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Ronin Is Essential for Embryogenesis and the Pluripotency of Mouse Embryonic Stem Cells

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SUMMARY

Pluripotency is a unique biological state that allows cells to differentiate into any tissue type. Here we describe a candidate pluripotency factor, Ronin, that possesses a THAP domain, which is associated with sequence-specific DNA binding and epigenetic silencing of gene expression. Ronin is expressed primarily during the earliest stages of murine embryonic development, and its deficiency in mice produces periimplantational lethality and defects in the inner cell mass. Conditional knockout of Ronin prevents the growth of ES cells while forced expression of Ronin allows ES cells to proliferate without differentiation under conditions that normally do not promote self-renewal. Ectopic expression also partly compensates for the effects of *Oct4* knockdown. We demonstrate that Ronin binds directly to HCF-1, a key transcriptional regulator. Our findings identify Ronin as an essential factor underlying embryogenesis and ES cell pluripotency. Its association with HCF-1 suggests an epigenetic mechanism of gene repression in pluripotent cells.

INTRODUCTION

Pluripotency, a biological state restricted to certain embryonic cells, enables development into any cell type in the body (Pedersen, 1986). Because this property can be exploited for genetic engineering and holds great promise for applications in regenerative medicine, an important goal is to understand the molecular pathways unique to pluripotent cells. Embryonic stem (ES) cells, derived from the inner cell mass (ICM) of blastocysts, are the most commonly used cell type in studies of early embryonic development and the pluripotent state (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998), largely because of their ability to self-renew in tissue culture for extended periods without differentiation.

Despite recent progress in reprogramming somatic cells to an embryonic-like state (so-called induced pluripotent stem, or iPS, cells) by manipulation of several key transcription factors (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007), the precise molecular mechanisms that underlie pluripotency remain elusive. It is proposed that a tightly balanced core set of specific transcription factors, able to promote self-renewal by repressing transcription factors that initiate differentiation programs, are the major driving forces in ES cell maintenance (Bernstein et al., 2006; Boyer et al., 2005, 2006; Lee et al., 2006). A second tier of control is likely achieved via enzyme-mediated modifications of chromatin (e.g., histone acetylation and methylation at specific residues and chromatin remodeling) that may "prime" critical differentiation genes for subsequent transcription (Boyer et al., 2006; Houlard et al., 2006; Klochendler-Yeivin et al., 2000). Whether the epigenetic status of ES cells directly reflects the actions of transcription factors known to be involved in pluripotency, or perhaps those yet to be linked to the pluripotent state, remains unclear.

Oct4, Sox2, and Nanog are considered part of the core set of pluripotency factors (Avilion et al., 2003; Chambers et al., 2003; Nichols et al., 1998). Although each of these proteins has been described as a "master regulator" of pluripotency, only Oct4 appears absolutely essential, while both Sox2 and Nanog appear dispensable, at least in certain molecular contexts (Masui et al., 2007; Chambers et al., 2007). Contributing to the complexity of ES cell regulation is the observation that ectopic expression of *Nanog*, but not *Oct4* and *Sox2*, will sustain self-renewal under unfavorable conditions but does not override the differentiation effects of forced downregulation of either *Oct4* or *Sox2* (Chambers et al., 2003; Matsui et al., 1992; Niwa et al., 2000). Moreover, the exact manner in which particular epigenetic modifiers, such as histone-modifying enzymes, influence the state of pluripotency and engage in crosstalk with other pluripotency factors is unclear.

We previously showed that certain components of the cell death system, Caspase-3 in particular, specifically cleave and deplete Nanog protein, compelling ES cells to exit their self-renewal phase and induce differentiation (Fujita et al., 2008). This discovery led us to hypothesize that Caspase-3 might recognize other pluripotency factors critical for ES cell function and to devise a yeast two-hybrid screen for Caspase-3 targets in ES cells that would fill this role. Here we describe a nuclear protein targeted by Caspase-3 that is expressed during the earliest stages of embryonic development, is essential for the maintenance of pluripotent stem cells both in vitro and in vivo, allows ES cells to self-renew under conditions that normally suppress self-renewal, and partly compensates for *Oct4* knockdown in ES cells. Designated Ronin (a masterless Japanese samurai) because of its lack of any apparent relationship to known "master" regulators of pluripotency, this factor contains a zinc-finger DNA-binding motif (THAP domain) common to many proteins associated with chromatin modification and silencing of gene expression (Roussigne et al., 2003a, 2003b; Macfarlan et al., 2005). Ronin binds directly to the host cell factor 1 (HCF-1) protein, a key regulator of transcriptional control that is associated with protein complexes involved in histone modification, suggesting that it acts through a previously unrecognized pathway of pluripotency control.

RESULTS

Identification of Ronin by Yeast Two-Hybrid Screening

Previous studies by our group showed that Nanog is targeted and cleaved by the proapoptotic enzyme Caspase-3 upon induction of ES cell differentiation (Fujita et al., 2008), leading us to hypothesize that other, still unknown factors critical for ES cell pluripotency may be Caspase-3 targets as well. We therefore performed yeast two-hybrid screening of a human ES cell cDNA expression library, using constitutively active Caspase-3 (mCasp3rev) as bait. mCasp3rev spontaneously folds into its active conformation and recognizes and binds to target proteins but no longer cleaves them owing to a C163S substitution. An estimated 32 million clones were screened, with 556 clones testing positive for interaction with the Caspase-3 mutant. Further study of a representative set of 286 clones, using rescued plasmids, digestion with restriction enzymes, and a validation assay, yielded 116 candidate genes. Subsequent sequencing and analysis with an in vitro transcription/translation Caspase-3 cleavage assay identified a cDNA whose protein product contained elements of a DNA-binding factor with striking similarities to the DNA-binding domain of the *Drosophila* P element transposase. This protein, termed Ronin for reasons given in the Introduction, proved to be an authentic target of Caspase-3 in further analyses (Figure S1A available online).

Characterization of the orthologous 305 residue Ronin protein encoded by the mouse cDNA (predicted length, 1809 bases) revealed a THAP domain at the N terminus (Figure 1A), which comprises a zinc-finger DNA-binding motif defined, in part, by a C_2CH signature (Cys-Xaa_{2-4}-Cys-Xaa_{33-50}-Cys-X-aa_2). There are also two polyalanine motifs, a polyglutamine tract (22 Qs), and a predicted coiled-coil structural domain at the C terminus. A nuclear translocation signal (NLS) is located toward the C terminus. A search for Ronin orthologs across multiple animal species showed exceptional conservation of the N and C termini, even among more distant species (e.g., humans versus zebrafish, Figures S1B and S1C). The most closely related nonvertebrate protein with a similar THAP domain is a transposase in the sea urchin (43% identity, seq XP_790851.2), which is related to several *Drosophila* transposases, including the P element transposase and the THAP domain-containing protein THAP9 in humans and other primates.

To determine the DNA sequence recognized by Ronin, we used the SELEX procedure (Bouvet, 2000) with a mouse Ronin-His/V5 recombinant protein (see Experimental Procedures for details) to select random oligonucleotides for sequencing. We identified a consensus sequence (Figure 1B, top panel) as well as a specific sequence (*3x*) that was represented three times in the sequenced pool. Gel-mobility shift experiments confirmed that the *3x* sequence is readily bound by Ronin (Figure 1B, bottom panel). Other related oligonucleotides were not able to abolish the gel shift, indicating that Ronin does not bind nonspecifically to either DNA or DNA ends (see Figure S1D). These data show that Ronin, like other proteins with a THAP domain, is a nuclear protein, as confirmed by immunofluorescence (see below), and binds to DNA in a sequence-specific manner. The coiled-coil motif at the C terminus may represent a second functional domain, as indicated by its capacity to bind directly to the HCF-1 protein (see below).

Ronin Expression Patterns

Northern blot analysis of multiple tissues in the mouse failed to detect appreciable expression of the *Ronin* gene, except in the mouse ES cell line used as a positive control (Figure 1C). To further clarify the expression patterns of Ronin, we generated a *lacZ* transgenic reporter mouse line in which a 3.3 kb genomic fragment representing the mouse *Ronin* promoter was ligated into the open reading frame of the *β-galactosidase* gene. The resultant mouse line expressed *β-galactosidase* in tissues where the *Ronin* promoter was active, in a pattern similar to the expression of wild-type (WT) *Ronin*. As in the Northern blot analysis, *Ronin* was not abundantly expressed in adult tissues, with two exceptions: (1) ovary, which showed very strong positive staining in oocytes (Figure 1D, left) and (2) some areas of the brain, including hippocampus, olfactory bulb, and Purkinje cells (Figure 1D, right). To establish the subcellular compartment in which Ronin is found, we raised an antibody against Ronin (Figure S2A). Immunostaining with this antibody in adult ovaries showed localization of Ronin mainly in the ooplasm without any evidence of its presence in the nucleus (Figure 1E, left). This pattern of staining contrasted with the detection of Ronin throughout the zygote (Figure 1E, right).

Using the *lacZ* animal model to assess *Ronin* expression during early embryonic development, we found that *lacZ* activity first appears at the 2-cell stage, intensifies during the 8-cell and compact morula stages, but subsides in the blastocyst (Figure 1F). However, immunofluorescence staining revealed that Ronin protein was still present at the blastocyst stage (Figure 1G), indicating that the lacZ reporter system is not as sensitive as antibody staining and therefore could underestimate Ronin transcription by comparison with the results of RT-PCR or microarray

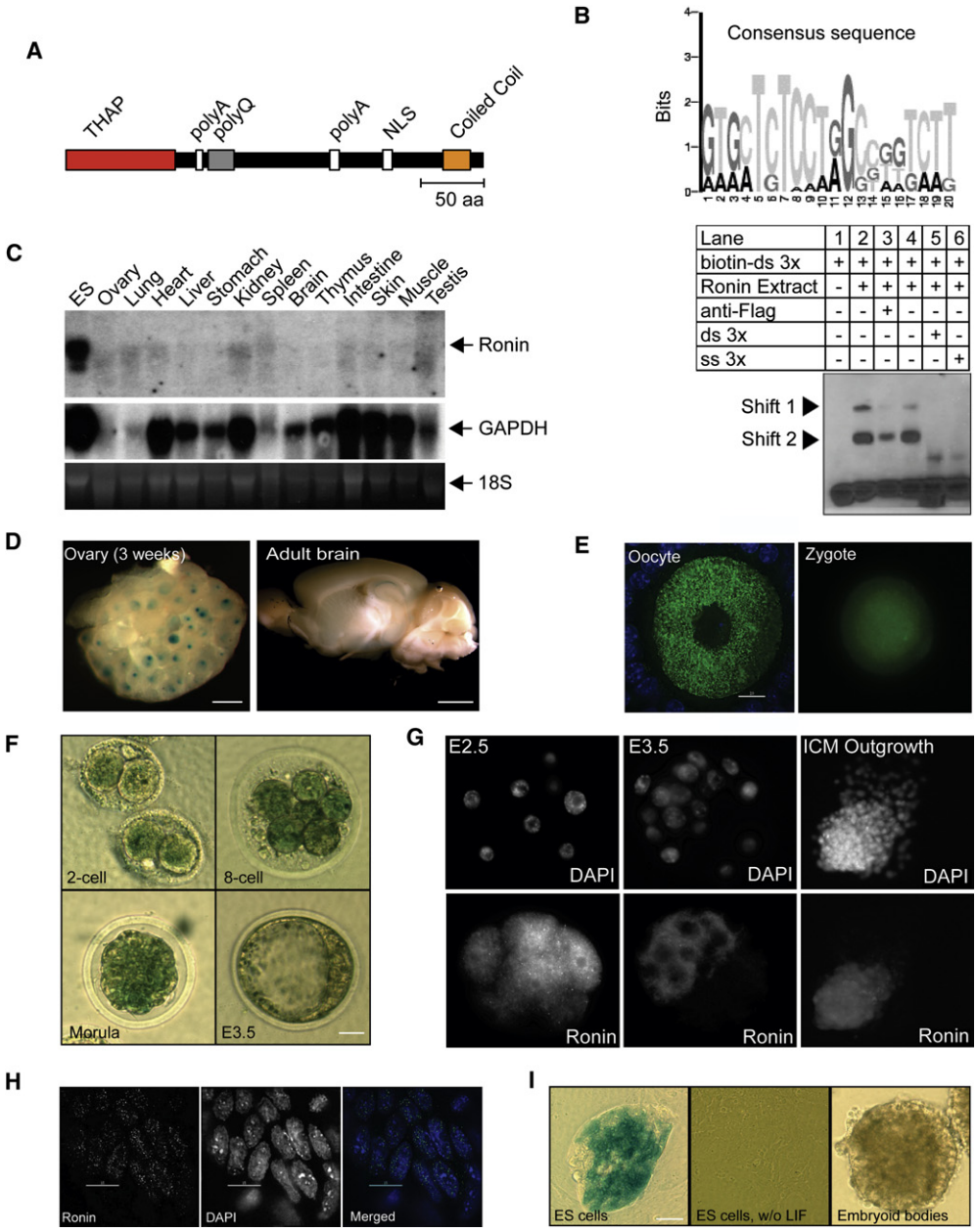

Figure 1. Ronin Is a Nuclear THAP Domain Protein Whose Expression Is Restricted to Early Embryonic Cells and Undifferentiated ES Cells

(A) Schematic diagram of the Ronin protein showing a THAP domain at the N terminus. NLS, nuclear translocation signal; polyQ, polyglutamine tract; polyA, polyalanine sequence; THAP, Thanatos-associated protein; aa, amino acid.

(B) SELEX identification of the consensus sequence recognized by Ronin DNA-binding motif (top) and gel-shift experiments with a specific DNA sequence (bottom). A motif search identified a specific consensus (top) and a "*3x* sequence." Binding of Ronin-Flag to the biotinylated *3x* sequence was verified by gel-shift experiments that could be inhibited by the Flag antibody and either double-stranded or single-stranded unlabeled competitor *3x* molecules. Triangles indicate gel-shift complexes.

(C) Northern Blot analysis of multiple mouse tissues.

(D) X-Gal staining of tissues isolated from *mpRonin-lacZ* reporter mice.

(E) Immunostaining of oocytes and zygotes isolated from adult females with a Ronin antiserum. (bar = 10 μm).

(F) *Ronin* promoter-driven *lacZ* expression during embryo development.

(G) Immunostaining of Ronin in morula and blastocyst-stage embryos and in in vitro inner cell mass (ICM) outgrowth.

(H) Confocal images of mouse ES cells stained with Ronin antibody.

(I) *lacZ* expression in undifferentiated ES cells, in the absence of MEFs and LIF or after EB formation for 3 days (scale bars: D left 30 μm, D right 80 μm, E left 30 μm, E right 20 μm).

analysis. Although present in both the cytoplasm and the nucleus of blastomeres in morula-stage embryos, Ronin appeared mainly in the cytoplasm of cells within the blastocysts, suggesting that its function may be regulated by shuttling of the protein between the cytoplasm and nucleus, similar to its fate at the oocyte/zygote transition. Once the blastocysts were placed in culture, the Ronin protein was again mostly localized in the nucleus with only scant amounts detected in the cytoplasm (Figure 1G).

Immunostaining for Ronin protein was strongly positive in the nucleus of undifferentiated mouse and human ES cells and was distributed in an uneven pattern that primarily exluded DAPI-positive areas, suggesting that Ronin is an abundant nuclear protein associated with open chromatin (Figures 1H and S2B). These results led us to study reporter gene activity in ES cells isolated from transgenic blastocysts to determine the temporal pattern of *Ronin* expression upon induction of differentiation. *lacZ* activity was detected in undifferentiated ES cells grown in the presence of a mouse embryonic fibroblast (MEF) feeder layer (Figure 1I, left). When these cultures were transferred to gelatin-coated dishes and maintained in the absence of leukemia inhibitory factor (LIF) and MEFs or incubated as hanging drops to form embryoid bodies (EB), *lacZ* activity was virtually undetectable in the differentiated cells (Figure 1I, middle and right). Overall, these findings indicate that *Ronin* expression is mainly restricted to pluripotent cells of the developing embryo, to oocytes, and to certain regions of the adult brain.

Ronin Knockout Leads to Periimplantational Lethality

To test whether Ronin plays a critical role in early embryonic development, we knocked out a single allele of the gene in mouse ES cells, generating $Ronin^{+/-}$ mice (see Experimental Procedures). When $Ronin^{+/-}$ male and female littermates were crossed, none of the 98 offspring at weaning age were $Ronin^{-/-}$, demontrating that a Ronin null genotype is embryonically lethal. An estimated two-thirds of the offspring (67 animals) were $Ronin^{+/-}$ and one-third (31 animals) were $Ronin^{+/+}$, supporting a lethal phenotype. To determine the embryonic stage of lethality, we crossed $Ronin^{+/-}$ animals and dissected and genotyped embryos at E7.5. Of the 26 decidua examined, 7 (27%) were empty, 8 (31%) contained embryos that were $Ronin^{+/+}$, and 11 (42%) contained embryos that were $Ronin^{+/-}$. Empty swollen decidua were similar in size to those containing embryos, an indication that implantation and decidualization had proceeded normally (Figure 2A, left). Crossing $Ronin^{+/-}$ females with $Ronin^{+/+}$ males did not yield empty decidua. Approximately half of the resultant embryos were $Ronin^{+/-}$, while the other half were $Ronin^{+/+}$, as expected. We propose that the $Ronin^{-/-}$ embryos die either during or shortly after implantation, an outcome that was confirmed by the presence of residual embryonic tissue in empty decidua from uteri examined after crosses with $Ronin^{+/-}$ mice (Figure 2A, right).

Next, superovulated immature $Ronin^{+/-}$ females were crossed with $Ronin^{+/-}$ males, and 45 blastocyst-stage embryos were isolated. Genotyping of these blastocysts identified nine (20%) as $Ronin^{-/-}$. Crossing $Ronin^{+/-}$ females with WT males produced the expected ratio of $Ronin^{+/-}$ and $Ronin^{+/+}$ blastocysts. Upon gross examination, the $Ronin^{-/-}$ blastocysts were indistinguishable from $Ronin^{+/+}$ and $Ronin^{+/-}$ blastocysts (Figure 2B, left). The vast majority (90%) of the ICMs of embryos resulting from additional $Ronin^{+/-}$ and $Ronin^{+/+}$ crosses showed outgrowth when cultured on gelatin-coated culture plates, in contrast to those from $Ronin^{-/-}$ embryos, which either failed to proliferate or, in one instance, produced only a residual mass (Figure 2B, right). We propose that Ronin is essential for maintenance and proliferation of the ICM.

Ronin Knockout ES Cells Are Not Viable

The severe defects in ICM outgrowth in $Ronin^{-/-}$ embryos implicated Ronin activity as a critical factor in both the derivation and propagation of ES cells. Hence, we sought to derive ES cells from crosses of $Ronin^{+/-}$ mice. Although $Ronin^{+/-}$ ES cell lines could be readily generated, it was not possible to obtain $Ronin^{-/-}$ lines despite repeated attempts, indicating that Ronin activity is essential for generating ES cell lines in vitro. Even so, a knockout phenotype characterized by defects in the ICM would not necessarily militate against the growth and viability of cultured ES cells with conditionally deleted alleles. Thus, we generated $Ronin^{flox/flox}$ ES cells, transfected them with a *Cre* expression vector, and sorted for Cre recombinase-positive cells (see Experimental Procedures). Among 110 genotyped subclones, most (90%) were $Ronin^{flox/-}$, with none lacking both alleles. Further testing of the Cre-transfected ES cells revealed a high rate of a phenotype resembling apoptotic death (Figure 2C, left), suggesting that *Ronin* knockout was lethal to ES cells under standard culture conditions. In contrast, when $Ronin^{flox/flox}$ MEFs derived from E14.5-old embryos were isolated and treated with *Cre* adenovirus, nearly 100% of the cells were transduced, resulting in complete knockout of *Ronin* (Figure 2C, right). Finally, the *Ronin loxP* allele was crossed into the *Mx1-Cre* background to generate $Ronin^{flox/flox}$—*Mx1-Cre* ES cell lines (Whyatt et al., 1993), but the induction of *Mx1-Cre* did not lead to deletion of the *Ronin* allele in any experiment (data not shown). Interestingly, knockdown of Ronin using siRNA did not result in any overt phenotype in the colony formation assay (Figures 2D and 2E); however, colony formation and cell proliferation assays revealed a small but reproducible decrease in the colony number formed (Figure 2F) and a significant decrease in the proliferation rate (Figure 2G), in agreement with our knockout data. The most likely interpretation is that the 50%–80% knockdown efficiency that we achieved (Figure 2H) is not sufficient to fully unmask the phenotype. Together, these findings demonstrate a stringent requirement for Ronin in maintenance of the self-renewal property of ES cells, as well as in the generation of the ICM during early embryogenesis.

Forced Expression of *Ronin* Inhibits Differentiation of ES cells

Because Ronin possesses several of the critical features of a pluripotency factor, we asked if its ectopic expression in ES cells would render them independent of LIF for self-renewal. In these experiments, we established stable ES cell lines expressing *loxP*-flanked *Ronin* under the influence of a constitutive promoter, *EF1α*. Western blot analyses were used to select several clones that expressed *Ronin* in ES cells and had normal

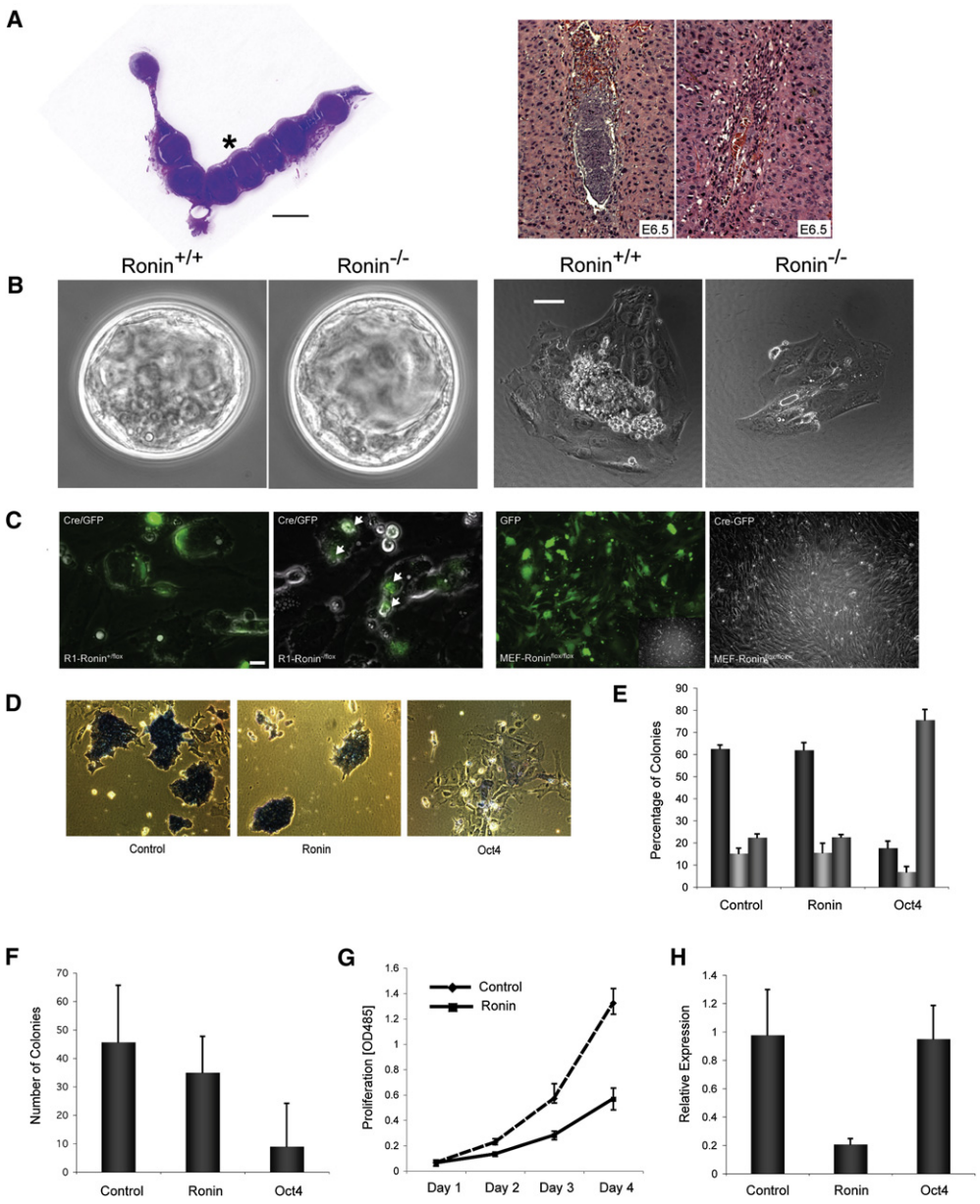

Figure 2. Ronin Is Essential for Normal Embryogenesis and for ES Cell Survival

(A) H&E staining of mouse uterine sections following *Ronin*$^{+/-}$ crosses. Empty swollen decidua (asterisk, bar = 1 cm, left), WT embryos (middle), and residual resorbed embryonic tissue (right) at day E6.5 are shown.

(B) Phase contrast images of blastocysts and ICM outgrowth. *Ronin*$^{-/-}$ and *Ronin*$^{+/-}$ blastocysts (left) and ICM (right) (bar = 30 μm).

(C, left) Immunofluorescent images of *Cre-GFP* transfected ES cells (arrow indicates typical morphology of apoptotic cells [bar = 20 μm]); (C, right) Immunofluorescent images of MEFs isolated from a *Ronin*$^{flox/flox}$ animal after transfection with *GFP* adenovirus; insert shows phase contrast image of transduced MEFs.

(D–H) siRNA-mediated knockdown of *Ronin* in ES cells. R1 cells were transfected with siRNA and the differentiation level assessed 4 days later (AP staining). (D) Phase contrast images of ES cells transfected with control siRNA, *Ronin* siRNA, and *Oct4* siRNA. (E) Quantification of experiment shown in (D). (F) Quantification of colony number (independent of differentiation level). (G) Proliferation rate of ES cells after treatment with siRNA. (H) Quantification of *Ronin* expression by real-time PCR of ES cells 18 hr after treatment with siRNA. All values are means ± SD from triplicate experiments.

morphology. To test the effects of *Ronin* overexpression on ES cell self-renewal, we plated control ES cells and those ectopically expressing *Ronin* (maintained without LIF or with a LIF-blocking antibody) at clonal densities and analyzed colony formation 4 days later. The vast majority of colonies overexpressing *Ronin* appeared morphologically unaffected by LIF removal or LIF inhibition, in contrast to ES cell controls, which were fully differentiated (Figure 3A). To quantify this result, we performed

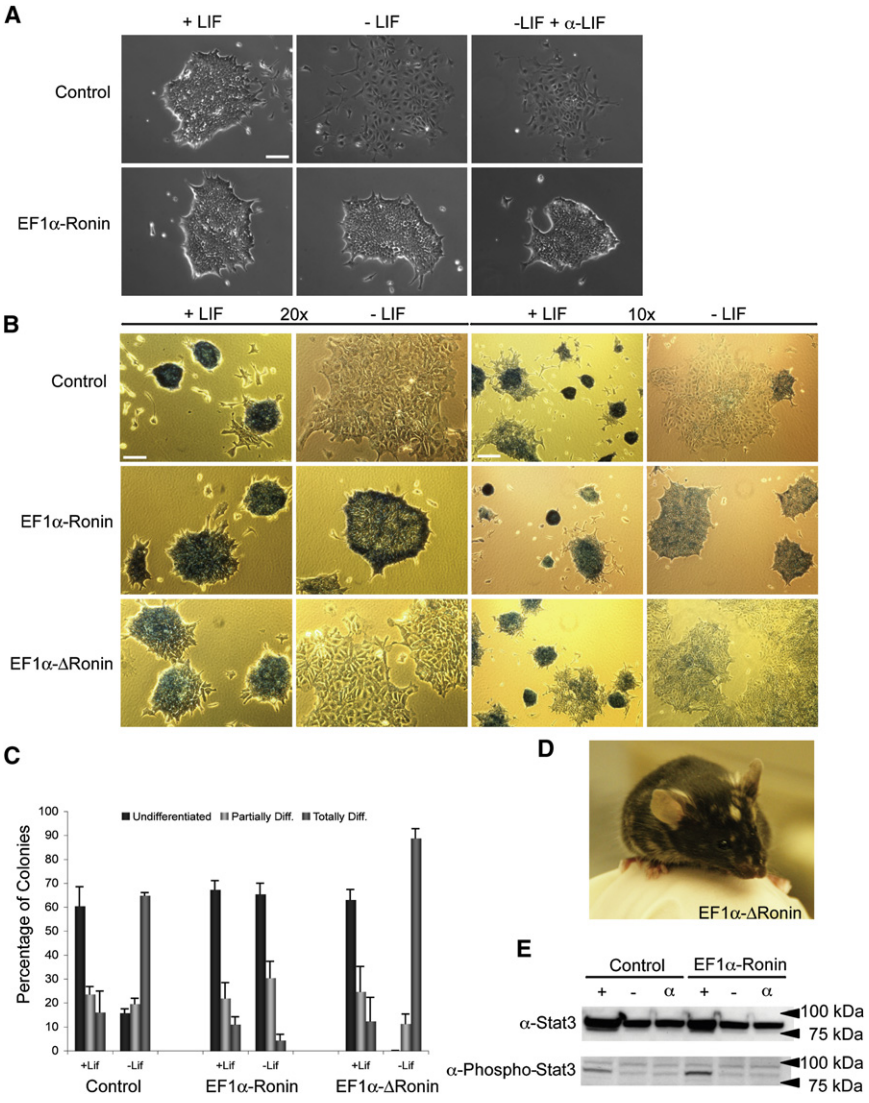

Figure 3. Forced Expression of Ronin Inhibits Differentiation of ES Cells

(A) Phase contrast images of control ES cells and *EF1α-Ronin* ES cells, stably overexpressing *Ronin*, after differentiation in monolayer cultures in the absence or presence of LIF for 4 days. Cells were split at clonal density under the same conditions. Cells that remained pluripotent after 8 days of selection were expanded in the presence of LIF on MEFs and, in a second step, *Ronin* expression was silenced by Cre-mediated recombination to obtain *EF1α-ΔRonin* ES cells (bar = 20 μm).

(B) Alkaline phosphatase staining of cells treated as in (A) at two different magnifications (bar 10× = 80 μm, bar 20× = 40 μm).

(C) Quantification of 3 × 50 colonies from the experiment described in (B). The values are means ± SD from triplicate experiments.

(D) Chimeric mice generated by injection of *EF1α-ΔRonin* ES cells into blastocysts.

(E) Western blot analysis of Stat3 and Phospho-Stat3.

alkaline phosphatase staining 4 days after clonal plating and determined the percentages of undifferentiated, partially differentiated, and fully differentiated ES cell colonies. As expected, in the absence of LIF, most of the control ES cell colonies were either partially (24%) or entirely (60%) differentiated, whereas two-thirds (65%) of the *EF1α-Ronin* ES cell colonies remained undifferentiated under the same conditions (Figures 3B and 3C). Furthermore, there was essentially no background differentiation in *EF1α-Ronin* ES cell cultures. This remarkable example of LIF-independent maintenance of pluripotency was further evaluated at the functional level by culturing ES cells without LIF for 8 days and subsequently removing the *Ronin* transgene with Cre recombinase. All control ES cells differentiated relatively quickly, to the extent that no cells with typical ES cell morphology remained in the culture when they were split after 4 days. In sharp contrast, the *Ronin*-expressing ES cells formed abundant colonies and could be split after 4 days of culture without LIF. After a total of 8 days in the absence of LIF, clones were expanded and the ectopic *Ronin* allele was removed by *Cre* transfection of expanded clones (*EF1α-ΔRonin*). These cells displayed properties indistinguishable from those of WT ES cells, including monolayer differentiation in medium without LIF (Figures 3B and 3C) and the ability to generate chimeric animals upon injection into blastocysts, similar to control cells (Figure 3D). These results indicate that the absolute differentiation block was not due to a secondary mutation in *EF1α-Ronin* ES cells. They also suggest that the pluripotency sustained by ectopic expression of *Ronin* is reversible.

We also asked whether Ronin can stimulate phosphorylation of Stat3 in the absence of LIF. Western blot analysis (Figure 3E) revealed that in ES cells ectopically expressing *Ronin*, Stat3 phosphorylation was not sustained after omission of LIF, indicating that the observed effects of Ronin are independent of LIF and Stat3 signaling.

To assess the effects of constitutive expression of *Ronin* on (1) known pluripotency factors, (2) marker genes for all three germ layers, and (3) extraembryonic tissues, we isolated RNA from ES cells on days 1 through 5, after they were plated at low densities in medium without LIF. RT-PCR analysis revealed two provocative but conflicting results (Figure 4A): (1) virtually all differentiation markers were inhibited upon withdrawal of LIF, indicating that forced expression of *Ronin* inhibits differentiation, similar to findings with the teratocarcinoma formation assays (see below), whereas (2) the amount of RNA for some housekeeping genes, such as *β-actin*, but not for others, such as *Gapdh*, was significantly reduced in repeated experiments (Figures 4A and S4). In agreement with our observation that these two genes respond differently to Ronin, we identified the

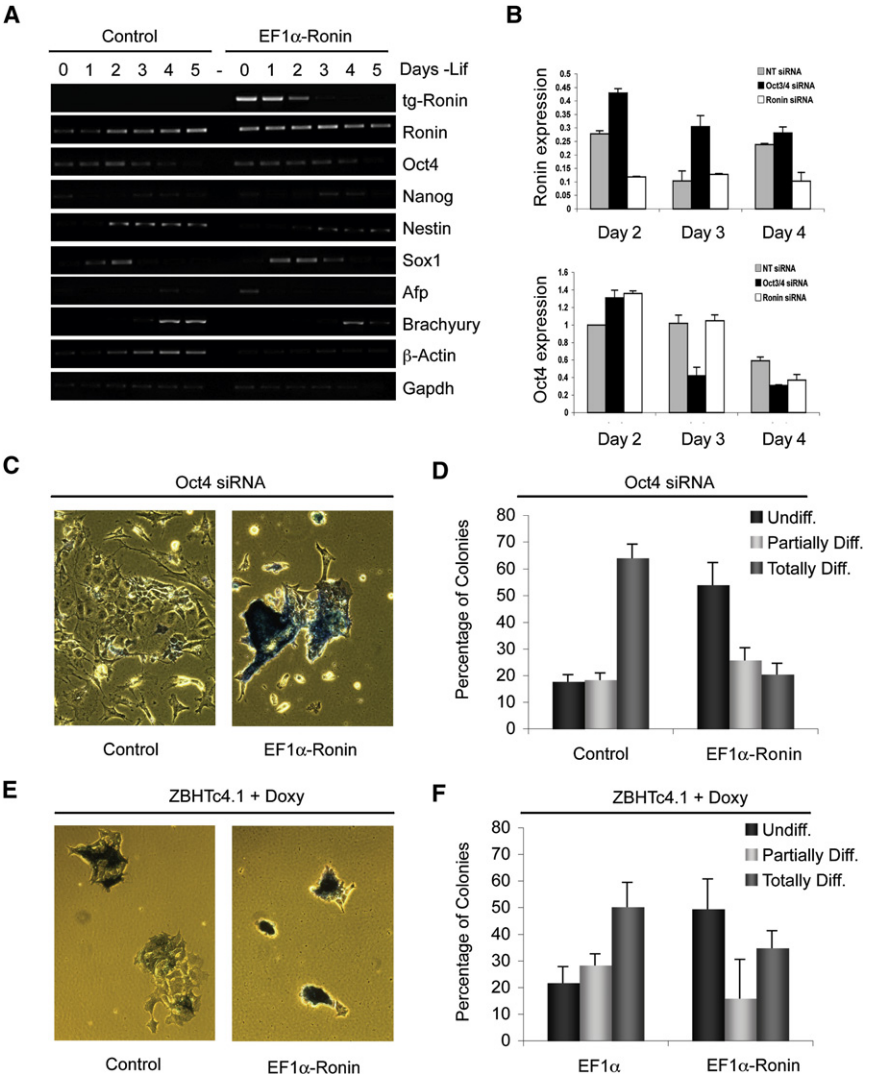

Figure 4. Ronin Inhibits Differentiation Independently of Canonical Pluripotency Factors

(A) Semiquantitative RT-PCR analysis of pluripotency factors and marker genes for all three germ layers.

(B) siRNA against *Oct4* and *Ronin* was transfected into ES cells and the expression of their mRNA determined at the indicated time points by real-time quantitative PCR. Data are reported as means and SD of triplicate experiments.

(C) Alkaline phosphatase staining of ES cells after siRNA knockdown of Oct4.

(D) Quantification of result in (C). Bars represent means and SD of results from triplicate experiments.

(E) Response of ZBHTC4.1 [EF1α] ES cells and ZBHTC4.1 [EF1α-Ronin] ES cells to doxycyclin.

(F) Quantification of (E). All values are means ± SD from triplicate experiments.

Ronin DNA-binding sequence in the *β-actin* gene but not in the *Gapdh* gene. Furthermore, we noticed that *Ronin* mRNA did not decrease as much as Oct4 (Figure 4A). This finding suggests that the repressive function of Ronin is not limited to specific developmental genes but extends widely over the transcriptome, a prediction we test in Figure 6.

To determine if knockdown of *Oct4* affects the expression of Ronin, we performed siRNA experiments in which *Oct4* was rapidly downregulated by day 3 while Ronin was upregulated by comparison with the control. When siRNAs against both genes were tested, Ronin was downregulated one day earlier than Oct4 (Figure 4B). These results suggest that Ronin may act independently of Oct4 and Nanog to maintain pluripotency, an interpretation supported by the findings of Ivanova et al. (Figure S5; Ivanova et al., 2006). We wish to point out that *Ronin* expression was not significantly affected and even appeared to be slightly upregulated by knockdown of *Oct4/Sox2/Nanog*, indicating that *Ronin* may not be regulated at the RNA level as stringently as other pluripotency factors. Functional proof that *Ronin*-expressing cells can self-renew independently of *Oct4* expression came from experiments in which we transfected control and *Ronin*-expressing cells with siRNA against *Oct4*. In contrast to controls, the reduction of *Oct4* expression had no effect on cell morphology or differentiation (Figures 4C and 4D). We also stably overexpressed *Ronin* in the ZBHTc4.1 ES cell line, in which *Oct4* expression can be downregulated with use of doxycycline. As in the preceding *Oct4* knockdown experiments, we found that ES cells from this line continue to self-renew and are capable of forming colonies after downregulation of *Oct4* (Figures 4E, 4F, and S6). Hence, to maintain pluripotency, Ronin does not require the LIF/Stat3 pathway and may not depend on direct interaction with the Oct4/Sox2/Nanog axis.

Ectopic Expression of *Ronin* Is Tumorigenic

If Ronin truly acts as an antidifferentiation factor in ES cells, its overexpression should be associated with strong tumorigenicity. Thus, to assess teratocarcinoma formation, we injected control ES cells and *EF1α-Ronin* ES cell lines into the hindleg quadriceps muscle of SCID immunocompromised mice. Animals injected with control ES cells displayed teratocarcinomas of the expected size by 17 days, while those injected with *EF1α-Ronin* had substantially larger tumors (2.8 cm versus 1.8) in two independent experiments, suggesting that increased Ronin activity triggers expansion of the stem cell pool, leading to more robust teratocarcinoma formation prior to differentiation (Figure 5A). Histologic examination of the teratocarcinomas derived from both control ES and *Ronin*-expressing *EF1α-Ronin* ES cells revealed differentiation into all three germ layers in both contexts (Figure 5B). However, we noticed a substantial number of

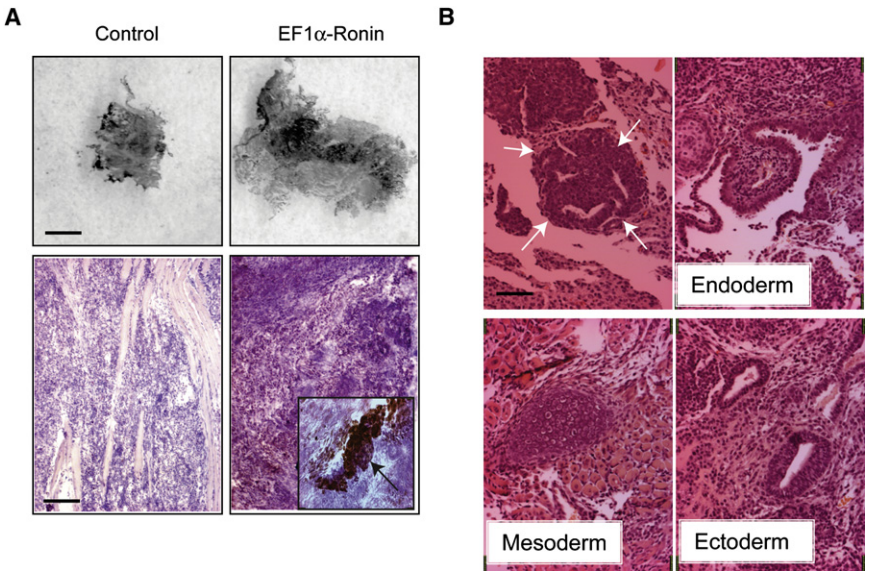

Figure 5. Ectopic Expression of Ronin Is Tumorigenic

(A) Immunohistochemical staining of Oct4 in teratocarcinoma sections. Tumors formed by *EF1α-Ronin* ES cells (top) and those formed by R1 control cells (bottom); insert: 40× magnification of typical Oct4-positive cell cluster in *EF1α-Ronin*-derived tumor; bar (top) = 0.5 cm, bar (bottom) = 0.1 mm.

(B) H&E staining of *EF1α-Ronin* teratocarcinoma sections; white arrows indicate clusters of undifferentiated cells, bar = 50 μm.

undifferentiated cell clusters in the *EF1α-Ronin* tumors that resembled embryonic carcinoma cells (Figure 5B, top left). This impression was supported by immunostaining results indicating Oct4-positive cell clusters among the *EF1α-Ronin* ES cells but not the control ES cell line (Figure 5A, bottom panels). Thus, the enhanced tumorigenicity of ES cells constitutively expressing *Ronin* appears to stem from the antidifferentiation effects of this factor, supporting its candidacy as a key regulator of the pluripotent state.

Ronin Is a Transcriptional Repressor That Acts through a Multimeric Protein Protein Complex Containing HCF-1

How does Ronin maintain the pluripotency of ES cells? The most plausible mechanism, based on Ronin's antidifferentiation effects and the epigenetic silencing activity of other THAP domain proteins (Roussigne et al., 2003a, 2003b; Macfarlan et al., 2005), is transcriptional repression of multiple genes that are either directly or indirectly involved in differentiation. To test this hypothesis, we performed gene expression profiling of control ES cells versus ES cells transiently transfected with a *Ronin*-overexpressing construct. This comparison (Figure 6A) showed a striking repression of the transcriptome of Ronin-transfected cells, reflecting either a large decrease in RNA stability or in the synthesis rate of new RNA. To distinguish between these possibilities, we generated a *Ronin*-inducible cell line by inserting *Ronin*-encoding cDNA, under the control of a tetracycline-inducible promoter, upstream of the *Hprt* locus in *A172loxP* ES cells and compared the kinetics of RNA transcription in control versus *Ronin*-expressing cells stained with a bromodeoxyuridine antibody against 5-fluorouracil (5-FU). There was a clear and rapid loss of newly synthesized RNA in cells that overexpressed *Ronin* (Figure 6B), indicating broad transcriptional repression. This outcome was confirmed by the results of a ^{3}H-uridine pulse-chase incorporation assay (Figure 6C). Finally, western blot analysis to detect histone H3 dimethylation at lysine 9 (H3K9me2), a reliable marker of chromatin-mediated gene repression, showed a large and rapid increase in the methylation of this protein over time in our *Ronin*-inducible cell line (Figure 6D). We further tested the ability of Ronin to bind to its target sequence in undifferentiated ES cells. Evidence for direct repression of genes involved in differentiation is provided in Figure 6E, which shows that Ronin binds to the *3x* sequence present in the promoter regions of *GATA4* and *GATA6*. Both of these genes show H3K9 methylation in the same region, and neither gene is transcribed in ES cells. After induction of differentiation, *GATA4* and *GATA6* are no longer bound by Ronin, and their H3K9 methylation is diminished. Interestingly, we also identified a putative DNA-binding sequence for Ronin in the promoter region of *Oct4*, and indeed Ronin did bind to this region, but only in differentiated ES cells; *Nanog* did not possess a similar binding sequence and was not bound by Ronin (Figure 6E). These observations strengthen the argument that Ronin suppresses gene expression in ES cells by directly binding to key genetic loci and recruiting epigenetic modifiers.

To pursue the idea that Ronin exerts its antidifferentiation effects through epigenetic silencing of gene expression, we devised an immunoprecipitation strategy to identify protein complexes associated with FLAG-tagged Ronin in ES cells. Putative interaction partners were separated by SDS gel electrophoresis and the protein bands subjected to protein tandem mass spectrometry analysis. Of 80 candidate proteins, 32 were selected for further evaluation by a directional yeast two-hybrid system. In this approach, full-length Ronin as well as two truncated forms carrying the N terminus (Ronin-N) or the C terminus (Ronin-C) were tested for their ability to bind directly to selected putative interaction partners (Figure 7A). The only direct interaction that was identified was between Ronin-C and HCF-1. Retrospective analysis of the Ronin sequence (not shown) revealed a previously described HCF-1 interaction motif (Freiman and Herr, 1997) at the C terminus of the molecule, making this protein a likely direct target of Ronin. To confirm that HCF-1 is indeed a functional target for Ronin, we performed HCF-1 knockdown experiments in ES cells ectopically expressing Ronin. Knockdown of this gene in both WT and Ronin-overexpressing ES cells generated the same phenotype, suggesting that Ronin expression cannot compensate for loss of HCF-1 and hence that Ronin and HCF-1 are functionally related with respect to self-renewal (Figure 7B).

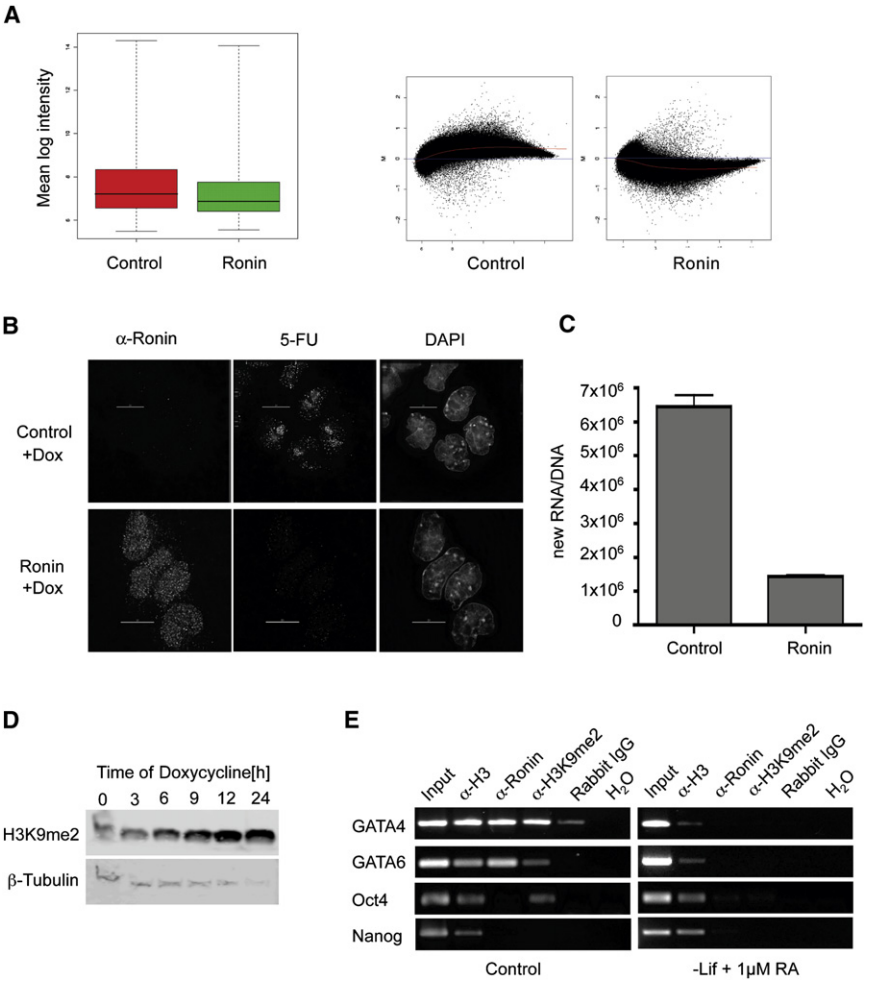

Figure 6. Ronin Acts as a Transcriptional Repressor and Epigenetic Modulator

(A, left) Box plots showing results of microarray analysis of control ES cells and ES cells 24 hr after transfection with p*EF1α-hRonin-Flag*. Each box represents median and 75th and 25th percentile values.

(B) Confocal images of 5-fluorouridine (5-FU) staining of newly transcribed RNA after induction of *Ronin* expression in a *Ronin*-inducible cell line (bar = 10 μm).

(C) Quantification of ^{3}H-uridine incorporation into newly transcribed RNA of control *A172loxP* cells and *A172LP-Ronin-Flag* cells after induction with 1 μg/ml doxycycline. Bars represent means and SD of triplicate experiments.

(D) Western blot analysis of H3K9me2 methylation.

(E) Chromatin immunoprecipitation and PCR of genomic regions upstream of *Gata4, Gata6*, *Oct4*, and *Nanog*.

Wysocka and coworkers (Wysocka et al., 2003) isolated a multimeric HCF-1-containing protein complex from HeLa cells by taking advantage of the glycoprotein properties of HCF-1. Hence, we applied a similar strategy to purify a Ronin protein complex from *EF1α-Ronin* ES cells. Comparison with the elution peaks of protein standards of known sizes, detected by Coomassie blue staining, suggested that Ronin functions within a very large (>2 MDa) protein complex (Figure 7C). To identify some of the components of this complex, we selected the same set of proteins used for the yeast two-hybrid evaluation and modified them for the use in cotransformation assays. Thus, 293 cells were cotransfected with GST-tagged variants of Ronin, Ronin-C or Ronin-N, and with Myc-tagged variants of the putative interaction partners. Using this strategy, we confirmed the binding of Ronin to HCF-1 via the C terminus. Other confirmed protein interaction partners (Figure 7D) were Ronin itself (via homodimerization through the C terminus); THAP7, another THAP domain protein (via heterodimerization through the N terminus); and Sin3A (C terminus) and HDAC3 (N terminus)—all factors associated with transcriptional repression or histone modifications. We therefore suggest that Ronin acts through a large HCF-1-containing protein complex that can modulate or repress gene expression over broad regions of the transcriptome.

DISCUSSION

Our findings promote the candidacy of Ronin as a pluripotency factor with functions that appear to differ from those of canonical pluripotency factors. Like *Oct4* and *Sox2*, but not *Nanog*, *Ronin* was highly expressed in the ooplasm of mature oocytes (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990; Avilion et al., 2003; Chambers et al., 2003), which may indicate its involvement in oocyte maturation. After fertilization, Ronin was found throughout the zygote, suggesting that it is essential for establishing the zygotic stage of the embryo. Indeed, the *Ronin* gene begins to be expressed at a time in embryonic development when zygotic gene transcription is generally initiated. *Ronin*'s expression pattern indicates differential requirements during early embryogenesis and in ES cells, a notion supported by the apparent ability of the Ronin protein to shuttle between the nucleus and the cytoplasm. Whether the nuclear and cytoplasmic proteins have different functions remains an open question; nonetheless, our data strongly suggest that Ronin is tightly regulated at the protein level.

Ronin was also expressed in specific areas of the adult brain, suggesting that it fills specific roles in these regions that may be related to specialized requirements for epigenetic and transcriptional regulation, as in ES cells. Otherwise, the *Ronin* expression level in adult animals was very low or nonexistent, although we cannot rule out the possibility that *Ronin* may be highly expressed in rare populations of stem or progenitor cells with exceptional needs for plasticity, analogous to expression of the newly recognized *Zfx* gene in both ES and hematopoietic stem cells (Galan-Caridad et al., 2007). Finally, *Ronin* transcription

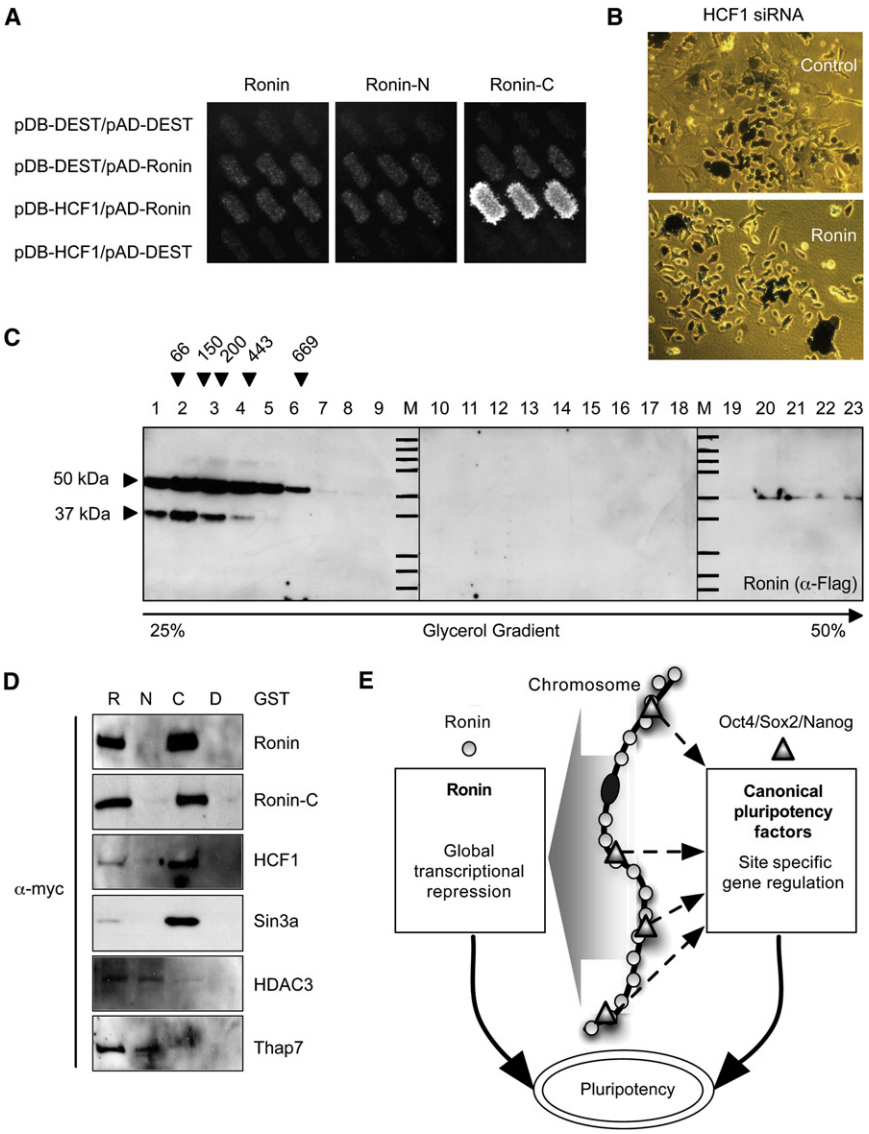

Figure 7. Ronin Binds Directly to HCF-1 and Is Associated with a Very Large Protein Complex

(A) Directional yeast two-hybrid approach to detect direct interaction of Ronin, Ronin-C, and Ronin-N with candidate interacting proteins identified by mass spectometry. Direct interaction of Ronin-C with HCF-1 is represented by growth of cotransfected MAV103 yeast on 50 mM 3AT-containing plates (AD = activation domain, DB = DNA-binding domain).

(B) Downregulation of HCF-1 by siRNA in control ES cells and Ronin ectopically expressing ES cells.

(C) Anti-Ronin-Flag western blot of glycerol gradient fractions of *EF1α-Ronin* ES cell nuclear extracts purified with wheat germ agglutinin beads. Ronin is present in the first 5 fractions and in fractions 20 to 23 with a peak in fraction 20. The elution peaks of protein standards with known sizes are compared (top).

(D) Western blot of Myc-tagged proteins after GST immunoprecipitation. Ronin interacts via its C terminus with itself, HCF-1, and Sin3A and via its N-terminus with HDAC3 and Thap7. R, Ronin; C, Ronin C terminus; N, Ronin N terminus; D, empty destination vector.

(E) Proposed model for the mechanism of Ronin function.

was turned off after induction of ES cell differentiation, but the disappearance of *Ronin* mRNA was delayed by comparison to the mRNA of other factors, such as *DPPA4* (Sperger et al., 2003), suggesting that Ronin may be required not only during the very early stages of embryonic development, but also during the differentiation stage. The results of our Ronin *lacZ* reporter assay argue strongly for selective expression of *Ronin* in a very limited number of cell types. If confirmed, this expression pattern might explain how Ronin has escaped detection by expression profiling of genes important for ES cell function and why it is represented by only marginal levels of expression in available databases (e.g., NIH SAGE).

The lethal defect in *Ronin* knockout mouse embryos likely resides within the ICM itself, as predicted by the striking similarities with the *Oct4* and *Nanog* knockout phenotypes (Nichols et al., 1998; Mitsui et al., 2003) and the finding that Ronin is an essential protein in ES cells. Nonetheless, we cannot rule out a defect in extraembryonic tissues required for the formation of the ICM. It is noteworthy that the expression pattern of Ronin does not necessarily contradict the lethal phenotype we observed in *Ronin* knockout embryos. Indeed, Ronin protein is present in the ICM (Figure 1G) and would be expected to function at that stage of development, so that its loss might well be lethal to the blastocyst. Whatever the explanation, our analysis of the *Ronin* expression pattern and the finding that *Ronin* knockout produces no phenotype in MEFs clearly indicate that the protein performs very specific functions in ES cells.

Although ES cells do not require Sox2 and Nanog for maintenance of the pluripotent state, at least in some contexts (Chambers et al., 2007; Masui et al., 2007), they do show an absolute dependency on Oct4, whose function cannot be replaced by other pluripotency factors tested to date (Ivanova et al., 2006). Hence, a major finding of our study is the ability of Ronin to override (at least partially) the requirement for Oct4 in the maintenance of pluripotency. To exclude the possibility that a small subfraction of cells might still be capable of undergoing self-renewal even in the absence of *Ronin*, we conditionally removed the endogenous *Ronin* allele in ES cells and observed that *Ronin* knockout seems to lead to rapid cell death. This result could reflect the inability of the tissue culture medium to support propagation of the particular cell type generated by *Ronin*-deficient ES cells, but this possibility seems unlikely because our medium

contains serum and supports all major lineages derived from ES cells. The most plausible explanation is that loss of *Ronin* activates large blocks of normally repressed genes, whose unscheduled expression leads to programmed cell death. However, we cannot rule out a direct effect of *Ronin* deficiency on the cell's apoptotic machinery. Moreover, *Ronin* was able to sustain the undifferentiated state of cultured ES cells even in the absence of LIF, an essential self-renewal factor that operates through the Jak-Stat pathway (Smith, 2001). A lack of complete dependence of Ronin function on canonical pathways was further indicated by its persistent expression upon knockdown of *Oct4*, *Sox2*, and *Nanog*. Taken together, these observations suggest a need to reconsider the prevailing Oct4/Sox2/Nanog-centric view of ES cell pluripotency.

We think it is important that in the teratocarcinoma model, Ronin acts as a tumor-promoting factor. Given that Ronin is expressed in some cells in the adult animal, it may possess a tumor-promoting function as well as the ability to regulate pluripotency. Indeed, the human *RONIN* gene is located on chromosome 16q22.1, a locus that has been associated with several forms of cancer, including leukemias, squamous cell carcinomas, and breast and prostate cancers, and *RONIN* was found to be overexpressed in tumors (Frengen et al., 2000; Yan et al., 2007).

A defining feature of Ronin is its THAP domain (Roussigne et al., 2003a, 2003b), whose zinc-finger DNA-binding motif is shared with a large family of cellular factors (more than 100 distinct members) in the animal kingdom. It has been proposed that THAP-containing proteins act at the level of chromatin regulation because of their frequent interaction with chromatin- modifying proteins (Macfarlan et al., 2005, 2006). The DNA sequence recognized by Ronin is unusually long, in agreement with data on Thap1, another THAP domain-containing protein that is responsible for regulating pRB-E2F target genes (Bessiere et al., 2007; Cayrol et al., 2007). However, the DNA sequence recognized by Thap1 differs from that recognized by Ronin, suggesting that each THAP domain may recognize a different DNA sequence, an idea supported by recent elucidation of structures within the THAP domain of Thap1 (Bessiere et al., 2007). Among the THAP family members, proteins that interact with chromatin-modifying elements, Thap7 and HIM-17 are perhaps the best characterized. Thap7 associates with both histone tails and HDACs (Macfarlan et al., 2005, 2006), while HIM-17 is involved in recruitment of the methyltransferase activity to histone H3 at lysine 9 (Reddy and Villeneuve, 2004; Bessler et al., 2007).

We hypothesize that Ronin suppresses the activity of multiple genes by binding directly to DNA and then recruiting HCF-1 and thus chromatin-modifying proteins. The association of HCF-1 with both activating and repressive epigenetic modifications raises the intriguing possibility that Ronin interaction with HCF-1 could introduce conflicting chromatin marks (so-called bivalent domains) at specific sites. An alternative hypothesis is that Ronin may affect RNA stability.

Our model (Figure 7E) predicts that Ronin acts broadly on transcription in pluripotent cells, in contrast to the canonical pluripotency factors (Oct4, Sox2, and Nanog), which modulate very specific particular genes that are required for either pluripotency or differentiation events. Thus, the major difference between Ronin and other pluripotency factors may lie in the scope of its action. If so, Ronin could be functionally compared in the broadest sense with the *C. elegans* PIE-1 protein, which globally represses transcription in germ cells as an integral step in its normal function (Blackwell, 2004). This model does not exclude the possibility that Ronin and the canonical pluripotency factors might act in parallel, perhaps on the same genes. Indeed, the DNA sequence bound by Ronin (*3x*) is upstream of many key development genes known to be targets of established pluripotency factors (e.g., *GATA4* and *GATA6*), and Ronin seems to bind to genomic regions associated with epigenetic silencing marks. This hypothesis is further strengthened by the fact that depending on the differentiation status of ES cells, Ronin can bind to the *Oct4* promoter. Identification of other key genes recognized by Ronin will help to elucidate the specific functions of this factor.

Our discovery and analysis of Ronin has several implications for the mechanisms that underlie pluripotency. Despite numerous investigations of factors that participate in the control of pluripotency, this state is still defined purely in functional terms. Thus, whether a particular cell is pluripotent (differentiation to all cells in the body), capable of contributing to the germline only (germline transmission), multipotent (differentiation to some but not all cell types), or unipotent (differentiation to a single cell type only) cannot be addressed in molecular terms with any degree of certainty. Our findings identifying Ronin as a novel type of pluripotency factor suggest a new tier of control in addition to the transcriptional circuit now believed to regulate ES cell pluripotency and contribute importantly to delineation of the role of epigenetic factors in this regulation.

EXPERIMENTAL PROCEDURES

ES Cell Culture

MEFs and HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO), L-Glutamine (GIBCO), 100 nM nonessential amino acids (GIBCO), and 100 μM beta-mercaptoethanol (Fluka). ES cells were cocultured with MEFs (or in 0.1% gelatin-coated dishes) in Knock-Out DMEM (GIBCO) containing the same supplements as MEF medium, plus 1000 U/ml LIF (Chemicon).

Targeted Deletion of the Mouse Ronin Gene

A targeting vector specific for the mouse Ronin allele was created in a four-step cloning procedure (see Supplemental Experimental Procedures) using the pfrt-loxP plasmid as a backbone (a gift from Dr. James Martin, Texas A&M Institute of Biotechnology).

Generation of *EF1α-Ronin* ES Cells

EF1α-Ronin mouse ES cells, in which constitutive, ectopic expression of FLAG-tagged human Ronin could be eliminated by a Cre recombination event, were generated by introducing FLAG tag and *loxP* sites and the desired restriction sites using PCR, as described in the Supplemental Experimental Procedures. The resulting vector was electroporated into R1 ES cells, and individual ES cell colonies were screened for Ronin expression.

siRNA Knockdown Experiments

To induce differentiation by knocking down *oct3/4*, R1 cells, or *EF1α-Ronin* ES cells, cells were plated and transfected with Smart Pool siRNA *oct3/4* using Lipofectamine2000. GFP duplex siRNA (Dharmacon) served as a negative control in a parallel experiment. Cell morphology was assessed by alkaline phosphatase staining after 3 days.

5-Fluorouridine Staining of Newly Transcribed RNA

A172loxP or A172LP-Ronin-FLAG cells were plated on an MEF feeder layer in 2-chamber slides. After 8 hr, Ronin expression was induced with doxycycline, and 12 hr later nascent RNA was labeled by incubation with 100 mM 5-fluorouridine (5-FU) for 1 hr. 5-FU was detected with an anti-BrdU primary antibody. Ronin was detected with the Ronin antiserum, G4275. Cells were mounted and examined by fluorescence microscopy.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cell.com/cgi/content/full/133/7/1162/DC1/.

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