIII Tubulin, also known as Tubb3). (C) Western blot analysis of E14Teg2a and Erk2+/−/+3X-FLAG-ERK2 showing phosphorylation of exogenously expressed ERK2. (D) qRT-PCR analysis of FGF2-mediated induction of Egr1 in E14Teg2a, embryo-derived Erk2−/− ES cells, and ERK2-rescued Erk2+/− ES cells. Transgenic add-back of ERK2 efficiently rescues normal Egr1 expression to Erk2+/− ES cells in three independent clones. (E) qRT-PCR analysis of embryoid bodies at 6 days of differentiation for markers of pluripotency (Nanog and Oct4), mesoderm (Pdgfra), and endoderm (Gata6).

Figure S3 Nanog-GFP targeting. (A) Schematic depicting Nanog targeting strategy. Black bars denote the position of the 5′ external probe used for Southern analysis. (B) Southern blot targeting strategy. Black bars denote the position of the 5′ Nanog probe used for Southern analysis. (C) Down-regulation of Nanog expression was observed in the absence of ERK2. (D) qRT-PCR analysis of Nanog and Oct4, mesoderm (Pdgfra), and endoderm (Gata6).

Figure S4 Tcfcp2l1-Venus reporter shows increased expression in Erk2−/− ES cells. (A) Schematic (not to scale) depicting the final Tcfcp2l1-Venus reporter construct. An 8.5 kb fragment of the mouse Tcfcp2l1 gene was cloned upstream of a splice-acceptor (SA) and GTX-RES (IRE5), followed by a destabilized Venus protein (Venus-PEST), an ECMV-RES (IRE5), a puromycin-resistance gene (PAC), and a polyadenylation signal (pA). (B) FACS analysis of Erk2−/− ES cells and ERK2-rescued Erk2+/− ES cells with the Tcfcp2l1-Venus reporter showed that Venus expression in self-renewing conditions was highest in the absence of ERK2. (C) Down-regulation of Venus expression over 48 h of differentiation followed similar kinetics in the presence or absence of ERK2.

Table S1 PCR primers for qRT-PCR analysis. (Forward and reverse (R) primer sequences (5′−3′) and Roche Universal Probe Library (UPL) probes used in qRT-PCR assays.

| Primer | Sequence (5′−3′) | UPL Probe | Description |
|--------|-----------------|-----------|-------------|
| Forward | Reverse | | |

References
1. Brook FA, Gardner RJ (1997) The origin and efficient derivation of embryonic stem cells in the mouse. Proc Natl Acad Sci USA 94: 5709–5712.
2. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154–156.
3. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78: 7634–7638.
4. Robertson E, Bradley A, Kuehn M, Evans M (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323: 445–448. doi:10.1038/323445a0.
5. Ying Q-L, Wray J, Nichol J, Battle-Morera L, Doble B, et al. (2008) The ground state of embryonic stem cell self-renewal. Nature 453: 519–523. doi:10.1038/nature06968.
6. Najm EJ, Chenoweth JG, Anderson PD, Nadeau JH, Redline RW, et al. (2011) Isolation of epiblast stem cells from preimplantation mouse embryos. Cell Stem Cell 16: 318–325. doi:10.1016/j.stem.2011.01.016.
7. Ying Q-L, Nichol J, Chambers I, Smith A (2005) BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115: 281–292.
8. Avilion AAA, Nikols SKS, Pevny LHL, Perez LI, Vivian NN, et al. (2005) Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17: 126–140. doi:10.1101/gad.224503.
9. Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24: 372–376. doi:10.1038/74199.
10. Leb Y-H, Wu Q, Chew J-L, Vega VB, Zhang W, et al. (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38: 431–440. doi:10.1038/ng.1670.
11. Niwa H, Ogasawara K, Shimosato D, Adachi K (2009) A parallel circuit of LIF signaling pathways maintains pluripotency of mouse ES cells. Nature 460: 118–122. doi:10.1038/nature08113.
12. Silva J, Smith A (2008) Capturing pluripotency. Cell 132: 353–366. doi:10.1016/j.cell.2008.02.006.
13. Arman E, Haffner-Krausz R, Chen Y, Heath JK, Lonai P (1998) Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc Natl Acad Sci USA 95: 5082–5087.
14. Chazaud C, Yamakawa Y, Pawson T, Rossant J (2006) Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2/SMAPK pathway. Developmental Cell 10: 615–624. doi:10.1016/j.devcel.2006.02.020.
15. Cheng AM, Saxton TM, Sakai R, Kulkarni S, Mhamadu G, et al. (1998) Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. Cell 95: 793–803.
16. Feldman B, Pousymiou W, Papaioannou V, DeChiaro TM, Goldfarb M (1995) Requirement of FGF-4 for postimplantation mouse development. Science 267: 246–249.
17. Yuan H, Corbi N, Basilico C, Dailey L (1995) Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev 9: 2635–2645.
18. Kunath T, Saba-EI-Leil MK, Almoussailleh M, Wray J, Melsce S, et al. (2007) FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development 134: 2059–2069. doi:10.1242/dev.012208.
19. Wilder PJ, Kelly D, Bridgman K, Peterson CL, Nowling T, et al. (1997) Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or the survival of their early differentiated progeny. Dev Biol 192: 614–629. doi:10.1006/dbio.1997.8277.
20. Nichols J, Jones K, Phillips JM, Newland SA, Roode M, et al. (2009) Validated germline-competent embryonic stem cell lines from nonobese diabetic mice. Nat Med 15: 814–818. doi:10.1038/nm.2102.
21. Buehr M, Merik S, Blair K, Yang J, Ure J, et al. (2008) Capture of authentic embryonic stem cells from rat blastocysts. Cell 135: 1287–1298. doi:10.1016/j.cell.2008.12.007.
22. Li P, Tong C, Mehrian-Shai R, Jia L, Wu N, et al. (2008) Germ-line competent embryonic stem cell derived from rat blastocysts. Cell 133: 1299–1310. doi:10.1016/j.cell.2008.12.006.
23. Saba-EI-Leil MK, Vella FDJ, Vernay B, Voisin L, Chen L, et al. (2003) An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. EMBO Rep 4: 964–968. doi:10.1038/sj.embor.embor939.
24. Yao Y, Li W, Wu J, Germann UA, Su MSS, et al. (2003) Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. Proc Natl Acad Sci USA 100: 12739–12744. doi:10.1073/pnas.2134514100.
25. Fischer AM, Katayama CD, Pages G, Poussemogu J, Heckrick SM (2005) The role of erk1 and erk2 in multiple stages of T cell development. Immunity 23: 431–443. doi:10.1016/j.immuni.2005.08.013.
26. Dalby KN, Morriss N, Caudwell FB, Averch J, Cohen P (1998) Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/g90thks that are inducible by MAPK. J Biol Chem 273: 1496–1505.
27. Santos SDM, Verveer PJ, Baatenburg PH (2007) Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate. Nat Cell Biol 9: 324–330. doi:10.1038/ncl Cell 112207.
28. De Cesare D, Jacquot S, Hanauer A, Sassone-Corsi P (1998) Rsk-2 activity is necessary for epidermal growth factor-induced phosphorylation of CREB protein and transcription of c-fos gene. Proc Natl Acad Sci USA 95: 12202–12207.
29. Nakakuki T, Birtwistle MR, Saeki Y, Yamoto N, Ide K, et al. (2010) Ligand-specific c-Fos expression emerges from the spatiotemporal control of ErbB network dynamics. Cell 141: 884–896. doi:10.1016/j.cell.2010.03.054.

30. Choudhury GG (2001) Akt serine threonine kinase regulates platelet-derived growth factor-induced DNA synthesis in glomerular mesangial cells: regulation of c-fos AND p27kip1 gene expression. J Biol Chem 276: 35630–35643. doi:10.1074/jbc.M109462000.

31. Brondello JM, Pouyssegur J, McKenzie FR (1999) Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. Science 286: 2514–2517.

32. Brondello JM, Brunet A, Pouyssegur J, McKenzie FR (1997) The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44MAPK cascade. J Biol Chem 272: 1368–1376.

33. Murphy LO, Smith S, Chen R-H, Fingar DC, Blenis J (2002) Molecular interpretation of ERK signal duration by immediate early gene products. Nat Cell Biol 4: 556–564. doi:10.1038/ncb822.

34. White J, Dalton S (2005) Cell cycle control of embryonic stem cells. Stem Cell Rev 1: 131–138. doi:10.1385/SCR:1:2:131.

35. Ying Q-L, Stavridis M, Griffiths D, Li M, Smith A (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. 4 doi:10.1038/nbt80.

36. Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, et al. (2007) Nanog safeguards pluripotency and mediates germline development. Nature 450: 1230–1234. doi:10.1038/nature06403.

37. Singh AM, Hamazaki T, Hankowski KE, Terada N (2007) A heterogeneous expression pattern for Nanog in embryonic stem cells. Stem Cells 25: 2534–2542. doi:10.1634/stemcells.2007-0126.

38. Ivanova N, Dohrn R, Lu R, Kosterko I, Levine J, et al. (2006) Dissecting self-renewal in stem cells with RNA interference. Nature 442: 533–538. doi:10.1038/nature04915.

39. van den Berg DLC, Snoek T, Mullin NP, Yates A, Broustoski K, et al. (2010) An Oct4-centered protein interaction network in embryonic stem cells. Cell Stem Cell 6: 369–381. doi:10.1016/j.stem.2010.02.014.

40. Chambers I, Colby D, Robertson M, Nichols J, Lee S, et al. (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643–653.

41. Kholodenko BN, Hancock JF, Kolch W (2010) Signalling ballet in space and time. Nat Rev Mol Cell Biol 11: 414–426. doi:10.1038/nrm2903.

42. Voisin L, Saba-EI-Leil MK, Julien C, Frémin G, Meloche S (2010) Genetic demonstration of a redundant role of extracellular signal-regulated kinase 1 (ERK1) and ERK2 mitogen-activated protein kinases in promoting fibroblast proliferation. Mol Cell Biol 30: 2918–2932. doi:10.1128/MCB.00131-10.

43. Canham MA, Sharov AA, Ko MSH, Brickman JM (2010) Functional heterogeneity of embryonic stem cells revealed through translational amplification of an early endodermal transcript. PLoS Biol 8: e1000379. doi:10.1371/journal.pbio.1000379.

44. Davies OR, Lin C-Y, Radziszewska A, Zhou Y, Tabb J, et al. (2013) Tcf15 primes pluripotent cells for differentiation. Cell Rep 3: 472–484. doi:10.1016/j.celrep.2013.01.017.

45. Burdon T, Sracey C, Chambers I, Nichols J, Smith A (1999) Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. Dev Biol 210: 30–43. doi:10.1006/dbio.1999.9265.

46. Niwa H, Yamamura K, Miyazaki J (1999) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 186: 193–199.

47. Tsakiridis A, Tzouanacou E, Rahman A, Colby D, Axton R, et al. (2009) Expression-independent gene trap vectors for random and targeted mutagenesis in embryonic stem cells. Nucl Acids Res 37: e129. doi:10.1093/nar/gkp640.

48. Rogers S, Wells R, Rechsteiner M (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science 234: 364–368.

49. Gurtu V, Yan G, Zhang G (1996) IRES bicistronic expression vectors for efficient creation of stable mammalian cell lines. Biochemical and Biophysical Research Communications 229: 295–298. doi:10.1006/brpc.1996.1795.

50. Hooper M, Hardy K, Handyke A, Hunter S, Monk M (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germ-line colonization by cultured cells. Nature 332: 292–295. doi:10.1038/332292a0.

51. Kiyatkin A, Aksamitene E (2009) Multistrip western blotting to increase quantitative data output. Methods Mol Biol 536: 149–161. doi:10.1007/978-1-59745-542-8-17.