Exit from Pluripotency Is Gated by Intracellular Redistribution of the bHLH Transcription Factor Tfe3

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SUMMARY
Factors that sustain self-renewal of mouse embryonic stem cells (ESCs) are well described. In contrast, the machinery regulating exit from pluripotency is ill defined. In a large-scale small interfering RNA (siRNA) screen, we found that knockdown of the tumor suppressors Folliculin (Flcn) and Tsc2 prevent ESC commitment. Tsc2 lies upstream of mammalian target of rapamycin (mTOR), whereas Flcn acts downstream and in parallel. Flcn with its interaction partners Fnip1 and Fnip2 drives differentiation by restricting nuclear localization and activity of the bHLH transcription factor Tfe3. Conversely, enforced nuclear Tfe3 enables ESCs to withstand differentiation conditions. Genome-wide location and functional analyses showed that Tfe3 directly integrates into the pluripotency circuitry through transcriptional regulation of Esrrb. These findings identify a cell-intrinsic rheostat for destabilizing ground-state pluripotency to allow lineage commitment. Congruently, stage-specific subcellular relocalization of Tfe3 suggests that Flcn-Fnip1/2 contributes to developmental progression of the pluripotent epiblast in vivo.

INTRODUCTION
Stem cell fate is determined by a balance between pro-self-renewal and pro-differentiation signals. To initiate differentiation, stem cells have to be forced out of self-renewal, the transcriptional networks conferring stem cell identity need to be dissolved, and lineages have to be chosen. How these interdependent tasks are coordinated is poorly understood. Mouse embryonic stem cells (ESCs) provide a tractable system for dissecting this process because intrinsic and extrinsic signals regulating self-renewal are reasonably well defined.

Genetic and biochemical studies have shown that activity of mitogen-activated protein kinase (MAPK) (Kunath et al., 2007) and glycogen synthetase kinase-3 (GSK3) (Doble et al., 2007) prime ESCs for differentiation. Chemical inhibition of both (2i) promotes robust self-renewal (Ying et al., 2008). It is therefore hypothesized that shielding ESCs from differentiation cues is sufficient for stabilization and propagation of a naive pluripotent ground state (Nichols and Smith, 2009).

Pluripotency is governed by a transcription factor network that contains numerous autoregulatory loops, suggestive of self-propagation (Jaenisch and Young, 2008). How this recursive circuitry is extinguished in an orderly manner to enable developmental progression and eventual lineage commitment is currently unclear. Endogenous repressors Tcf3 (encoded by Tcf711) and the NuRD complex (Kaji et al., 2006; Nichols and Smith, 2012; Pereira et al., 2006) play a role, but other key drivers of exit from naive pluripotency have yet to be identified.

Delineation of mechanisms that steer ESCs into differentiation may reveal predetermined breaking points in the pluripotent transcription factor network. Furthermore, factors regulating ESC fate transition are candidates to drive progression of the naive preimplantation into the lineage-poised postimplantation epiblast in vivo (Nichols and Smith, 2009).

Here, we exploited the ground-state culture system to screen functionally for genes promoting exit from naive pluripotency.

RESULTS
A Large-Scale siRNA Screen Identifies Genes Required for Exit from Ground-State Pluripotency
To uncover genes that are required for progression from naive pluripotency into a state primed for differentiation, we used a simple monolayer differentiation protocol (Ying et al., 2003). Efficient loss of ESC identity over 3 days (Figures 1A and 1B) is a time window compatible with perdurance of transient knockdown by siRNA. We assayed for resistance to exit the ESC state by ability to proliferate in selective 2i culture conditions plus retained expression of the Oct4 locus. After transfecting siRNAs in 2i, differentiation was enabled by withdrawal of the inhibitors. Resistance to commitment was then assayed by reapplying inhibitors and selecting for Oct4 expression (Figure 1A). After
72 hr, cells transfected with GFP, Klf4, or Nr0b1 siRNAs lost ESC properties, but upon knockdown of FGF4, GSK3α/β, or Tcf3, significant numbers of alkaline phosphatase (AP) expressing ESCs were recovered (Figure 1B).

To allow quantitation at high throughput, commitment resistance was measured by cell survival. We screened roughly 9,900 genes in duplicate experiments with pools of four independent siRNAs. Z scores were determined for each run (Figure 1C and Figure S1A available online). Seventy genes that stringently scored positive (Z > 3 and Z > 2.5 in the two trials) were intersected with RNA-sequencing data from ESC differentiations (T. Kalkan and A.S., unpublished data), and 17 genes were excluded due to lack of expression. Another 7 genes were manually eliminated as likely false-positives (see Extended Experimental Procedures). The remaining 46 primary hits were retested, and 28 validated with at least two different siRNAs (Figure S1B). These include Tcf3 and other members of the pathways inhibited in 2i culture conditions (Figure 1D), indicating that the screen successfully identified genes regulating exit from ESC pluripotency.

**Folliculin Regulates Exit from Pluripotency**

We focused on the tumor suppressor Folliculin (Flcn) (Nickerson et al., 2002) because a role in pluripotency has not been described, but homozygous knockout alleles show early embryonic lethality (Chen et al., 2008a; Hasumi et al., 2009). Rex1GFPd2 ESCs that quantitatively report differentiation (Marks et al., 2012; Wray et al., 2011; Yang et al., 2012) were engineered to express a small hairpin RNA (shRNA) targeting Flcn, resulting in reduced transcript and protein (Figures S2A and S2B). Twenty-four hours after 2i withdrawal, control cells downregulated GFP, whereas Flcn knockdown lines maintained expression (Figure 2A). We used colony-forming assays to quantify 10%–15% of cells in

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**Figure 1. A Large-Scale siRNA Screen for Genes Regulating ESC Commitment**

(A) Outline of the screening procedure; O4GIP ESCs were transfected with siRNAs in 2i, differentiation enabled by inhibitor removal, and resistance to commitment assayed by restoring 2i with puromycin selection for Oct4 expression.

(B) Exit from pluripotency in differentiating O4GIP ESCs transfected with indicated siRNAs assayed after 24 hr, 48 hr, and 72 hr and stained for AP.

(C) Average screen Z scores. Red and green triangles show validated hits (see Figure S1B); gray triangles show duplicates within the transcription factor subset.

(D) O4GIP ESC resistance to commitment after transfection with siRNAs was quantified with a cell viability assay and normalized to no siRNA transfection controls. Pools and individual siRNAs are shown. Note that Tsc1 was not recovered in the primary screen. For siRNA pools, the average and standard deviation (SD) of two technical replicates is shown.

See also Figure S1.
Flcn shRNA knockdown cell lines as uncommitted 3 days after inhibitor withdrawal (Figure 2C). This is a conservative estimate because cloning efficiency of ESCs is typically only 50% (Wray et al., 2010). The phenotype could be rescued with the transgene (Figures 2D and S2E). Resistance to differentiation was also observed in the presence of Activin A, fibroblast growth factor 4 (FGF4), and bone morphogenetic protein 4 (BMP4) or 10% fetal bovine serum (Figure 2B), suggesting that Flcn is important for exit from pluripotency under different inductive regimes.

To confirm these findings in a genetic null background, we derived ESCs from Flcn flox/+ intercrosses (Hasumi et al., 2009). Cells were stably transfected with a plasmid expressing Cre fused to the ligand-binding domain of the estrogen receptor (ERT2), and single-cell clones expanded. After treatment with 4-Hydroxytamoxifen (Tam) to activate Cre, Flcn protein was undetectable in two independently derived knockout ESC lines (Figure S2F). After 3 days of differentiation, wild-type and heterozygous cells lost colony-forming ability, whereas 20%–30% of the knockout cells generated undifferentiated colonies (Figure 2E). These observations confirm that Flcn expedites exit from the ESC state.

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Flcn has been linked to mammalian target of rapamycin (mTOR) kinase signaling (Baba et al., 2006; Hartman et al., 2009; Piao et al., 2009). The mTOR inhibitor Tsc2 scored as a commitment regulator in our screen (Figure 1D). Tsc2 functions as a heterodimer with Tsc1 (Laplante and Sabatini, 2012), and knockdown of Tsc1 also inhibited exit from pluripotency (Figure 1D). However, we found that mTOR was strongly activated after inhibitor withdrawal, leading to increased phosphorylation of ribosomal protein S6 (S6), S6 kinase, and the translational regulator 4EBP1 (Figure 3A). The phosphorylation kinetics differed; in particular, S6 and 4EBP1 phosphorylation peaked at 24 hr and 48 hr, respectively. These effects are not due to starvation in N2B27 as they were also observed in serum. To test whether activation of mTOR is important for ground-state exit, we quantified undifferentiated ESCs at 24 hr, 36 hr, and 48 hr of 2i withdrawal in the presence or absence of the mTOR inhibitor rapamycin (Rapa) (Figure 3B). No difference was detected. As expected, knockdown of Tsc2 increased S6 phosphorylation (Figure S3A) and impaired Rex1GFPd2 downregulation.
Cell gated the differentiation defects (Figure 3C). These data indicate that elevated activity of mTOR in ESCs impedes exit from self-renewal, but that activation during differentiation is not required for cell-fate transition. Rapa did not affect renewal, but that activation during differentiation is not required for exit from pluripotency quantified with a cell-viability assay and normalized to no siRNA transfection controls. Average and SD are of two technical replicates.

Figure 3. Flcn Acts downstream of or in Parallel to mTOR and Interacts with Fnip1/2

(A) Rex1GFPd2 cells were differentiated in N2B27 with and without 20 nM Rapa (left panel) or in N2B27/10%FBS (right panel), and cell lysates probed with indicated antibodies.

(B) Rex1GFPd2 cells differentiated in N2B27 with and without 20 nM Rapa were replated at single-cell density in 2i including Rex1-expression selection at the indicated time points. The average percentage of uncommitted cells forming AP-positive colonies relative to the number of cells plated and SD are of two technical replicates.

(C) Rex1GFPd2 cells transfected with indicated siRNAs were differentiated for 72 hr in N2B27 with and without 20 nM Rapa and replated in 2i with Rex1-expression selection, and resulting colonies were stained for AP.

(D) Proteins were immunoprecipitated with FLAG antibodies from stably transfected Rex1GFPd2 cells cultured in 2i or differentiated for 40 hr and probed with indicated antibodies.

(E) mRNA levels were quantified during differentiation and normalized to 2i-cultured cells. Average and SD are of two cell lines.

(F) O4GIP ESCs were transfected with indicated siRNAs, and after differentiation for 3 days, exit from pluripotency quantified with a cell-viability assay and normalized to no siRNA transfection controls. Average and SD are of two technical replicates.

See also Figure S3.

Flcn has been reported to bind to two Flcn-interacting proteins, Fnip1 and Fnip2 (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008). To identify binding partners in ESCs, we immunopurified (IP) Flcn from cells expressing functional 3×FLAG-Flcn (data not shown). Silver staining revealed the presence of one specific copurifying protein at approximately 130 kDa that was identified as Fnip1 by mass-spectrometry (Figure S3B, arrowhead). Vice versa, 3×FLAG-Fnip1 copurified one specific protein with a molecular weight similar to that of Flcn (Figure S3B, open arrowhead). Endogenous Flcn and Flcn were detected in 3×FLAG-Flcn and 3×FLAG-Fnip1 IPs, respectively (Figure 3D), confirming the presence of a Flcn-Fnip1 protein complex in ESCs.

Flcn, Tsc1, Tsc2, and Fnip1 messenger RNA (mRNA) levels did not change markedly during early differentiation as compared to downregulation of Nanog and upregulation of FGF5 (Figure 3E). Furthermore, Flcn or Fnip1 overexpression did not induce transcriptional changes indicative of differentiation (Figure S3C) or accelerate ESC commitment (Figure S3D). This suggests that Flcn and Fnip1 levels are not limiting for exit from pluripotency.

To test the functional significance of the Flcn-Fnip1 interaction, we knocked down Fnip1 and its homolog Fnip2 and assayed loss of ESC identity after 2i withdrawal. Although neither Fnip1 nor Fnip2 single knockdown had a strong impact, knockdown of both had a comparable effect to loss of Flcn (Figures 3F and S3E). Consistent with this, Rex1GFPd2 cells transfected with Fnip1/2 siRNAs retained GFP expression after 24 hr of withdrawal from 2i (Figure S2D).

To investigate whether the functions of mTOR and Flcn in regulation of pluripotency are ESC specific, we turned to postimplantation epiblast stem cells (EpiScs) (Brons et al., 2007; Tesar et al., 2007). EpiScs express Oct4, Flcn, Fnip1/2, Tcf3, and Tsc1/2 (Hayashi et al., 2011). O4GIP EpiScs were induced to differentiate for 3 days through withdrawal of growth factors and assayed for commitment by reapplying EpiSC culture conditions and selecting for Oct4 expression. Neither Tcf3, Tsc2, Flcn, nor Fnip1/2 knockdown impaired EpiSC differentiation (Figure S3F), suggesting that the role of these factors is specific to the cell context.

Flcn Regulates Tfe3 Localization and Activity

Flcn regulates nuclear translocation of the bHLH transcription factor Tfe3 in human kidney cells and mouse embryonic fibroblasts (Hong et al., 2010). Tfe3 showed heterogeneous...
subcellular localization in ESCs in 2i and could be detected in the nucleus (Figure 4A, left panel, arrowhead), cytoplasm (Figure 4A, left panel, open arrowhead), or both compartments. Staining was specific as it was reduced in Tfe3 shRNA knockdown cells (Figure 4A, lower panel). Tfe3 mRNA levels did not change distinctly during ESC differentiation (Figure 3E), but we observed

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a marked cytoplasmic relocalization (Figure 4A, right panel). In contrast, most Flcn shRNA cells featured exclusively nuclear Tfe3 in 2i (Figure 4A, left panel), and many retained nuclear protein after inhibitor withdrawal (Figure 4A, right panel, arrowhead). Consistently, biochemical nuclceocytoplasmic fractionations showed more Tfe3 in the nuclear fraction of Flcn shRNA knockdown cells compared to control EScs (Figure 4B). Tfe3 was also enriched in the nuclei of Flcn null ESCs (Figure S4A). We used automated image quantification to determine nuclear-cytoplasmic ratios of Tfe3 in siRNA-treated cells (see Extended Experimental Procedures). Compared to controls, knockdown of Flcn or Fnip1/2 lead to a significant increase in nuclear Tfe3 both in 2i conditions and 24 hr after inhibitor withdrawal (Figure 4C). Importantly, this was not an indirect consequence of maintained ESC identity, as Tcf3-depleted cells showed cytoplasmic Tfe3. Knockdown of Tsc2 increased nuclear Tfe3 concentration in an mTOR-dependent manner (Figures 4C and S4B), although less pronounced than after knockdown of Flcn or Fnip1/2. These observations suggest that Flcn-Fnip1/2 and Tsc2 activities converge on subcellular localization of Tfe3 and that nuclear exclusion of Tfe3 normally correlates with loss of ground-state pluripotency.

In EpiSCs, Tfe3 is exclusively cytoplasmic (Figure S4C). We therefore examined Tfe3 localization in the embryo. Tfe3 was localized in the nuclei of both inner cell mass (ICM) (Figure 4D, arrowhead) and trophectoderm (TE) cells at embryonic day (E) 3.5. At E4.5, Tfe3 could be detected in nuclei and cytoplasm of Nanog-positive epiblast cells (Figure 4E, arrowhead) but was exclusively nuclear in GATA4-positive hypoblast (Figure 4E, open arrowhead) and TE cells. In postimplantation E5.5 embryos, Tfe3 was strongly expressed but excluded from the nucleus in Oct4-positive epiblast cells (Figure 4F, arrowhead), whereas it remained nuclear in the extraembryonic endoderm (Figure 4F, open arrowhead). Thus, nuclear exclusion of Tfe3 in the epiblast correlates with progression from the naive preimplantation state to the postimplantation egg cylinder.

**Tfe3 Acts in ESC Commitment**

To test whether Tfe3 is functionally downstream of Flcn-Fnip1/2, we performed simultaneous siRNA transfections. Knockdown of the pluripotency-associated genes Klf4, Rex1, or Stat3 did not restore commitment in Tcf3-, Flcn-, Fnip1/2-, or Tsc2-depleted cells. However, knockdown of Tfe3 completely suppressed the Flcn, Fnip1/2, and Tsc2 phenotypes although only mildly affecting Tcf3-dependent commitment (Figures S5A and S5A). Conversely, transfection of Flcn or Fnip1/2 siRNAs had little impact in Tfe3 shRNA knockdown cells (Figure S5B). Thus, Tfe3 is epistatic to Flcn, Fnip1/2, and Tsc2.

Upon depletion of Tfe3, we saw no effect on self-renewal in 2i but a reduction in ESC clonogenicity 24 hr after inhibitor withdrawal (Figures 5B and SSC). Interestingly, knockdown of the Tfe3 family member Tfeb had little effect on its own. We conclude that Tfe3 is not essential for naive pluripotency but constrains exit from ESC self-renewal.

To examine whether nuclear Tfe3 is sufficient to prevent loss of pluripotency, we generated ESCs expressing Tfe3 fused to ERT2 to allow for induction of nuclear translocation by Tam. Tfe3 and Tfe3-ERT2 transfectants were withdrawn from 2i in the absence or presence of Tam and, after 3–4 days, replated in 2i with selection for Rex1 expression. Whereas Tfe3-expressing cells exhibited only mild commitment defects, Tam-induced Tfe3-ERT2-expressing cells showed significantly reduced exit from the ESC state (Figures 5C and 5D). To address the timing of Tfe3 action, we pulsed exposure to Tam. Induction during the first 24 hr after 2i withdrawal was sufficient to impair commitment, whereas later Tam treatment gave weaker phenotypes (Figure 5E). Rex1GFPd2 cells expressing Tfe3-ERT2 maintained GFP after 24 hr in N2B27 in the presence of Tam (data not shown). Even after 76 hr, Rex1GFPd2 expression was maintained in a substantial subset of cells (10%–20%) (data not shown). To test whether this heterogeneous response is due to Tfe3-ERT2 expression variation within the pool of transfec-
tants or due to variable phenotypic penetrance, we picked clones with defined expression levels. Indeed, higher expres-
sion and, consequently, more nuclear Tfe3 (Figure S5D) corre-
lated with a higher frequency of GFP maintenance after 3 days of differentiation (Figure S5E). These data are consistent with nuclear Tfe3 inhibiting initial events in the exit from naive pluripotency.

To test for possible crosstalk between the Flcn-Fnip1/2-Tfe3 axis and the differentiation-promoting activities of GSK3 or MAPK signaling, we analyzed downstream targets. FGF4 induces Etv4 and Spry4 (Lanner et al., 2010), whereas GSK3 inhibition derepresses Axin2 and Cdk1 (Martello et al., 2012; Wray et al., 2011). Upon removal of the respective inhibitor, mRNA levels changed similarly to those in controls in Flcn shRNA, Flcn knockout, or ectopic nuclear Tfe3 cell lines (Figure S5F and data not shown). Thus, Tfe3 is unlikely to generally inhibit GSK3 or MAPK signaling. Overlap between pathways regulating ESC self-renewal is well documented (Martello et al., 2012; Nichols and Smith, 2012; Niwa et al., 2009; Wray et al., 2010), and we therefore tested whether Flcn and Tfe3 could compensate for GSK3 or MAPK inhibition. Flcn shRNA or ectopic nuclear Tfe3 ESC lines cultured in the presence of either inhibitor alone maintained higher Rex1GFPd2 expression compared to the respective controls even after serial passaging (Figure S5G). This indicates that Flcn-Fnip1/2-Tfe3 operates independently of GSK3 or MAPK signaling as a third module to enable ESC differentiation.

ESC differentiation products can be converted into EpiSCs by continuous exposure to FGF2 and Activin A (Guo et al., 2009). After three passages in these culture conditions, both Tfe3-ERT2-expressing and Flcn knockdown cell populations acquired a transcriptional signature similar to that of EpiSCs (Figure S6H, upper panel). However, a small percentage of cells remained clonogenic in 2i (Figure S6H, lower panel), suggesting that nuclear Tfe3 reduces transition into a postimplantation cell fate even in a prolonged and highly inductive context.

Ablation of signals mediating exit from the ESC state during differentiation might also facilitate reprogramming. However, neither depletion of Flcn, Fnip1/2, or Tsc2 nor nuclear translocation of Tfe3 enhanced reprogramming of O4GIP EpiSCs into naive pluripotent ESCs, even in sensitized backgrounds overexpressing Klf4 (Guo et al., 2009) and Nanog (Silva et al., 2009), or hyperactive LIF-STAT3 signaling (Yang et al., 2010) (Figures S5I and S5J and data not shown). Thus, Flcn-Fnip1/2-Tfe3 acts
specifically in exit from pluripotency during differentiation and not in acquisition of pluripotency during reprogramming.

**Identification of Tfe3 Downstream Targets**

To explore the molecular function of Tfe3 downstream of Flcn, we performed chromatin immunoprecipitation coupled to deep sequencing (ChIP-Seq). We identified 6,789 peaks in control and 8,941 peaks in Flcn shRNA cell lines. Cluster analysis showed a high overlap between both Tfe3 ChIP data sets (Figures S6A and S6B), and de novo motif recognition identified a modified E box in more than 80% of bound loci in both experiments (Figure S6C). This suggests that Flcn-Fnip1/2 regulates the amount of chromatin-bound Tfe3 rather than binding specificity. Consistent with this, normalized read numbers per peak region were greater in Flcn shRNA compared to control cells (Figure S6D).

We compared Tfe3 binding with published ESC ChIP data sets (Martello et al., 2012). Cluster analysis of peak regions (Figure S6A) and associated genes (Figure S6B) showed that the Tfe3-binding profile is distinct and well separated from that of a group containing the bHLH transcription factor n-Myc but closer to the core pluripotency factor family including Oct4, Nanog, and Sox2. The majority of sites occupied by Oct4, Sox2, Nanog, and Tfe3 are distant from sites bound by Tfe3 (Figure S6E). However, a significant fraction of common target genes of Oct4, Sox2, Nanog, and Tcf3 are associated with Tfe3 sites (Figure S6F). This group is more likely transcriptionally up- or downregulated during ESC differentiation compared with genes exclusively bound by Tfe3 (Figure S6G), suggesting a functional relationship between Tfe3 and the pluripotency network.

Because pluripotency transcription factors are strongly relayed onto themselves and exhibit pronounced colocation (Chen et al., 2008b), we examined the overlap of Tfe3, Oct4, Sox2, Nanog, and Tcf3 peaks (Table S1). Co-occupied loci included putative cis-regulatory elements in the pivotal pluripotency regulators Klf2, Esrrb (Figure 6A), and Tbx3 (data not shown), suggesting that these may be nodes where Tfe3 connects to the pluripotent circuitry.
To assess functionality of Tfe3 binding, we analyzed mRNA levels after 3 hr of Tam addition to Tfe3-ERT2-expressing cells. We included ApoE and Trpm1, which are bound by Tfe3 (data not shown) and have been described to be regulated by Flcn (Hong et al., 2010) and the Tfe3 homolog MiTF (Miller et al., 2004), respectively. In 2i, we detected induction of Trpm1 and ApoE and a modest but significant upregulation of Esrrb, though not Tbx3 or Kit2 (Figure 6B). Although only about 2-fold, the upregulation of Esrrb is notable on top of the induction by GSK3 inhibition (Martello et al., 2012). In fact, less than 2-fold overexpression of Esrrb was sufficient to impede exit from pluripotency (Figures S6H and S6I). To test whether Flcn regulates this activity of Tfe3, we analyzed target genes after Flcn siRNA transfection. Indeed, Esrrb, ApoE, and Trpm1 were upregulated after Flcn knockdown but not after codepletion of Tfe3. In contrast, an unrelated Tfe3-binding target, Smad7, was not
regulated in ESCs (Figure 6Q). These observations suggest that Flcn may drive exit from pluripotency by restricting nuclear access of Tfe3 and consequently reducing transcriptional activation of the core pluripotency factor Esrrb.

To test for functional relevance, we performed epistasis experiments and knocked down Esrrb, Klf2, Tbx3, and other pluripotency-associated transcription factors including Nanog in Tfe3-ERT2-expressing cells. Only knockdown of Esrrb reduced Tam-dependent resistance to differentiation, by approximately 50% (Figure 6D). Likewise, cotransfection of Esrrb siRNAs together with Flcn or Fnip1/2 depletion restored commitment (Figure 6E). Conversely, nuclear Tfe3 failed to inhibit exit from the ES cell state in Esrrb knockout cells (Figure S6J). These results suggest that Esrrb acts epistatically to Flcn-Fnip1/2-Tfe3. We note, however, that knockdown of Esrrb alone destabilized self-renewal (Figures 6D and 6E), consistent with its pivotal role in the pluripotency circuit (Nichols and Smith, 2012).

**Nuclear Tfe3 Maintains an ESC State**

Tfe3-ERT2-expressing ESCs differentiated into neural progenitors and neurons in N2B27 (Figure 7A, left panel). In the presence of Tam, however, many cells resisted differentiation. These cultures acquired stable morphology within 2–4 passages (Figure 7A, right panel) and could be propagated in N2B27 containing Tam for at least 25 passages. Tfe3-ERT2-Tam (TET) cells derived from Rex1GFPd2 and O4GIP ESCs maintained GFP expression in approximately 60%–90% and 90% of cells in the population, respectively (Figure S7A and data not shown). Transcript profiling showed that, with the exception of Nanog, TET cells maintained expression of pluripotency genes at levels similar to those in 2i cells (Figure 7B) and were distinct from EpiSCs (Figure S7B). Flcn knockout but not wild-type or heterozygous ESCs could be similarly sustained in N2B27 alone, although expression levels of pluripotency genes appeared lower (Figure S7C). The differentiation markers FG5, GATA3, and GATA4 were upregulated in both cases (Figures 7B, 7C, and S7D). To test whether this is because of ongoing differentiation in the cultures, we sorted GFP-high and -low Rex1GFPd2 TET cells. Indeed, GFP-low cells expressed lower levels of pluripotency and higher levels of differentiation markers compared to GFP-high cells (Figure S7E), indicating that TET cell cultures show heterogeneity, similar to ESCs in serum/LIF (Marks et al., 2012).

Withdrawal of Tam caused downregulation of pluripotency gene expression, whereas the neural progenitor markers Sox1, Pax6, Hes5, and Ascl1 were induced (Figures 7C and 7D). Both Flcn knockout cells and TET cells in the presence of Tam self-renewed at single-cell level, generating AP-positive colonies with a skirt of negative cells (mixed) but very few AP-negative, differentiated colonies (Figures 7E and S7F). Addition of a Janus kinase (JAK) inhibitor did not alter colony composition, indicating that LIF-JAK signaling is not required for TET cell self-renewal. In this regard, it is also noteworthy that TET cells self-renew without GSK3 inhibition.

Plating TET cells in 2i conditions yielded similar numbers of colonies compared to the presence of Tam, but the majority were undifferentiated and homogeneously positive for AP (Figure 7E). This suggests that the TET cell state is fully responsive to conversion into the naïve ground state.

To confirm that TET cells retain embryonic identity and developmental potential in vivo, cells kept in Tam for six passages were stably transfected with a GFP-expression plasmid, sorted for high GFP and after two further passages in Tam injected into mouse blastocysts. Resulting embryos showed widespread contribution of GFP-positive cells at E11.5 (Figure 7G). Furthermore, two independently generated TET cell lines contributed to adult tissues. Extensive coat color chimerism was obtained with or without pretreatment in 2i or 4/5 and 6/7 or 2/7 mice, respectively (Figures 7H and S7H and data not shown).

**DISCUSSION**

Exit from the ESC state requires dissolution of the core pluripotency transcription-factor circuit, but how the differentiation machinery breaks down this network is unclear. Recovery of several activators in the GSK3 and MAPK pathways (Figure 1D) indicated that our screen was effective in identifying drivers of commitment. GSK3, via β-catenin and Tcf3, represses the orphan nuclear receptor Esrrb, a central member of the pluripotent circuitry (Festuccia et al., 2012; Ivanova et al., 2006; Martello et al., 2012; Percherade et al., 2012). Besides Tcf3 and the GSK3-scaffold gene APC, we recovered CtBP2, a Tcf3 interactor that may act as a transcriptional corepressor (Brannon et al., 1999; Tarleton and Lemischka, 2010). Autocrine FGF4 signaling drives ESC differentiation, and we recovered two kinases, Kras and Raf1, in this pathway as well as the MAPK-scaffold protein Mapksp1/Mp1 (Westerman et al., 2011). Mp1 is required for MAPK signaling at late endosomes (Teis et al., 2002), and we also identified Hgs/Hrs, an ESCRT-0 complex member important for FGF receptor endocytosis and signaling (Chanut-Delalande et al., 2010).

We found that efficient exit from naïve pluripotency requires components not previously implicated in ESC biology, namely Flcn-Fnip1/2 and the mTOR regulators Tsc1 and Tsc2, that converge on relocalization of the bHLH transcription factor Tfe3. Conversely, enforced nuclear Tfe3 prevents commitment and maintains an ESC state. Interestingly, Tsc1/2 and Flcn are tumor suppressors, whereas Tfe3 is a protooncogene. Tfe3-gene fusions can be causal for renal cell carcinomas, which also occur in tuberosclerosis due to mutations in Tsc1 or Tsc2 and in Bird-Hogg-Dubé (BHD) syndrome due to Flcn mutations (Armah and Parwani, 2010; Linehan et al., 2010). There are differences in the histopathological classifications of these tumors, but there may be a common underlying pathway. Consistent with this, nuclear Tfe3 has been described in BHD kidney cancers (Hong et al., 2010).

**The Flcn Pathway**

The primary sequence of Flcn does not feature recognizable domains. A Flcn protein complex purified from HEK293 cells...
Figure 7. Nuclear Tfe3 Maintains an ESC State

(A) Tfe3-ERT2-expressing cells differentiated into neurons (arrowhead) in the absence (left panel) but retained undifferentiated morphology and could be serially passaged in the presence of 0.1 μM Tam (right panel).

(B) mRNA expression in Rex1GFPd2 and O4GIP TET cells relative to ESCs in 2i conditions. Average and SD are of three independent experiments.

(C) mRNA expression in Rex1GFPd2 TET cells after Tam withdrawal relative to presence of Tam. Average and SD are of two technical replicates.

(D) Immunohistochemistry in Rex1GFPd2 TET cells treated for 6 days with and without 0.1 μM Tam.

(E) TET cells were plated at single-cell density in indicated culture conditions. Resulting colonies were stained for AP (lower panel) and quantified. Average numbers relative to Tam and SD are from two independent experiments (left bar: Rex1GFPd2, right bar: O4GIP).

(F) Rex1GFPd2 TET cells were transfected with indicated siRNAs and replated after 2 days at clonal density in the presence of Tam. Average clonogenicity relative to negative siRNA and SD are of two independent experiments.

(G) Rex1GFPd2 TET cells stably transfected with a GFP-expressing plasmid were microinjected into blastocysts, and resulting embryos analyzed at E11.5. Widespread contribution was detected in 4/7 embryos. For comparison, a non-GFP-expressing embryo (arrowhead) of the same litter is shown.

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contained Fnip1 and AMP kinase (AMPK) (Baba et al., 2006). Fnip2 has been identified based on sequence homology and shown to bind to Fln, Fnip1, and AMPK (Hasumi et al., 2008; Takagi et al., 2008). Although we only identified Fnip1 in Fln IPIs, simultaneous knockdown of Fnip1 and Fnip2 was required for pronounced commitment defects. This may be due to redundancy or a Fln-independent function of Fnip1/2, but the similarity in phenotypes between Fln- and Fnip1/2-depleted ESCs is consistent with them acting in the same pathway. Whereas absence of Fln impedes embryogenesis (Hasumi et al., 2009), Fnip1-deficient mice are viable (Baba et al., 2012; Park et al., 2012), suggesting redundancy with Fnip2. However, Fnip1 and conditional Fln mutants show similar defects in B cell development (Baba et al., 2012), indicating that requirement for Fnip2 is facultative and cell type dependent.

Depletion of Tsc1 or Tsc2, similar to Fln-Fnip1/2, induced nuclear accumulation of Tfe3 and impaired exit from pluripotency. Epistasis experiments with rapamycin and Tfe3 are consistent with mTOR acting upstream of and/or in parallel with Fln-Fnip1/2 to inhibit Tfe3-dependent ESC commitment. Phosphorylation of Fln downstream of mTOR has been described (Baba et al., 2006; Piao et al., 2009), suggesting a linear relationship between Tsc1/2, mTOR, and Fln-Fnip1/2 (Figure 7I). However, the identity of the kinase and how phosphorylation affects Fln remains to be determined. In vivo, Tsc1 and Tsc2 knockout mice show defects after gastrulation at around E10.0 (Kobayashi et al., 1999, 2001), whereas Fln mutants are affected by E6.0 (Hasumi et al., 2009). Tsc mutant embryos feature phenotypic variability and developmental delay, though, which may suggest earlier defects that are compensated for by regulatory mechanisms.

We postulate that Tsc1/2 and Fln-Fnip1/2 phenotypes in ESCs are mediated by nuclear translocation and activation of Tfe3 (Figure 7I). Depletion of Tfe3 accelerated ESC commitment, indicating that Tfe3 resists breakdown of the pluripotency network. However, Tfe3 mutant mice are viable and fertile (Stein Grimsson et al., 2002). MTF, Tfe3, and Tfeb can interact with each other (Hemesath et al., 1994), and functional redundancy has been reported (Huan et al., 2006; Stein Grimsson et al., 2002). In fact, although Tfeb was not essential for Tsc2 or Fln-Fnip1/2 phenotypes (Figures 5A and 6E), its overexpression impaired differentiation (data not shown). Thus, redundancy could explain why Tfe3 mutants have no overt developmental phenotype. It should also be noted that several factors important for in vitro ESC self-renewal are dispensable in the embryo (Nichols and Smith, 2012).

**Regulation of Pluripotency**

However, early lethality of Fln mutant embryos is consistent with a role of the Fln-Fnip1/2-Tfe3 module in progression of pluripotency in vivo. We observed a change in subcellular localization of Tfe3, suggesting that Fln-Fnip1/2 is inactive in the early ICM and then becomes active in epiblast but not hypoblast cells. Fln, Fnip1, Tsc1, and Tsc2 mRNA levels persisted during ESC differentiation (Figure 3E) and the transition from ICM to epiblast cell identities (Tang et al., 2010). Furthermore, Fln-Fnip1 complex stoichiometry was maintained during exit from the ESC state (Figure 3D), suggesting posttranscriptional regulation.

Nuclear exclusion of Tfe3 in postimplantation epiblast cells appears to coincide with epithelialization prior to gastrulation and loss of ESC derivation potential (Nichols and Smith, 2011). Tfe3 similarly translocates into the cytoplasm at the onset of ESC differentiation and is cytoplasmic in EpiSCs. These observations suggest that the Fln-Fnip1/2-Tfe3 module acts as a cell-fate switch that steers naive early epiblast and ESCs into a primed postimplantation state in vitro and in vivo.

Fln-Fnip1/2-Tfe3 activity requires an ESC-specific context, as nuclear translocation of Tfe3 or knockdown of Tsc2 and Fln-Fnip1/2 in EpiSCs did not induce naive pluripotency. Similarly, ectopic activation of Tfe3 efficiently impeded ESC differentiation in the first 24 hr after inhibitor withdrawal, but less so afterward (Figure 5E).

The ability of nuclear Tfe3 to maintain an ESC state in the complete absence of pro-self-renewal or antiderivation signals is unprecedented and implies potent input to the transcriptional circuitry sustaining self-renewal (Nichols and Smith, 2012). Functional analysis indicates that transcriptional upregulation of Esrrb is a relevant output of nuclear Tfe3. It is likely that there are additional Tfe3 targets because TET cells self-renew without requirement for either MAPK or GSK3 inhibition, whereas forced expression of Esrrb confers independence only from the latter (Martello et al., 2012). Nonetheless, it is striking that transcription of Esrrb is regulated by Fln-Fnip1/2-Tfe3, GSK3/i-catenin/Tcf3 (Martello et al., 2012), and Nanog (Festuccia et al., 2012) (Figure 7I). We surmise that repression by Tcf3 combined with withdrawal of Tfe3 and downregulation of Nanog act to ensure prompt and efficient silencing of this pivotal pluripotency factor at the onset of differentiation. It remains to be tested whether distinct regulatory inputs act in parallel or in sequence, but it is striking that absence of Tcf3 does not infringe development prior to gastrulation (Merrill et al., 2004), suggesting compensatory dependence of Esrrb on Nanog or Tfe3 in vivo.

In summary, this study has identified a module that was previously undescribed in ESCs and mediates a switch from naive to primed pluripotent states. Our findings suggest that nuclear exclusion of Tfe3 acts as a rheostat for controlled disequilibration of the regulatory network that establishes ground-state pluripotency in the embryo and sustains ESCs in vitro.

**EXPERIMENTAL PROCEDURES**

Full details are provided in the Extended Experimental Procedures.
Mice and Embryos
Chimeras were generated by microinjection into C57BL/6 blastocysts. Animal experiments were approved by the University of Cambridge Ethical Review Committee and authorized by the UK Home Office.

Cell Culture
ESCs were maintained in serum-free N2B27 medium containing 2i and, where indicated, LIF (Wray et al., 2011; Ying et al., 2008). EpiSCs were cultured as described (Guo et al., 2009).

Commitment Assay
ESCs were plated on gelatin-coated plates at 1.5 × 10⁶ cells/cm². The next day, cells were washed with PBS (PAa) and medium changed to N2B27 without 2i or LIF. Commitment of O4GIP ESCs was determined by restoring 2i plus 1 μg/ml puromycin (Sigma). Uncommitted ESCs were quantified after 2-3 days by adding Alamar Blue (Invitrogen), diluted 1:10 in 2i medium, and read out on a SpectraMax M2 (Molecular Devices). Commitment of other genotypes was quantified by replating on laminin-coated plates in ESC medium. For REX1GFPd2 cells, 10 μg/ml blasticidin (PAa) was added to select for Rex1 expression.

siRNA Transfection
Cells were reverse transfected with 16.7 nM siRNA in 2i or FGF2/Activin A containing N2B27 medium with transfection mixes prepared with RNAiMax or Lipofectamine 2000 (Invitrogen) in OptiMEM (Invitrogen). All siRNAs with the exception of Tcf7l1/Tcf3 (Dharmacon, L-048614-01-0005) and Tbx3 (Ambion, 223884 and 223885) were purchased from Qiagen (FlexiTube GeneSolution siRNA pools; AllStars Negative Control siRNA, 1027281; GFP-22 siRNA, 1022064). Knockdown of Tcf3 is included as a reference.

ACCESSION NUMBERS
ChIP-Seq data sets have been deposited in the GEO database with the ID number GSE39815.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.03.012.

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