TCF/LEF regulation of the topologically associated domain ADI promotes mESCs to exit the pluripotent ground state

Highlights

- Generation of TCF/LEF-KO, β-catenin-KO, and TCF/LEF+β-catenin-KO mESC lines

- Quadruple TCF/LEF-KO (qKO) mESCs self-renew in the absence of 2iL

- Aire, Dnmt3l, and IcosL are targets of TCF/LEF but not of β-catenin

- Aire and Dnmt3l are functional TCF/LEF effectors mediating the exit from pluripotency

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In brief

Doumpas et al. show that mESCs with genetic deletion of all four TCF/LEF transcription factors self-renew in the absence of GSK and MEK inhibition. TCF/LEFs drive mESCs to exit their state of self-renewal through Wnt/b-catenin-independent transcriptional regulation of the ADI domain that contains three genes, Aire, Dnmt3l, and IcosL.
**TCF/LEF regulation of the topologically associated domain ADI promotes mESCs to exit the pluripotent ground state**

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**SUMMARY**

Mouse embryonic stem cells (mESCs) can be maintained in vitro in defined N2B27 medium supplemented with two chemical inhibitors for GSK3 and MEK (2i) and the cytokine leukemia inhibitory factor (LIF), which act synergistically to promote self-renewal and pluripotency. Here, we find that genetic deletion of the four genes encoding the TCF/LEF transcription factors confers mESCs with the ability to self-renew in N2B27 medium alone. TCF/LEF quadruple knockout (qKO) mESCs display dysregulation of several genes, including Aire, Dnmt3l, and Icosl, located adjacent to each other within a topologically associated domain (TAD). Aire, Dnmt3l, and Icosl appear to be regulated by TCF/LEF in a β-catenin independent manner. Moreover, downregulation of Aire and Dnmt3l in wild-type mESCs mimics the loss of TCF/LEF and increases mESC survival in the absence of 2iL. Hence, this study identifies TCF/LEF effectors that mediate exit from the pluripotent state.

**INTRODUCTION**

Mouse embryonic stem cells (mESCs) are derived from pre-implantation blastocyst cells that, when cultured in vitro, display unrestrained self-renewal while preserving differentiation potential (Huang et al., 2015; Evans and Kaufman, 1981). mESCs can be propagated in a chemically defined medium termed N2B27, supplemented with two specific inhibitors (2i) to secure pluripotency: CHIR90921 (hereafter referred to as CHIR) inhibits glycogen synthase kinase 3 (GSK3), and PD0325901 (referred to as PD) blocks mitogen-activated protein kinase kinase (MEK, also known as MAPKK) (Yang et al., 2008). Further addition of LIF (leukemia inhibitory factor) activates JAK/STAT3 signaling to stimulate self-renewal (Smith et al., 1988). “2iL” in N2B27 is the standard cocktail to allow mESCs to divide perpetually, to preserve the features of the inner cell mass of the blastocyst (Blair et al., 2011; Morgani et al., 2017), and to sustain stable expression of the transcription factors typical of the naive pluripotency gene regulatory network (Blair et al., 2011; Dunn et al., 2014; Morgani et al., 2017; Mulas et al., 2019).

Many efforts have been devoted to identify genes that promote exit from the pluripotent state and the transition to a primed state of differentiation (Betschinger et al., 2013; Guo et al., 2011; Kinoshita et al., 2020; Li et al., 2018; Villegas et al., 2019). The Wnt/β-catenin effector TCF7L1 (previously referred to as TCF3), belonging to the family of four TCF/LEF transcription factors, was one of the first identified players in this process (Pereira et al., 2006). Mechanistically, TCF7L1 acts as pro-differentiation factor by repressing pluripotency genes such as Nanog and Klf2 (Dunn et al., 2014; Pereira et al., 2006; Yeo et al., 2014), and its activity is counteracted by CHIR-mediated GSK3 inhibition (Martello et al., 2012; Qiu et al., 2015). CHIR stabilizes β-catenin that, in turn, is thought to abrogate TCF7L1 repression of pluripotency genes thereby allowing a Wnt signaling response mediated by other TCF/LEF factors (Wray et al., 2011; Yi et al., 2011). Hence, it has been hypothesized that β-catenin stabilization is a key mechanism by which CHIR promotes mESC self-renewal (Aulicino et al., 2020; Kelly et al., 2011; Wray et al., 2011; Yi et al., 2011). On the other hand, β-catenin stabilization could induce the activation of differentiation genes upon interaction with TCF7 and LEF1 (Chatterjee et al., 2015; Chen et al., 2013), indicating that fine-tuning of this pathway is crucial for the balance between self-renewal and differentiation of mESCs (Habib et al., 2013).

Of note, there is little evidence that the genetic abrogation of the mechanisms required for the exit from the pluripotent state...
is sufficient to enable self-renewal in the absence of 2iL (Guo et al., 2011; Wray et al., 2011). For example, homozygous Tcf7l1 deletion confers enhanced self-renewal but does not substitute for LIF (Guo et al., 2011). Recent efforts identified a number of factors that contribute to the transition from pluripotency to differentiation, such as the transcription factor ETV5 (Kalkan et al., 2019), or the plasma membrane Ca²⁺ pump ATP2b1 (MacDougall et al., 2019). Loss-of-function mutations of Etv5 or Atp2b1, when introduced in a Tcf7l1 knockout background, allow maintenance of mESC self-renewal in the absence of 2iL.

Here, we found that complete loss-of-function of Tcf7l1, Lef1, Tcf7l1, and Tcf7l2, the genes encoding for the four TCF/LEF transcription factors, allows mESCs to become fully 2iL-independent and to propagate in basal N2B27 ad libitum. Transcriptomics profiles (RNA sequencing [RNA-seq]) and chromatin immunoprecipitation (ChIP-seq) data pointed to a TCF/LEF-regulated locus containing three genes, Aire, Dnmt3l, and Icosl. These genes are clustered within a topologically associated domain (TAD), and their downregulation functionally contributes to the acquired 2iL-independent self-renewal. Importantly, this mechanism appears to be independent of β-catenin, thus suggesting that an alternative TCF/LEF-dependent regulatory mechanism is employed at this locus.

RESULTS

We generated quadruple knockout (qKO) mESCs bearing loss-of-function mutations in the four genes encoding the TCF/LEF family of transcription factors (Figure 1A). qKO mESCs did not display any detectable TCF/LEF protein (Figure 1B) and were incapable of transducing Wnt/β-catenin-dependent transcription upon pathway stimulation (Figure S1). Surprisingly, qKO mESCs acquired the ability to proliferate in N2B27 basal medium without 2iL supplementation, a condition in which wild-type mESCs (WT) are lost after only a maximum of two passages (Morgani et al., 2017) (Figure 1C). Systematic testing of cell lines carrying different combinations of TCF/LEF knockout revealed that survival in the absence of 2iL is possible only when all Tcf/.Lef genes are nullizygous (Figure 1D). Of note, a recent report provided evidence that compound deletion of only Tcf7l1 and Lef1 induced similar 2iL-independent renewal properties (Ye et al., 2017). Combined with our observation, this suggests that TCF/LEF proteins possess non-redundant functions in mESCs, and that TCF7L1 and LEF1 likely represent the key effectors that induce the exit from the pluripotent state.

To test whether qKO mESCs maintained stem cell features in the absence of 2iL—in addition to cell proliferation—we exploited three classic approaches. First, we performed staining for alkaline phosphatase (AP), a key marker of pluripotency in ESCs (Stefková et al., 2015). 6 days after single-cell plating in basal N2B27, qKO cells remained AP positive and were indistinguishable from WT colonies grown in 2iL medium, both in shape and number (Figure 1E). Second, we measured the expression of the pluripotency genes, Oct4, Nanog, Sox2, and Klf4 via quantitative reverse transcriptase PCR (qRT-PCR). Even after 14 days in basal N2B27 medium, qKO mESCs still expressed levels of Oct4, Nanog, and Sox2 comparable to WT control cells grown in presence of 2iL (Figure 1F). Klf4 expression was decreased, suggesting possible alternative regulation of this gene, which did not perturb self-renewal. Finally, we allowed spontaneous differentiation into embryoid bodies (EBs), embryo-like aggregates containing cells of all three germ layer lineages (Doetschman et al., 1985). qKO cells formed EBs that expressed general differentiation markers (Figure 1G). While TCF7L1 is known to repress pluripotency genes (Dunn et al., 2014), TCF7 was shown to promote mESC differentiation in a β-catenin-dependent manner (Chatterjee et al., 2015). It is
Figure 2. Aire, Dnmt3l, and IcosL are TCF/LEF effectors

(A) Volcano plot of differentially expressed genes (DEGs) in the qKO versus WT comparison (without additional CHIR stimulation). The x axis shows logarithmically (log2) transformed fold change of expression, while the y axis represents the –log10-transformed FDR-adjusted p values. Each dot represents an individual gene. Blue dots correspond to downregulated genes and red dots to upregulated genes. Gray dots denote genes whose differential expression was not statistically significant based on an FDR-adjusted p value threshold of 0.05.
plausible that the mere inability of activating canonical Wnt/β-catenin signaling locks qKO cells in a perpetual self-renewing state. We tested this by inducing loss-of-function mutations in Ctnnb1, the gene encoding for β-catenin, both in WT mESCs (to obtain β-catenin knockout, icatKO cells), and in qKO cells (to obtain penta-knockout, penKO cells). The use of guide RNA (gRNA) targeting exons 4 and 12 resulted in several clones displaying loss of detectable β-catenin protein (Figure 1H). As expected, icatKO and penKO clones were incapable of activating Wnt-dependent transcription, as seen by their failure to induce endogenous Wnt target gene expression upon CHIR stimulation (Figure 1I).

To understand the genetic changes that follow deletion of TCF/LEF and might allow qKO cells to achieve 2iL-independent self-renewal, we performed RNA-seq of qKO and WT mESCs cultured in standard 2iL and identified 512 upregulated and 348 downregulated genes in qKO cells (Figure 2A; Figures S3A and S3B; Table S1). In agreement with previous data (Moreira et al., 2017), qKO cells had reduced expression of endoderm and mesoderm markers (Figure S3B). Gene Ontology (GO) scrutiny revealed that genes involved in embryonic development and cell differentiation were enriched among the downregulated groups (Figure 2B). In particular, we noticed that close to the top of the list of genes ranked based on their fold change, three displayed similar levels of downregulation and encode: autoimmune regulator (Aire), DNA methyltransferase 3 like (Dnmt3l), and inducible T cell co-stimulator ligand (IcosL) (Figure 2C; Figure S3C). Surprisingly, these genes are located adjacent to each other within the same genomic locus at 10qC1 (Figure 2D), and their chromosomal arrangement is conserved between mouse and human. By exploring Cohesin-ChIA-PET datasets obtained from mESC studies (Downen et al., 2014), we found that the genomic borders of this locus are marked by Cohesin complexes, likely representing the boundaries of a topologically associated domain (TAD, Dixon et al., 2013) (Figure 2D), to which we refer as the ADI locus (for Aire-Dnmt3l-IcosL).

We hypothesized that TCF/LEF family members could contribute to the regulation of the genes in the ADI locus and used chromatin immunoprecipitation followed by deep DNA sequencing (chromatin immunoprecipitation sequencing [ChIP-seq]) to assess potential physical association of these transcription factors with this locus. We identified TCF7L1 ChIP-seq peaks in the proximity of several genes differentially expressed in qKO cells, including one at the center of the ADI locus, downstream of the IcosL coding sequence (Figure 2F; Table S2; Moreira et al., 2018). The size of the TCF7L1 ChIP-seq peak at this location was present but slightly reduced upon increase of CHIR concentration (Figure 2F, bottom track). Of note, the observed reduction in peak intensity is likely due to a CHIR-induced decrease in TCF7L1 protein levels (Figure S3D), corroborating previous reports showing that CHIR causes Tcf7l1 downregulation in mESCs (Ye et al., 2017). TCF7, which did not confer dependency on 2iL, showed no association with this locus (Figure 2G). Consistent with our genetic evidence, no β-catenin ChIP-seq peak was ever detected within the borders of the ADI locus across three independent experiments in CHIR-stimulated mESCs. (Figure 2H). On the other hand, β-catenin association was always observed at classic Wnt responsive elements (WREs) present in the Axin2 and Sp5 promotors, where it requires TCF/LEF for its physical association with DNA targets (Figure 2H; Figure S4; Table S3).

Notably, this TCF7L1-bound DNA region has been previously annotated as a typical enhancer in mESCs (Figure 3A; Downen et al., 2014), and it also displayed simultaneous presence of H3K4me1 and H3K27ac histone marks (Figure 3A; Joshi et al., 2015); we refer to this regulatory region as the ADI enhancer. To test whether the ADI enhancer is responsive to TCF7L1, we PCR-amplified the genomic region of ~200 bp underlying the...
TCF7L1 ChIP-seq peak and cloned it into a Luciferase expression plasmid upstream of a minimal thymidine kinase promoter (Figure 3B). Remarkably, TCF7L1 overexpression produced an ~15-fold activation of the ADI enhancer reporter, while it did not induce any transcriptional activity of the minimal promoter control reporter (Figure 3B, right). In agreement with our previous observation that CHIR induced a mild TCF7L1 downregulation, CHIR quantitatively, but not completely, decreased the induction of the ADI enhancer by TCF7L1 (Figure 3B). Of note, overexpression of TCF7L1 in HEK293T cells induced a significant upregulation of Aire, Dnmt3l, and Icosl (Figure 3C), supporting the notion of a direct transcriptional regulation of the genes in the ADI locus by TCF7L1. The ability of TCF7L1 to induce transcription in vitro from the ADI enhancer raised the prediction that removal of CHIR from the culture medium would allow TCF7L1 to activate the ADI genes in mESCs. Indeed, we found that the expression of the ADI genes is suppressed by CHIR, while removing CHIR from the culture medium, a condition that still allows mESCs to divide, results in significant transcriptional upregulation of the ADI genes (Figure 3D). This regulation is dependent on TCF/LEF, as the upregulation of the ADI genes did not occur in qKO cells (Figure 3D). Notably, we detected significant upregulation of Aire also in 6catKO cells upon CHIR removal (as it occurs in WT cells), while Dnmt3l and Icosl remained unchanged (Figure S3E), indicating that the CHIR-mediated, TCF7L1-dependent regulation of Aire is likely β-catenin independent. To further test the notion that the ADI enhancer regulates the expression of Aire, Dnmt3l, and Icosl, we analyzed the Capture Hi-C data from Joshi and colleagues, which recorded the DNA-DNA interactions occurring in mESCs with high resolution (Joshi et al., 2015), and focused on the ADI locus. When mESCs are cultured in DMEM containing 10% fetal calf serum and LIF, the ADI enhancer displays several long-range chromatin contacts within this locus, including significant ones with the promoters and coding sequences of Aire, Dnmt3l, and Icosl (Figure 3E). Consistent with our observations, these interactions appear to be decreased when 2i is added to the culture medium (Figure 3E), supporting the notion that CHIR inhibits the functional association between Aire, Dnmt3l, and Icosl and the ADI enhancer.

Our observations raise the possibility that ADI genes are relevant TCF/LEF targets that induce the exit from the pluripotent state. Therefore, we tested whether their decreased expression in qKO could contribute to the sustained 2iL-independent self-renewal. We induced downregulation of the ADI genes expression in WT mESCs and assessed the capability of treated cells to divide in 2iL-deprived N2B27 basal medium (Figure 4A). By means of small interfering RNAs (siRNAs), we achieved efficient and relatively stable downregulation of Aire and Dnmt3l and, albeit to a lesser extent, of Icosl (Figure 4B). WT mESCs electroporated with scrambled siRNAs could not divide in basal N2B27 and died after 4 days (Figure 4C). Notably, simultaneous downregulation of the ADI genes extended cell self-renewal over 7 days of culture (Figure 4D). Strikingly, individual downregulation of Aire or Dnmt3l had a similar effect on colony viability (Figure 4C), suggesting that these two genes might contribute to the balance between self-renewal and differentiation via parallel, non-redundant mechanisms. In addition to the proliferation/viability assay, we tested the expression of the pluripotency genes Oct4, Nanog, Sox2, and Klf4. Depletion of 2iL, in WT cells, caused loss of self-renewal accompanied by a drop in the expression of these genes (Figure 4D). Consistent with their sustained ability to divide, and similarly to qKO cells, mESCs with decreased expression of Aire, Dnmt3l, and Icosl, maintained high expression of the pluripotency transcription factors to levels comparable to WT cells (Figure 4D). Taken together, these data support a model in which decreased expression of the genes in the ADI locus in qKO cells is responsible, at least in part, for the acquired independence of the 2iL inhibitors.

**DISCUSSION**

Here, we report the surprising observation that the loss of all TCF/LEF transcription factors confers to mESCs the ability to self-renew in the absence of CHIR, PD, and LIF. This effect is likely achieved through downregulation of three key genes physically located within the same genomic locus: Aire, Dnmt3l, and Icosl. These genes belong to a TAD, suggesting that they are co-regulated in mESCs. In particular, Aire and Dnmt3l have overlapping, yet directionally opposing transcriptional start sites, hinting

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**Figure 3. TCF7L1 binds enhancer in a TAD influencing ADI gene expression**

(A) Genomic tracks with focus on the TCF7L1 binding site downstream of the ADI locus. First track: TCF7L1 ChIP-seq showing the binding peak emphasized by a semi-transparent red box. Second track: typical enhancers predicted by Dowen et al. (2014). One such enhancer region is seen overlapping the TCF7L1 peak within the ADI locus. Third and fourth tracks: histone modifications (H3K4me1 and H3K27ac, respectively) from Joshi et al. (2015). The histone modification peaks corresponded the DNA-DNA interactions occurring in mESCs with the promoters and coding sequences of Aire, Dnmt3l, and Icosl (Figure 3C). Third and fourth tracks: histone modifications (H3K4me1 and H3K27ac, respectively) from Joshi et al. (2015). The histone modification peaks corresponded the DNA-DNA interactions occurring in mESCs with the promoters and coding sequences of Aire, Dnmt3l, and Icosl (Figure 3C). Relevant interactions whose anchors points the ADI enhancer overlap with ADI genes’ promoters are annotated with thick red lines. For each of these tracks, the data correspond to two merged experimental replicates.

(B) Left panel: schematic representation of the TCF7L1 enhancer peak tested in a luciferase assay (ADI enhancer reporter). Right panel: ADI enhancer reporter response in different stimulation conditions: +/- CHIR, +/- TCF7L1 or LEF1 overexpression. Samples were compared using Student’s t test. *p < 0.05; **p < 0.01; ns, not significant (p > 0.05).

(C) Left panel: qPCR validation of TCF7L1 overexpression in HEK293T. Right panel: expression of the ADI genes was measured via qRT-PCR upon overexpression of TCF7L1 or LEF1. The data represent the mean ± SEM obtained from three independent experiments (n = 3). Samples were compared using Student’s t test. *p < 0.05; ns, not significant (p > 0.05).

(D) Expression of the ADI genes was measured via qRT-PCR upon treatment or removal of CHIR in mESCs. The data represent the mean ± SEM obtained from three independent experiments (n = 3). Samples were compared using Student’s t test. *p < 0.05; **p < 0.01; ns, not significant (p > 0.05).

(E) Genomic tracks displaying long-range chromatin interactions obtained by Capture Hi-C (Joshi et al., 2015). First track: TCF7L1 ChIP-seq showing the TCF7L1 ChIP-seq peak, on the right of the ADI locus, marks the ADI enhancers. Second track: annotation of the Aire, Dnmt3l, and Icosl promoters. Third and fourth tracks: long-range chromatin interactions (serum and 2i, respectively) shown as arches connecting the associated regions. Relevant interactions whose anchors points the ADI enhancer overlap with ADI genes’ promoters are annotated with thick red lines. For each of these tracks, the data correspond to two merged experimental replicates.

Cell Reports 36, 109705, September 14, 2021 7
to the possibility that their coordinated regulation and function might be a general phenomenon also important in other tissues. Aire, Dnmt3l, and IcosL seem to be activated downstream of TCF/LEF and contribute to the balance between self-renewal and differentiation of mESCs. Of note, AIRE and Dnmt3L have previously been implicated in mESC function. In line with the notion that AIRE could contribute to the exit of mESCs from the pluripotent state, knockdown of Aire in mESCs resulted in attenuated cell cycle but also in the reduction of differentiation-associated genes (Gu et al., 2010). Dnmt3L, an enzymatically inactive DNA methyltransferase that regulates enzymatically functional de novo methyltransferases Dnmt3a and Dnmt3b through direct protein-protein interactions (Suetake et al., 2004). Dnmt3L was shown to function as regulator of DNA methylation in mESCs, and, consistent with our findings, its expression has been shown to decrease upon CHIR administration (Wu et al., 2013). The third gene, IcosL, encodes a secreted ligand that regulates B cell function (Liu et al., 2015); more work is needed to understand its yet-unknown role in mESCs. Intriguingly, IcosL expression in cancer patients correlates with decreased survival, possibly indicating a function in sustained cell proliferation or self-renewal (Wang et al., 2019). Overall, our data support a model in which the Aire genes are activated by Tcf7l1 and act as pro-differentiation factors.

Among different signaling pathways, Wnt/b-catenin has been shown to play a major role in the regulation of self-renewal and pluripotency of mESCs (Ogawa et al., 2006; Sato et al., 2004; Söderholm and Cantù, 2020). More specifically, Wnt/b-catenin signaling is required for the induction and patterning of endoderm and mesoderm during gastrulation, a process that can be mimicked in vitro in mESCs (Baillie-Johnson et al., 2015). Surprisingly, despite diverging functions of TCF/LEF members in mESCs (Lyashenko et al., 2011; Wray et al., 2011; Yi et al., 2011), a single TCF/LEF transcription factor has been shown to be capable of driving trilineage differentiation of mESCs (Moreira et al., 2017). Of note, the genes in the Aire locus that we identify in this work appear to be directly regulated by Tcf7l1, in response to CHIR, but neither by Tcf7 nor by b-catenin. Their expression is decreased when TCF/LEF are mutated but increases in response to CHIR removal. This is compatible with the notion that CHIR dampens the activation of Aire by Tcf7l1, thereby contributing in sustaining the pluripotency network (Dunn et al., 2014). However, the Aire genes are unaffected by b-catenin deletion both in WT as well as in a qko background. Consistent with this notion, no b-catenin was found to physically bind to the Aire locus. An intriguing possibility is that Plikogobin/b-catenin substitutes b-catenin in our bcatKO cells. b-catenin has been shown to play a role for the activation of TCF/LEF target genes in b-catenin-deficient cells (Maeda et al., 2004). b-catenin is also stabilized by CHIR, but its endogenous levels are not normally sufficient to promote the regulation of classic Wnt targets (Mahendram et al., 2013). While a possible regulation of the Aire locus by b-catenin remains to be tested, we could not detect any upregulation of the Jup mRNA (encoding for b-catenin) in our bcatKO cells (Figure S3F), that might hint at such a compensatory mechanism. Taken together, our data suggest that an alternative, b-catenin-independent but TCF/LEF-dependent regulatory mechanism is operating at the Aire locus in mESCs.

**Figure 4. Knockdown of Aire renders mESCs 2iL independent**
(A) Schematic description of siRNA-mediated downregulation of Aire genes.
(B) Efficiency of downregulation of Aire, Dnmt3b, and IcosL measured by qRT-PCR performed on WT mESCs 3 and 5 days after specific siRNA treatment. The data represent the mean ± SEM obtained from three independent experiments (n = 3). Samples were compared using Student’s t test. **p < 0.01; ***p < 0.001.
(C) Cell viability after 7-day culture in N2B27 for scrambled siRNA-treated control mESCs or cells treated with specific Aire gene siRNAs. A black sad smile is used as placeholder for an otherwise empty plate as consequence of cell death. The black scale bar displayed on the top-left image represents approximately 200 μm.
(D) qRT-PCR analysis of Oct4, Nanog, Sox2, and Klf4, performed after siRNA treatment for each of the annotated gene (Aire, Dnmt3l, Icosl), cultured in N2B27 medium without 2iL. The data represent the mean ± SEM obtained from three independent experiments (n = 3). Samples were compared using Student’s t test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant (p > 0.05).
Limitations of the study
Our work leaves two questions unsolved. The first concerns the relevance of the interaction between TCF7L1 and the ADI enhancer, and how this is interrupted or attenuated by CHIR in a manner that likely does not involve β-catenin. Novel assays need to be employed to test the functional role of this physical association. For example, CRISPR/Cas-mediated deletion of the ADI enhancer, or mutation of the TCF7L1 binding motif contained in it, might impact TCF7L1 binding and the consequent regulation of Aire, Dnmt3l, and IcosL. Our model would predict that such genomic mutations would extend the lifespan of mESCs in the absence of 2iL.

Second, we did not address the mechanism by which the ADI genes contribute to the exit from the pluripotent state. Further loss-of-function and gain-of-function approaches in mESCs will be required to tackle this. In particular, whether IcosL contributes to this process at all remains unclear. It is plausible that the siRNA-mediated partial downregulation that we achieve is sufficient to affect pluripotency genes (Figures 4B and 4D) but not to extend renewal in the absence of 2iL (Figure 4C).

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Culture of cell lines
- METHOD DETAILS
  - Generation of knockout mESC lines
  - Embryoid body formation
  - Clonogenicity assays
  - Quantitative (q)RT-PCR
  - Western blot analyses
  - Antibodies
  - Electroporation
  - Transcriptomics analysis (RNA-seq)
  - Chromatin immunoprecipitation
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Statistical analysis
  - Western blot quantification
  - RNA-seq data analysis
  - β-catenin ChIP-seq data analysis

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109705.

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AUTHOR CONTRIBUTIONS
N.D., S.S., C.C., and K.B. designed the experiments. N.D. conducted the experiments and prepared the figure panels. S.S. performed the bioinformatics analyses and composed the figures. S.N. and B.W.D. generated the βcatKO and penKO cell clones. S.M. and B.W.D. performed ChIP-seq experiments of TCF7 and TCF7L1, and C.C. performed the ChIP-seq experiments of β-catenin. All authors assisted with data interpretation. C.C. and K.B. supervised the research teams and provided financial support for the study. C.C. wrote the manuscript with input from all authors. All authors contributed to multiple rounds of manuscript revision.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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**STAR METHODs**

**KEY RESOURCES TABLE**

| Reagent or resource            | Source                          | Identifier       |
|--------------------------------|---------------------------------|------------------|
| Antibodies                     |                                 |                  |
| α-tubulin                      | Sigma-Aldrich                   | Cat# T6074; RRID:AB_477582 |
| TCF7                           | Cell Signaling                  | Cat# C63D9; RRID:AB_2199302 |
| LEF1                           | Cell Signaling                  | Cat# C12A5; RRID:AB_823558 |
| TCF7L1                         | Abcam                           | Cat# ab86175; RRID:AB_1925494 |
| TCF7L2                         | Cell Signaling                  | Cat# C48H11; RRID:AB_2199816 |
| β-catenin                      | R&D system                      | Cat# AF1329; RRID:AB_354736 |
| β-catenin                      | Santa Cruz                      | Cat# SC-7963; RRID:AB_626807 |
| HRP anti-mouse                 | Jackson                          | Cat# 115-035-003; RRID:AB_10015289 |
| HRP anti-rabbit                | Jackson                          | Cat# 111-035-144; RRID:AB_2307391 |
| Bacterial and virus strains    |                                 |                  |
| DH5a Competent Cells           | ThermoFisher (eBioscience)      | Cat# 18265017    |
| Chemicals, peptides, and recombinant proteins | | |
| DMEM (1X) + GlutaMAX           | GIBCO                           | 31966-021        |
| DMEM (1x)                      | GIBCO                           | 41966-029        |
| Opti-MEM                       | GIBCO                           | 31985082         |
| Heat Inactivated FBS           | GIBCO                           | 10500-064        |
| TrypLE Express                 | GIBCO                           | 12604-013        |
| Trypsin-EDTA                   | GIBCO                           | 25200056         |
| Tween 20                       | Sigma                           | P1379-250ML      |
| PBS, pH 7.4 (1X)               | GIBCO                           | 10010-015        |
| Tris(hydroxymethyl)aminomethane| Biosolve                        | 200923           |
| Triton X-100                   | Sigma-Aldrich                   | 93443            |
| SDS                            | Sigma-Aldrich                   | 11667289001     |
| HEPES                          | Millipore                       | 391340           |
| EDTA                           | Sigma-Aldrich                   | 93283            |
| EGTA                           | Millipore                       | 324626           |
| Pen/strep                      | ThermoFischer                   | 15140-122        |
| B27 (50x)                      | ThermoFischer                   | 17504-044        |
| N2 (100x)                      | ThermoFischer                   | 17502-048        |
| Gelatine                       | Sigma-Aldrich                   | G1890            |
| CHIR99021                      | STEMCELL TECHNOLOGIES           | 72052            |
| PD0325901                      | STEMCELL TECHNOLOGIES           | 72184            |
| ESGRO                          | Merck                           | ESG1107          |
| Puromycin                      | Sigma-Aldrich                   | P8833-10MG       |
| SYBR Green SuperMix            | BioRad                          | 170880           |
| Bovine Serum Albumin Fraction V| Sigma                           | 10735086001     |
| ethylene glycol-bis            | Merck                           | E3257-250MG      |
| Laminin                        | Sigma-Aldrich                   | L2020            |
| NuPAGE                         | ThermoFischer                   | NP0004           |
| formaldehyde                   | Sigma-Aldrich                   | 47608-1L-F       |
| Critical commercial assays     |                                 |                  |
| AP kit                         | Sigma-Aldrich                   | SCR004           |
| Lipofectamine® 3000            | ThermoFischer                   | L3000001         |

(Continued on next page)
## Deposited data

| Description                                                                 | Source/Identifier               |
|-----------------------------------------------------------------------------|---------------------------------|
| Raw and processed RNA-seq data                                              | This paper, ArrayExpress: E-MTAB-10564 |
| Raw and processed ChIP-seq data (β-catenin)                                 | This paper, ArrayExpress: E-MTAB-10575 |
| ChiA-PET data                                                               | Downen et al., 2014, GEO: GSE57913 |
| Capture Hi-C data                                                           | Joshi et al., 2015, GEO: GSE72164 |
| Mouse reference genome and annotation, release M24, GENCODE                 | hhttps://www.gencodegenes.org/mouse/ |
| Mouse reference genome GRCh38/mm10, UCSC                                    | http://hgdownload.cse.ucsc.edu/goldenpath/mm10/bigZips/ |
| ENCODE blacklisted regions, v2                                               | Amemiya et al., 2019, https://github.com/Boyle-Lab/Blacklist/tree/master/lists |
| UCSC Liftover chain data, mm9 to mm10                                       | UCSC, http://hgdownload.soe.ucsc.edu/goldenPath/mm9/liftOver/mm9ToMm10.over.chain.gz |

## Experimental models: Cell lines

| Cell line          | Source/Identifier |
|--------------------|-------------------|
| HEK293T            | ATTC, CRL-3216    |
| E14TG2a            | ATTC, CRL-182     |

## Oligonucleotides

| PCR primers and guide RNAs designed in this study | See Table S4 for oligonucleotide information |
|---------------------------------------------------|---------------------------------------------|
|                                                  | N/A                                         |

## Software and algorithms

| Software and algorithms                                                                 | Source/Website                                                                 |
|----------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| ImageJ (Fiji) v. 2.0.0                                                                   | Schneider et al., 2012, https://imagej.net/software/imagej/                    |
| Prism 9                                                                                | Graphpad, https://www.graphpad.com/scientific-software/prism/                |
| R programming language, v. 3.4.4                                                          | R Core Team, 2017, https://www.r-project.org/                                 |
| Rstudio, v. 1.1.463                                                                      | Rstudio Team, 2020, https://www.rstudio.com/                                  |
| FastQC, v. 0.11.5                                                                       | Babraham Bioinformatics, Babraham Institute, https://anaconda.org/bioconda/fastqc |
| MultiQC, v. 1.7                                                                         | Ewels et al., 2016, https://anaconda.org/bioconda/multiqc                     |
| SamTools, v. 1.9                                                                        | Li et al., 2009, https://anaconda.org/bioconda/samtools                      |
| BedTools, v. 2.26.0                                                                     | Quinlan and Hall, 2010, https://anaconda.org/bioconda/bedtools                |
| ggplot2, R-package, v. 3.2.1                                                             | Wickham, 2016, https://ggplot2.tidyverse.org/                                 |
| Integrative Genomic Viewer (IGV), v. 2.4.17                                             | Robinson et al., 2011, https://anaconda.org/bioconda/igv                     |
| BBduk, part of the BBTools suite, v. 38.58                                              | Bushnell, n.d., https://sourceforge.net/projects/bbmap                        |
| Spliced Transcripts Alignment to a Reference (STAR), v. 2.7.3a                           | Dobin et al., 2013, https://anaconda.org/bioconda/star                       |
| DESeq2, R-package, v. 1.1.8.1                                                             | Love et al., 2014, https://bioconductor.org/packages/3.6/bioc/html/DESeq2.html |
| Limma, R-package, v. 3.34.9                                                               | Ritchie et al., 2015, https://bioconductor.org/packages/3.6/bioc/html/limma.html |
| Benjamin-Hochberg FDR correction (DESeq2)                                                | Benjamin and Hochberg, 1995, http://www.math.tau.ac.il/~ybenja/MyPapers/benjamin_hochberg1995.pdf |
| REVIGO                                                                                | Supek et al., 2011, http://revigo.irb.hr/                                     |
| Bowtie2, v. 2.3.4.1                                                                      | Langmead and Salzberg, 2012, https://anaconda.org/bioconda/bowtie2           |
| MACS2, v. 2.2.6                                                                         | Zhang et al., 2008, https://anaconda.org/bioconda/macs2                       |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Claudio Cantu (claudio.cantu@liu.se).

Materials availability
All newly generated plasmids and cell lines in this study are available by contacting the lead contact.

Data and code availability
The RNA-seq and ChIP-seq datasets generated during this study have been deposited at the ArrayExpress Archive of Functional Genomic Data and are publicly available as of the date of this publication. Dataset accession numbers are listed in the key resources table (ArrayExpress: E-MTAB-10564, E-MTAB-10575). Microscopy and quantitative (q)RT-PCR data reported in this paper are shared by the lead contact upon request.

This study did not generate any novel software or algorithms. Published and freely available software and algorithms used for analysis in this study are listed in the key resources table. Original codes are shared by the lead contact upon request.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Culture of cell lines
E14TG2a (ATCC CRL-182) mESCs were obtained from Doble lab. mESCs were cultured in media containing N2B27 (GIBCO) supplemented with 2i/LIF, unless otherwise indicated, at the following final concentrations: PD0325901 (MEKi, Stem Cell), 1 μM; CHIR99021 (GSK3i, Stem Cell), 3 μM; LIF (Millipore), 1:1000.

For passaging, cells were dissociated with TrypLE express (GIBCO) for 30 s, pelleted by centrifugation (250 g), and resuspended in fresh 2i/LIF. mESCs were maintained in filtered T25 flasks (TPP) coated with 0.1% Gelatine (Sigma-Aldrich, G1890). Cells were passaged every 2 days.

Human embryonic kidney 293 cells were cultured in media consisting of DMEM, high glucose (41966-029 GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 1x penicillin-streptomycin according to the manufacturer’s recommendation.

METHOD DETAILS

Generation of knockout mESC lines
Knockout cell lines were created using pSpCas9-PX459 V2.0 plasmid. 200,000 cells were transfected using Lipofectamine® 2000 DNA Transfection Reagent protocol (Invitrogen). After 24 hours, cells were subjected to 2 μM puromycin to kill untransfected lacking puromycin resistance conferred by the vector. Puromycin selection was only maintained for 24 hours after which cells were allowed to grow in standard medium until single colonies were visible. Single colonies were isolated and allowed to expand.

Embryoid body formation
To differentiate cells to embryoid bodies (EB), cells were suspended in N2B27 medium supplemented with 2i without LIF and 10% of Fetal Bovine Serum (hereafter referred to as EB medium). Droplets of cells, each containing 800 mESCs / 30 μL EB medium were plated on the lid of 10 cm² Petri dishes. The Petri dishes were placed in incubator (37°C, 5% CO₂) for 3 days. Each droplet was then transferred to ultra-low adhesion 96-well plate, containing 200 μL of EB medium. The EB medium was replenished every two days.

Clonogenicity assays
For clonogenicity assays, cells were dissociated at 40-48h post-2i withdrawal, and following resuspension in appropriate media. Cells were plated at single cell density (500 cells / 6-well) in 2i/LIF or N2B27 on plates coated with 1% Laminin (Sigma, Cat.

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L2020) in triplicate. At day 6, alkaline phosphatase staining was performed using an AP Kit (Sigma-Aldrich). Colonies were scored manually.

**Quantitative (q)RT-PCR**
Total RNA (1 μg) isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific) was used to generate cDNA with Transcription High Fidelity cDNA Synthesis Kit (Roche) and then treated with DNA-free Kit (Thermo Fisher Scientific). SYBR Green SuperMix (Bio-Rad) was used for qPCR reactions using 3 μL of diluted cDNA (1 μg RNA equivalent in 100 μL). GAPDH was used as the reference gene. QuantStudio 3 (Thermo Fisher Scientific) instrument and software were used to determine relative gene expression levels using the delta-delta Ct method. Primer sequences were designed using Roche’s online primer design software (https://lifescience.roche.com/en_ch/brands/universal-probe-library.html) or were obtained from prior publications (Table S4). Statistical analyses (two-tailed Student’s t test) were performed in Microsoft Excel considering both independent experiments and technical repetitions. Each experiment was performed at least three times.

**Western blot analyses**
Cell lysates were mixed with LDS sample buffer supplemented with Sample Reducing Agent (Invitrogen) and heated for 5’ at 95C. 10–15 μg of protein/lane was run through 10% Bis-Tris gels (120V, 90’). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes with a Bio-Rad Mini Trans-Blot System (35V,3h). Membranes were blocked with 5% milk/PBS-T (Phosphate-buffered saline supplemented with tween 20) for 45’ in room temperature (RT) and incubated with primary antibodies diluted in 5% Bovine Serum Albumin/PBS-T overnight at 4C. Blots were washed with PBS-T (four times, 15’/wash), and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1hr, RT). After four washes with 15’ in PBS-T, blots were incubated in WesternBright Quantum (Advastan) and imaged by using a Fusion SL imaging System (Vilmer).

**Antibodies**
The following primary antibodies were used for western blots: mouse anti-α-tubulin (T6074, Sigma-Aldrich); rabbit anti-TCF7 (C63D9, Cell Signaling); rabbit anti-LEF1 (C12A5, Cell Signaling); rabbit anti-TCF7L1 (ab86175, Abcam); rabbit anti-TCF7L2 (C48H11, Cell Signaling); mouse anti-β-catenin (AF1329: R&D system); mouse anti-β-catenin carboxy (SC-7963; Santa Cruz). Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies for western blotting were purchased from Jackson.

**Electroporation**
mESCs were collected in a 15 mL tube and pelleted at 145 g for 5’. For each electroporation experiment, the cell pellet was resuspended in Opti-MEM medium to a final concentration of 10^6 cells per 100 μL. The corresponding siRNAs (Sigma-Aldrich) in 300 nM final were added to the cell mix.

For the electroporation a NEPA electroporator was used with the following set up; poring pulse: 135(V), 5(ms), 50(ms), 10(D.rate) + (polarity); transfer pulse: 20(V), 50(ms), 59(ms), 40(D.ate)+-(polarity).

After the cells were electroporated, they were transferred to a 12-well plate supplemented with 1 mL of the appropriate cell medium and monitored for at least 8 days.

**Transcriptomics analysis (RNA-seq)**
Library preparation and sequencing was performed by the Functional Genomics Center Zurich (FGCZ), Switzerland. Quality of isolated RNA was determined with a Qubit® (1.0) Fluorometer (Life Technologies, California, USA) and a Fragment Analyzer (Agilent, Santa Clara, California, USA). Only those samples with a 260 nm/280 nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were further processed. The TruSeq Stranded mRNA (Illumina, Inc, California, USA) was used in the succeeding steps. Briefly, total RNA samples (100-1000 ng) were polyA enriched and then reverse-transcribed into double-stranded cDNA. The cDNA samples were fragmented, end-repaired and adenylated before ligation of TruSeq adapters containing unique dual indices (UDI) for multiplexing. Fragments containing TruSeq adapters on both ends were selectively enriched with PCR. The quality and quantity of the enriched libraries were validated using Qubit® (1.0) Fluorometer and the Fragment Analyzer (Agilent, Santa Clara, California, USA). The product is a smear with an average fragment size of approximately 260 bp. The libraries were normalized to 10nM in Tris-C1 10 mM, pH8.5 with 0.1% Tween 20. The Novaseq 6000 (Illumina, Inc, California, USA) was used for cluster generation and sequencing according to standard protocol. Sequencing was single end at 100 bp. The RNA-seq datasets (raw and processed files) have been deposited at ArrayExpress under accession number E-MTAB-10564.

**Chromatin immunoprecipitation**
β-catenin ChIP on mESC was performed using previous procedures and antibodies as described before for HEK293T (Doumpas et al., 2019). Briefly, mESC were treated with increased CHIR up to 10 μM for 24 h to stimulated Wnt/β-catenin signaling. Ca. 10 x 10^6 cells per samples were cross-linked in 20 mL PBS for 40' with the addition of 1.5 mM ethylene glycol-bis(succinimidyl succinate) (Thermo Scientific, Waltham, MA, USA), for protein–protein cross-linking, and 1% formaldehyde for the last 20’ of incubation, to preserve DNA–protein interactions. The reaction was blocked with glycate and the cells were subsequently lysed in 1 mL HEPES buffer (0.3% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES). Chromatin was sheared using Covaris
S2 (Covaris, Woburn, MA, USA) for 8’ with the following set up: duty cycle: max, intensity: max, cycles/burst: max, mode: Power Tracking. The sonicated chromatin was diluted to 0.15% SDS and incubated overnight at 4°C with 10 μg of anti-β-catenin (SantaCruz sc-7199 or Cell Signaling) and 50 μL of protein A/G magnetic beads (Upstate). The beads were washed at 4°C with wash buffer 1 (0.1% SDS, 0.1% deoxycholate, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), wash buffer 2 (0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), wash buffer 3 (0.25 M LiCl, 0.5% sodium deoxycholate, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES) and finally twice with Tris EDTA buffer. The chromatin was eluted with 1% SDS, 0.1 M NaHCO₃, de-cross-linked by incubation at 65°C for 5 h with 200 mM NaCl, extracted with phenol–chloroform and ethanol-precipitated. The immunoprecipitated DNA was used as input material for DNA deep sequencing.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis**

Statistical analysis was performed in Prism v9. Statistical details for all experiments, including value and definition of n, error bars, and significance thresholds can be found in the Figure Legends.

**Western blot quantification**

TCF7L1 protein abundance in WT and βcatKO cell lines was quantified using the image analysis software ImageJ (version 1.52n). Integrated density was measured for the TCF7L1 band in each lane and divided by the value of the corresponding Tubulin band. Each lane was normalized to the first land (WT no CHIR) giving relative integrated density values.

**RNA-seq data analysis**

Raw data in the form of obtained fastq files were controlled for sequencing quality using the tool FastQC (0.11.5). Sequencing reads were trimmed to remove adaptor remnants using BBDuk, part of the BBTools suite (38.58). MultiQC (1.7) (Ewels et al., 2016) was used to summarize the quality results.

Mouse mm10 reference genome and corresponding annotation data were downloaded from GENCODE, release M24 (https://www.gencodegenes.org/mouse/) to which pre-processed sequenced reads were mapped using the Spliced Transcript Alignment to a Reference (STAR) software (2.7.3a) (Dobin et al., 2013) with –quantMode TranscriptomeSAM GeneCounts settings.

Downstream analysis, annotation and visualization was made with the R programming language and Rstudio. Differential gene expression analysis was performed using DESeq2 (1.18.1) (Love et al., 2014) and obtained p values were adjusted for multiple comparisons through the Benjamini-Hochberg false discovery rate (FDR) procedure (Benjamini and Hochberg, 1995). An FDR adjusted p value below 0.05 was used as cut-off when determining statistical significance. Functional enrichment analysis of differentially expressed genes (DEGs) was performed using the goana function in the limma package (3.34.9) (Ritchie et al., 2015), based on gene function information from the Gene Ontology (GO) knowledgebase. Obtained significant GO terms (p < 0.05) were further filtered by removing redundant GO terms based on semantic similarity (simRel score ≤ 0.7) using the online tool REVIGO (Supek et al., 2011).

**β-catenin ChIP-seq data analysis**

Raw sequencing data were quality checked using FastQC (0.11.5). Because all the samples showed high average phred quality scores > 30 and no detected sequencing adaptor remnants, no trimming step was deemed necessary. Reads were then mapped to the mouse reference genome (version GRCm38) by end-to-end alignment using the read alignment tool Bowtie2 (2.3.4.1) (Langmead and Salzberg, 2012). Aligned reads were sorted and indexed using samtools (1.9) (Li et al., 2009) and reads mapped to the mitochondrial chromosome were removed. Genomic enrichment of aligned reads was determined with the peak calling tool MACS2 (2.2.6) (Zhang et al., 2008), using the penta knockout sample as control. Finally, peaks within ENCODE blacklisted regions were removed using BedTools (2.26.0) (Quinlan and Hall, 2010). Overlap between called peaks in replicates was determined with Intervene (0.6.4) (Khan and Mathelier, 2017), a tool for intersection of genomic regions, and used as basis for the definition of consensus peak sets. Visualization of aligned read enrichment as genomic tracks was produced using the Integrative Genomics Viewer (IGV) (2.4.17) (Robinson et al., 2011).