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Supplemental Data

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

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Supplemental Experimental Procedures

Culture, differentiation, alkaline phosphatase staining and transfection of cells

For monolayer differentiation studies using the mpRonin-lacZ reporter ES cell line (clone C) and wild-type (wt) R1 ES cells, cells were plated onto gelatin-coated culture dishes at 6000 cells/cm^2 in ES cell medium without LIF and cultured for 3 days. Medium was replaced daily. Embryoid bodies (EB) were formed in hanging drops (20 μl mES medium) seeded with 600 cells and cultured for 3 days without LIF. Differentiation of R1, EF1*a*-Ronin and EF1*a*-ΔRonin R1 ES cells, detected by alkaline phosphatase staining, was induced by plating 1000 cells/cm^2 and culturing in the absence of LIF for 4 days. For alkaline phosphatase staining, cells were washed in phosphate-buffered saline (PBS), fixed for 30 minutes in 2% paraformaldehyde at room temperature (RT), washed once in PBS and stained in the dark using the AlkPhosIII Kit (Vector Laboratories), as described by the manufacturer. Plasmids were transfected using Lipofectamine2000 (Invitrogen) following the manufacturer's standard protocol, unless indicated otherwise.

Targeted deletion of the mouse Ronin gene

This vector contains loxP sites separated by a multiple cloning site, a PGK-neomycin-resistance cassette (NeoR) flanked by Flp recombinase recognition sites, and a downstream thymidine kinase gene for negative selection. The entire Ronin mRNA coding region was inserted between the loxP sites, which were placed in regions of low homology to create an inducible null genotype (Supplemental Figure 3A). After linearization with AscI, the Ronin targeting vector was introduced into R1 ES cells by electroporation followed by selection in the presence of G418 and ganciclovir; 960 G418- and ganciclovir-resistant colonies were isolated. After screening 56 individual ES cell colonies by PCR analysis, we identified four positive clones, designated Ronin$^{+/flox}$; three of these were subsequently microinjected into blastocyst-stage embryos and implanted into pseudopregnant female recipients to generate chimeric mice (Mouse Embryo Manipulation Services at Baylor College of Medicine). After confirming the genotypes of the resulting mice by Southern blotting and PCR analysis of genomic DNA (Supplemental Figure 3B), the mice were crossed with Zp3-Cre transgenic mice (The Jackson Laboratory). Female mice carrying the deleted Ronin allele and Zp3-Cre transgene were then crossed with B6 wild-type males to obtain Ronin$^{+/-}$ (heterozygous) offspring.

Generation of *EF1a-Ronin* ES Cells

EF1a-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 Supplementary Experimental Procedures. The resulting PCR product was ligated into BglII and XbaI sites of the p*EF1-luciferase-IRES-Neo* vector (a generous gift from David Spencer, BCM), replacing the luciferase gene with the Ronin coding sequence to generate the p*EF1-hRonin-Flag-loxP* vector. Twenty micrograms of circular vector were linearized with the restriction enzyme, NdeI, and electroporated into 10 x 10^6 R1 mouse ES cells, which were then grown on a layer of neomycin-resistant MEF feeder cells. Transfectants were selected over a period of 8 to 10 days using 200 μg/ml Geneticin (Invitrogen), after which individual ES cell colonies were screened for Ronin expression by Western blot analyses using an antibody against the FLAG epitope (Sigma). Under denaturing conditions, Ronin migrated as a 50 kD protein. A control cell line expressing the p*EF1/His/C* vector (Invitrogen) was produced in a similar manner using NruI-linearized vector.

siRNA knockdown experiments

To induce differentiation by knocking down *oct3/4*, R1 cells (1 x 10^5/well) or *EF1a-Ronin* ES cells (2 x 10^5/well), plated in 6-well plates (10 cm^2/well), were transfected with Smart Pool siRNA *oct3/4* (Dharmacon, M-046256-00-0005) using 5 μl of Lipofectamine2000 and following the siRNA transfection protocol for D3 cells (Invitrogen). Briefly, Lipofectamine2000 and siRNA were diluted in 250 μl OptiMEM, incubated for 15 minutes, mixed, incubated for an additional 15 minutes, and then added to cells. GFP duplex siRNA (Dharmacon) served as a negative control in a parallel experiment. Cell morphology was assessed by alkaline phosphatase staining after 3 days. Knockdown of HCF-1 was accomplished with SMARTpool siRNA HCFC1 (Dharmacon, M-051186-00-0005).

5-Fluorouridine staining of newly transcribed RNA

A172loxP or A172LP-Ronin-FLAG cells (1.5 x 10^5 cells/well) were plated on an MEF feeder layer in 2-chamber slides. After 8 hours, Ronin expression was induced with doxycycline (1 μg/ml), and 12 hours later nascent RNA was labeled by incubation with 100 μM 5-fluorouridine (5-FU, Sigma, F5130) for 1 hour, as described by Boisvert et al. (2000). 5-FU was detected with an anti-BrdU primary antibody (Sigma, 1:500) and goat anti-mouse Alexa Fluor 488 secondary antibody (Molecular Probes, 1:1000). Ronin was detected with the Ronin antiserum, G4275 (1:2000), and the secondary antibody, AlexaFluor 594 (Molecular Probes, 1:1000). Cells were mounted in Vectashield Mounting medium with DAPI (Vector Laboratories) and examined by fluorescence microscopy using a Zeiss 63x/1.40 objective. Images were deconvoluted using the Resolve3D software

Generation of *EF1a-ΔRonin* ES cells

To eliminate ectopic Ronin expression and generate *EF1a-ΔRonin* ES cells, *EF1a-Ronin* ES cells were plated at a density of 3600 cells/10-cm dish and cultured without LIF. After 4 days, cells grown on 10-cm dishes were re-plated at the same density in 15-cm dishes and selected in medium without LIF for 4 more days. Colonies were then selected and expanded on MEF feeder cells in medium supplemented with LIF. After expansion, 5 x 10^6 cells were plated on MEFs in a 10-cm dish and transfected 4 hours later with a 1:4 mixture of p*CMV-GFP* (Stratagene) and p*Salk-Cre* (a kind gift of Dr. Michael Kyba). After culturing for 18 hours, 5 x 10^6 GFP positive cells were sorted by fluorescent activated cell sorting (FACS; BD Biosciences FACSaria) and 2,500 green fluorescent cells/cm^2 were plated on MEFs in a 10-cm dish. After passaging twice, the GFP signal had faded completely, indicating that Cre activity was absent. Passaged cells were then plated at 1,000 cells/cm^2 and cultured for 9 days to allow colonies to form. Selected colonies were expanded and genotyped for successful elimination of Ronin by Cre-mediated recombination. Thirty clones were genotyped using the oligos MAD221 (5'-CCG GCC TTA TTC CAA GCG GC-3') and MAD224 (5'-CTG ACT GCT GTC TAC AGT GGC CTG-3'). A second PCR, using oligos MAD221 and MAD222 (5'-AGT CAG GCT CCG GGA TCC GTA CAG -3'), was performed to exclude the presence of expanded mixed cultures containing non-revertant *EF1a-Ronin* ES cells. After culturing in the absence of LIF for 4 days, as described, six of these clones were shown to differentiate in a manner similar to wt R1 cells, three of which generated chimeric mice following injection into blastocysts, confirming pluripotency. Chimeric mice were identified at 3 weeks of age on the basis of coat color.

Generation of the Ronin-inducible mouse ES cell line, A172LP-Ronin-Flag

To generate an inducible cell line, a FLAG tag was first added to the 3' end of Ronin in a PCR reaction using the primers, (+) 5'-GTC GCA GCC ATG CCT GGC TTT ACG-3' and (-) 5'-TCA CTT ATC GTC GTC ATC CTT GTA ATC CAT GCC GTG CTT CTT ACG GAT G-3'. The purified product was cloned into pGEM-Teasy (Promega), digested with EcoRI and ligated into the corresponding EcoRI site of p*2Lox-EGFP*, replacing EGFP with mRonin. Twenty micrograms of the resulting vector, p*2Lox-Ronin-CFlag(C15)*, and 20 μg of pSalkCre were simultaneously electroporated into 1.5 x 10^6 A172LoxP cells, as described by Kyba et al., and plated onto neomycin-resistant MEFs (plasmids and cells were a kind gift of Dr. M. Kyba, UT Southwestern). Individual clones were selected and expanded in the presence of 400 μg/ml geneticin. Ronin expression was verified by Western blotting and immunofluorescence using an anti-FLAG antibody after induction with 100 ng/ml to 1 μg/ml doxycycline for 6 to 80 hours.

SELEX

The coding region of mouse Ronin was introduced into pET101/D/lacZ (Invitrogen) to create a mRonin-His/V5 fusion construct. Fusion protein expression was induced in the BL21Star strain of E. coli by treating with 1 mM IPTG for 5 hours. His-tagged Ronin was purified using Ni-NTA Fast Start (Qiagen) columns according to the manufacturer's protocol and concentrated using YM-10 centrifugal filters (Millipore). The SELEX procedure was performed essentially as described by Bouvet et al. (2000). Briefly, the randomized SELEX template, 5'-TGG GCA CTA TTT ATA TCA AC -N25- AAT GTC GTT GGT GGC CC-3' (where N25 is a 25-base sequence of randomly inserted nucleotides), was synthesized and amplified with the primers, (+) 5'-CCC GAC ACC CGC GGA TGG GCA CTA TTT ATA TCA AC-3' and (-) 5-CGC GGA TCC TAA TAC GAC TCA CTA TAG GG G CCA CCA AC GAC ATT-3' in a PCR reaction using 10 μM of each oligonucleotide. PCR cycling conditions used were: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 50°C (1 minute) and 72°C (1 minute), with a final 10 minute extension at 72°C. NTA agarose bead-conjugated Ronin was prepared by adding 1 μl (550 ng) recombinant Ronin-His protein to 3 μl washed Ni-NTA agarose beads in 100 μl NT2 buffer (20 mM Tris, pH 7.5; 100 mM NaCl; 0.05% NP40). After a 30 minute incubation at 4°C beads were washed twice and resuspended in 100 μl binding buffer (20 mM Tris, pH 7.5; 100 mM NaCl; 0.05% NP40; 0.5 mM EDTA; 100 μg/ml BSA; 50 μg/ml Poly dI-dC). One

hundred microliters (3 μg) of purified PCR product (PCR Purification Kit; Qiagen) was added to Ronin-conjugated beads and incubated for 5 minutes at RT, followed by 5 washes with NT2. Beads were resuspended in 100 μl H_2O, and Ronin-bound DNA was purified by phenol/chloroform extraction and ethanol precipitation, then resuspended in 10 μl H_2O. Two microliters of DNA isolated from the first round of Ronin binding were used in a second round of PCR amplification, purification and Ronin-binding using the same conditions. Seven rounds of PCR amplification were used in the Selex procedure, generating a population of PCR products enriched for specific Ronin binding. These products were then ligated into the pGEM-TEasy vector (Promega). One hundred and four randomly selected clones were sequenced and analyzed using the Multiple EM for Motif Elicitation software (meme.sdsc.edu).

Gel mobility shift experiments

Gel mobility shift experiments were performed using the LightShift Chemiluminescent EMSA kit, according to the manufacturer's instructions (Pierce). Nuclear extracts were prepared from A172LP-Ronin-Flag cells after induction with 1 μg/ml doxycycline for 12 hours. First, microscopically intact nuclei were obtained as described by Remboutsika et al. (1999). Intact nuclei were then lysed on ice for 20 minutes in NIB buffer (15 mM Tris pH 7.2; 60 mM KCl; 15 mM $MgCl_2$; 15 mM NaCl; 1 mM $CaCl_2$; 1 mM PMSF; 5 mM sodium orthovanadate; 5 mM sodium fluoride; 500 μM dithiothreitol; protease inhibitor cocktail) supplemented with 0.6% NP40. Lysates were centrifuged at 2000 x g for 5 minutes at 4°C and the supernatant, containing the nuclear contents, was stored in 25% glycerol at -80°C until ready for use. The biotin-labeled 3x sequence, 5-bio-ATC AAC TGT ATA CAA GCA GCT AGG ACA GCA CCC TAA TGT C-3', and the unlabeled 3x sequence, 5'-TGT ATT ACA AGC TAG GAC AGC ACC T-3', were used as probes. Sense and anti-sense strands were synthesized and hybridized in equimolar amounts for use as a double-stranded template. Two microliters of 20 nM biotin-labeled 3x double-stranded DNA was mixed with 4 μl (3 g) nuclear extract in a total volume of 20 μl. To block binding of Ronin to the template, 1 mg/ml anti-FLAG antibody (M2, Stratagene) was preincubated with 10 μM unlabeled double-stranded 3x or single-stranded 3x sequence and nuclear extract. Normal mouse IgG (Santa Cruz) was used in the control reaction. Oligos used in competition experiment: Oligo A: 5'-GGA CAG CAC CCT-3', Oligo B: 5'-CAA GCT AGG ACA G-3' and Oligo C: 5'-TGT ATT ACA AGC TAG GAC AGC ACC CT-3'

Generating probes for Northern and Southern blot hybridization

Ronin Probe 1 was generated by PCR using the GoTaq Green mastermix (Promega) and the primers, (+) 5'-GGA TCC GTG GTT CCC GTG -3' and (-) 5'-CCA TGG CAA GCA GAC GAT C-3' under the following cycling conditions: 98°C for 15 seconds, 40 cycles of 95°C for 10 seconds, 52°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Ronin probe 2 was amplified using the primers, (+) 5'-GTG GGT CGC TAA ACC TGA GAG -3' and (-) 5'-GTT CAA TGA GTT AGC TGT GTC -3' under the same conditions as used for Ronin probe 1. The GAPDH probe was generated using the primers, (+) 5'-ACC ACA GTC CAT GCC ATC AC-3' and (-) 5'-TCC ACC ACC CTG TTG CTG TA-3' under the same PCR cycling conditions, except that an annealing temperature of 55°C was used. Five nanograms of mouse genomic DNA was used as template for all probes. The PCR products were purified by agarose gel extraction (Qiagen) and labeled with ^{32}P using the Rediprime II Random Prime labeling system following the manufacturer's protocol (Amersham/GE Healthcare). Seventy-five nanograms of the labeled probes were used in subsequent Northern or Southern blot hybridizations.

Northern blot analysis

Total RNA from R1 ES cells and adult mouse tissues was extracted using the UltraSpec RNA reagent (Biotex). For Northern blot analysis, total RNA (10 μg) was separated on a 1% agarose gel in 1x MOPS buffer in the presence of 8% formaldehyde, transferred to a charged nylon membrane (Millipore) and crosslinked with UV light. The membrane was hybridized in Express Hyb Hybridization solution (Clontech) at 68°C overnight using ^{32}P-labeled Ronin probe 1. The membranes were washed three times for 1 hour with wash buffer 1 (2x SSC, 0.1% SDS), two times for 1 hour with wash buffer 2 (0.1x SSC, 0.1% SDS) followed by an overnight wash in wash buffer 2. All washes were carried out at 68°C. Membranes were then exposed to X-Ray film for 6 hours to 2 days at -80°C.

Southern blot analysis

Genomic DNA was isolated from tail biopsies following digestion with proteinase K using phenol-chloroform extraction. DNA (10 μg) was incubated with the indicated restriction enzyme overnight and then separated on 0.8% agarose gels. DNA was denatured in situ and then transferred to a nylon membrane (Millipore). After crosslinking DNA to the membrane, the membrane was hybridized with the indicated ^{32}P-labeled cDNA probe (see above). Probe labeling, hybridization and washes were done as described for the Northern blot hybridization (see above), but hybridization and washes were performed at 55°C. The additional overnight wash was only included when Ronin probe 1 was used.

Production of Ronin antiserum

Ronin antiserum was made by GeneMed Synthesis, Inc. Three peptides - VPG CYN NSH RDK ALH, TGS DHS YSL SSG TTE and LME VKM KEM KGS IRH - corresponding to sequences conserved between mouse and human were synthesized; each was KLH-conjugated and contained an N-terminal cysteine. Two separate rabbits were immunized with a mixture of all three peptides on day 1, 20, 40 and 60. Anti-sera were collected from each rabbit ten days after the fourth immunization. Rabbit anti-Ronin lot G4275 and the corresponding pre-immune serum were used in all experiments.

Immunofluorescence of mouse and human ES cells, ovarian tissue and zygotes

Ovaries from a superovulated C57B/6 wild-type female were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 *m*m) were stained with a 1:50 dilution of the anti-Ronin or pre-immune serum and detected with the secondary Alexa Fluor 488 anti-rabbit antibody (Molecular Probes; 1:2000 dilution). Sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories) and immunofluorescence was visualized using a deconvolution microscope.

For imunostaining of Ronin in mouse and human ES cells, H9 or R1 cells were grown on glass coverslips and fixed for 30 minutes at 4C in 2% paraformaldehyde dissolved in PBS. After three washes in PBS, the cells were permeabilized for 30 minutes at room temperature with 0.3% Triton-X-100 in PBS and blocked over night at 4C in Buffer G. The cells were washed one hour in PBS and then incubated for one hour at room temperature with the Ronin immune serum (described), and pre-immune serum as control, diluted 1:5000 in Buffer G. After three washes for 15 minutes in PBS/0.1% Triton-X-100, secondary antibody in Buffer G (1:2000), was added for one hour at room temperature. After three washes for 15 minutes in PBS/0.1% Triton-X-100 and two washes for 15 minutes in PBS, the cells were fixed in 0.2% formaldehyde in PBS for 30 minutes at 4°C and washed twice in PBS. Slides were mounted in Vectashield with DAPI (Vector Laboratories). For immunostaining of early embryos, they were isolated from wildtype 129X1/SVJ mice following the protocol described by Nagy et al (Nagy, 2003). E2.5 or E3.5 embryos were washed twice with 1X BD perm/wash buffer (BD Pharmingen) and then fixed with Cytofix/Cytoperm buffer (BD Pharmingen) at 4°C for 1 hour. Embryos were washed twice and then blocked in 1X BD perm/wash buffer for an additional hour at room temperature. The embryos were then incubated at 4°C overnight shaking gently in a 1:1000 dilution in 1X BD perm/wash buffer of anti-Ronin G4275 or the pre-immun serum control. Embryos were washed by moving them through small drops of 1X BD perm/wash buffer at room temperature and then incubated with the secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen) in a 1:2000 dilution in 1X BD perm/wash buffer at 4°C overnight shaking gently. Embryos were washed as before and mounted in Vectashield with DAPI (Vector Laboratories Inc.). Signals were visualized with a fluorescence microscope (Zeiss Axioplan 2), images were taken with the MetaVue (Version 6.1r1) software. E3.5 blastocysts were plated on 0.1% gelatinized 24-well culture dishes and grown for 3 days to detect Ronin expression in ICM outgrowth following the same staining protocol with a 1:5000 dilution of the primary antibodies.

Generation of mpRonin-lacZ reporter mouse line

An mpRonin-lacZ reporter construct was generated by PCR amplification of a 3.3 kb genomic region of the mouse Ronin gene that included a region upstream of the translation initiating ATG codon and the first 15 base pairs within the annotated coding region. PCR was performed using native Pfu Polymerase (Stratagene) in the presence of 4% DMSO and the primers (+) 5'-ACA AAG CTT AGT CTC GCG ATG CTG CCA C-3' and (-) 5'-ACA GCT AGC CGT AAA GCC AGG CAT GGC TG-3'. The PCR program consisted of an initial denaturation at 98°C for 1 minute, 35 cycles of 96°C for 15 seconds, 55°C for 1 minute and 68°C for 8 minutes, and a final extension at 68°C for 10 minutes. The PCR product was ligated into the HindIII and NheI sites of the pEF1/His/LacZ expression vector (Invitrogen), exchanging the p*EF1a-His* region for the Ronin promoter region. The resulting mpRonin-lacZ vector was linearized with Hind III and injected into the pronucleus of wt C57B/6 oocytes (Mouse Embryo Manipulation Services (MEMS),Baylor College of Medicine). The resulting mice were genotyped using GoTaq DNA Polymerase (Promega) and the primers, lacZ(+) 5'-CTT AGG ACG AGC TTC ATC TG-3' and lacZ(-) 5'-GAC GGG ATC AAC TCC AAG CTG-3' with the following cycling conditions: 95°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Three of seven PCR-positive transgenic mouse lines (8, 9 and 15) were analyzed for ovarian lacZ expression; post-implantation studies were done using line #8. For pre-implantation studies, a transgenic male from line #8 was crossed with 3-week-old superovulated wt C57B/6 females. Line #15 was used for X-Gal staining of adult mouse tissues.

X-Gal staining of adult tissues, tissue sections, cultured cells and EBs

Adult mice were perfused with 4% Paraformaldehyde, tissues were dissected and postfixed for 1 hour at 4°C, washed three times in X-Gal rinse buffer (PBS containing 2 mM $MgCl_2$, 0.25% sodium deoxycholate and 0.2% triton X-100) at RT for 30 minutes and then stained in X-Gal staining solution (40 μg/mL X-gal in 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6x3H_2O$ and 5 mM $K_3Fe(CN)_6$) overnight at 37°C. Specimens were then post-fixed in 2% paraformaldehyde for 1 hour and stored in 70% ethanol.

Cultured cells and EBs were fixed for 5 minutes at room temperature in 2% formaldehyde/0.2% glutaraldehyde, washed four times (10 minutes each) in wash buffer, submerged in X-Gal staining solution for 2 to 16 hours, post-fixed in 2% paraformaldehyde and stored in PBS at 4°C.

For X-Gal staining of sections from OCT embedded tissue, 10 μm sections were post-fixed for 15 minutes in 4% paraformaldehyde, rinsed in PBS and then washed (10 minutes) in PBS containing 2 mM $MgCl_2$, followed by two 10-minute washes in rinse buffer. All steps were preformed on ice. Sections were stained overnight at 37°C in X-Gal staining solution, washed twice (5 minutes each) in PBS, rinsed in H_2O, counterstained with Nuclearfast Red, dehydrated and mounted in Permount (Fisher Scientific).

Histology and embryo collection

Preimplantation embryos were isolated from 3.5- to 4.5-week-old mpRonin-lacZ or *Ronin*$^{+/-}$ female mice, superovulated using pregnant mare serum (5 units) followed 47 hours later by human coriogonadotropic hormone. These females were then mated with wt or *Ronin*$^{+/-}$ males and examined the following morning for vaginal plugs. Embryos were flushed from the uterus 3 days after

identifying vaginal plugs or removed from the oviduct the same day. After removal, embryos were either fixed or cultured. For histology, the uterus was fixed, paraffin-embedded and H&E-stained after sectioning (according to Behringer et. al., "Mouse phenotypes"). DNA for genotyping was isolated from embryos dissected from the deciduas and cleared of maternal tissue.

Embryo culture and ES cell derivation

For ICM outgrowth experiments, individual blastocysts isolated from *Ronin*$^{+/-}$ crosses were transferred to a well of a gelatin-coated 24-well culture dish in ES cell medium containing 20% FCS and cultured for 4 days. Cultures were genotyped using PCR. To derive ES cells, blastocysts were cultured for 3 days on gelatin, as above, and then expanded on an MEF feeder layer. Newly derived ES cell lines were genotyped using PCR and Southern blotting.

Generation of the Ronin targeting vector

A targeting vector specific for the mouse Ronin allele was created using the plasmid pfrt-loxP as a backbone (a gift from Dr. James Martin, Texas A&M Institute of Biotechnology) in a four step cloning procedure. Using genomic DNA isolated from R1 ES cell as template, four PCR fragments were generated with Pfu Polymerase (Stratagene), sequence-verified and ligated into pfrt-loxP. A 3' short homologous arm (1.5 kb), amplified using the primers, (+) 5'-ACA CTC GAG TAGA TAG GTA TTG GCC TAT TTG AAA GAA C-3' and (-) 5'-ACA CCT AGGT GGC ACA TAC CTT TAA TCC CAG CAC-3', was first ligated into the XhoI and AvrII restriction sites. The Ronin mRNA region (2 kb) was split into two fragments and sequentially ligated (3' end of the gene followed by the 5' end) into the NotI site in pfrt-loxP using the corresponding NotI site within the Ronin gene. The 3' end of the gene was amplified using the primers (+) 5'-AGG GC GGC CGC AAG ACC TAC ACG GTG-3' and (-) 5'-ACA CGG CCG ATC CCC ACA TTT CAA GGA CA CTT AGC T-3' and ligated using the EagI site (introduced into the (-) primer) to preserve NotI as a unique site. The 5' end of the gene was amplified using the primers (+) 5'-ACA GC GGC CGCT ACC TTT CGC TTA GGA CGAG CTT CATC -3' and (-) 5'-CTT GCG GCC GCC CTG AAA GTG GAC GCT GCA G-3'. Finally, a 5' long homologous arm (8 kb) was amplified using the primers (+) 5'-ACA GGC GCG CCA CGT CTA CGC TAA CTC TGG CAC TGG -3' and (-) 5'-ACA ACG CGT TGT ATT CGA ATG GAC ACG TTA TGG C-3' and ligated into the AscI site. To preserve AscI as a unique site, the 3' end of the long arm was ligated into the MluI site.

Generation of *Ronin*$^{flox/flox}$ mES cell line by gene targeting

Cells from the original *Ronin*$^{+/flox}$ -targeted ES cell clones used for generating the conditional Ronin mouse model (see above) were transfected with p*FLP-Cre-IRES-GFP* (a kind gift of Dr. Joerg Fehling, University of Ulm) and sorted by FACS to isolated GFP-positive cells. To screen for cells in which the Neor cassette had been deleted, individual colonies were selected and expanded on an MEF feeder layer, then tested for sensitivity to geneticin; Neo^r deletion was confirmed by PCR. The resulting *Ronin*$^{+/flox}$ Neo^r cells were electroporated with the Ronin targeting vector (see above) to introduce loxP sites into the second allele, followed by selection with geneticin and ganciclovir, and genotyping. Clone A3 was found to be successfully *Ronin*$^{flox/flox}$ -targeted and was used for subsequent studies.

Cre-mediated excision of loxP-flanked *Ronin* in *Ronin*$^{flox/flox}$ ES cells

Ronin$^{flox/flox}$ ES cells (see above) were plated onto MEFs at a density of 100,000 cells/cm^2 and co-transfected with a 4:1 mixture of p*Salk-Cre* or *pEF1a-C* (kind gift of Dr. Michael Kyba) and pGFP. Twenty hours after transfection, GFP-positive cells were isolated by FACS (with the expectation that GFP-positive cells would also express Cre-recombinase) and plated onto an MEF feeder layer in a 10-cm dish (30,000 cells/dish). After 7 days, 96 individual colonies were selected, expanded on MEFs and genotyped by PCR. After removing selected colonies, the remaining cells were fixed and stained with crystal violet.

Cloning of p*MAX-Cre-GFP*

To generate the pMAX-Cre-GFP expression vector, the Cre gene was amplified using the primers (+) 5'-ACA GCT AGC GCC ACC ATG TCC AAT TTA CTG ACC GTA CAC -3' and (-) 5'-ACA CCC GGG AGA TC GCC ATC TTC CAGC AGGC GCA C-3'. The PCR product was cut with NheI and AgeI and ligated into the corresponding sites of p*MAX-GFP* (Amaxa).

Nucleofection of *Ronin*$^{flox/-}$ and *Ronin*$^{+/flox}$ ES cells

A 6-cm plate of confluent cells was used for nucleofection, employing the Mouse ES cell Nucleofector Kit and following the manufacturers standard protocol in combination with electroporation program A-30 (Amaxa). *Ronin*$^{-/flox}$ ES cells were nucleofected with p*MAX-GFP* (Amaxa), and *Ronin*$^{+/flox}$ ES cells were nucleofected with p*MAX-Cre-GFP*. After nucleofection, cells were plated into one well of a 6-well plate on an MEF feeder layer, and Cre activity was assessed 48 hours later by GFP expression.

Cre virus transduction of MEFs

MEFs were isolated from embryos derived from Roninflox/floxP mice and split into one well of a 6-well dish at P1 or P2. When cells reached approximately 50% confluency, they were transduced with adenovirus containing *CMV-GFP* or *CMV-Cre-IRES-GFP* in the presence of the GeneJammer transfection reagent (Stratagene), according to the method of Fouletier-Dilling et. al. Adenoviral supernatant was obtained from the Vector Core Facility at the Center for Gene and Cell Therapy (Baylor College of Medicine). Four hours after transduction, cells were washed twice with medium and then cultured in fresh medium. Cells were split after reaching 90-100% confluency and genotyped by PCR.

RT-PCR analysis

For RT-PCR analysis, R1 cells (1500 cells/cm^{2}2) and *EF1a-Ronin* ES cells (2500 cells/cm^2) were cultured in the absence of LIF for 1, 2, 3, 4 and 5 days. RNA was isolated using the RNeasy tissue kit following the manufacturers standard protocol with on-column DNA digestion (Qiagen). Total RNA (1 μg) was reverse transcribed using the ImPromII Reverse Transcription System (Promega) in the presence of 4.8 mM $MgCl_2$ and oligo-dT to generate cDNA. The expression levels of the tested genes were assessed in subsequent PCR reactions using 1 l of each cDNA. PCR reactions (50 μl total volume) were performed using the GoTaq Green Mastermix (Promega) and 10 *m*M of each primer under the following cycle conditions: denaturation for 3 minutes at 94^{o}C, followed by variable numbers of cycles (see below) of 30 seconds at 94^{o}C, 30 seconds at 55^{o}C, 30 seconds at 72^{o}C, and a final extension for 7 min at 72^{o}C. Primers, cycle numbers and product size were as follows: *mRonin* (+) 5'-GCC TCA GAG CTA GAG GCT GCT ACG -3' (-) 5'-TGG AAG GAG TCA CGA ATT CTG CAG-3' (27 cycles, 400 bp), *Oct4* (+) 5'-GGC GTT CTCT TTGG AAA GGT GTT C-3' (-) 5'-CTC GAA CCA CAT CCT TCT CT-3' (27 cycles, 312 bp), *Nanog* (+) 5'-CCA GTGG AGT ATCC CAG C AT-3' (-) 5'- GAA GTT ATG GAG CGG AGC AG - 3' (40 cycles, 237 bp), *Fgf5* (+) 5'-ATA GCA GTT TCCA GTG GAG CCC TT-3' (-) 5'-TGG ATC GCG GAC GCA TAGG TAT TA-3' (35 cycles, 241 bp), Sox1 (+) 5'-TTAC TTC CCG CCA GCT CTT C-3' (-) 5'-TGA TGC ATT TTG GGG GTA TCT CTC -3' (30 cycles, 373 bp), *Afp* (+) 5'-TCG TAT TCC AAC AGGA GG -3' (-) 5'-AGG CTTT TGC TT CAC CAG-3' (35 cycles, 174 bp), *T-Brachyury* (+) 5'-ATG CC AAAG AAA GAA ACG AC-3' (-) 5'-AGA GGC TGT AGA ACA TGA TT-3' (35 cycles, 835 bp), (*b-Actin* (+) 5'-GGC CCA GAGC AAG AGA GGT ATC C-3' (-) 5'- ACG CA CGA TTT CCC TCT CAGC -3' (27 cycles, 460 bp). GAPDH(F) 5'- ACC ACA GTC CAT GCC ATC AC -3' GAPDH(R) 5'- TCC ACC ACC CTG TTG CTG TA-3'. To quantify the expression level of GAPDH and *b*-actin, we analyzed the RT-PCR signals of three independent PCRs after agarose gel electrophoresis and ethidium bromide staining using the AlphaEaseFC (FluorChem) Software Version 5.0.1 (Alpha Innotech). The expression level is displayed as the relative intensity in % based on the integrated density value. Ronin transgene specific primers: tgRonin(+) 5'- AGT CAG GCT CCG GGA TCC GTA CAG-3' (-) 5'-CCG GCC TTA TTC CAA GCG GC-3' (30 cycles, 605 bp). The relative (%) value shown in Figure S4 are calculated by band densitrometric analysis using (ChemiImager 4000) software gives tis the relative cumulative intensity of individual bands in comparison to the entire gel image analyzed. The value is directly linear to the pixel intensity value of each band.

Immunohistological staining of Oct-4 in teratoma sections

Teratoma sections (10 *m*m) were immunostained with the *oct3/4* H-134 rabbit polyclonal primary antibody (Santa Cruz Biotechnology; 1:200 dilution) and the biotinylated anti-rabbit IgG (H+L) secondary antibody (Vector Laboratories, Burlingame, CA; 1:200 dilution). The signal was developed using the Rabbit IgG Vectastain ABC-AP Kit (Vector Laboratories) following the manufacturer's recommendations. Sections were counterstained with hematoxylin and mounted in Vectashield mounting medium (Vector Laboratories).

Microarray Analysis

D3 mouse ES cells were transfected with p*EF1/His/C* or p*EF1a-hRonin-FLAG* in a 6-cm dish. Medium was changed 2 hours before and 4 hours after transfection and cells were harvested 24 hours later. RNA was isolated using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion, as described by the manufacturer. Microarray analysis of RNA was performed by the Microarray Core facility at Baylor College of medicine using the GeneChip Mouse Genome 430 2.0 Array (Affymetrix)

Methyl-^{3}H Uridine incorporation assay

R1 and *EF1a-Ronin* clones were plated in a 96-well plate at a density of 5000 cells/well, incubated overnight and then stimulated with 1 g/ml retinolic acid for the indicated times. Cells were then pulsed with methyl-^{3}H uridine (Perkin Elmer, NET027) for 3 hours and harvested (Packard Filtermate harvester). Methyl-3H uridine incorporation was measured using a Packard Topocount-NXT Microplate Scintillation and Luminescence Counter.

Detection of histone H3K9 di-methylation after induction of Ronin expression

Ronin expression was induced in A172LP-mRonin-FLAG cells, plated 24 hours previously, by treating with 1 *m*g/ml doxycycline for the indicated times. After induction, cells were resuspended in lysis buffer (0.5 M NaCl , 20 mM Trizma , 0.5% Triton X-100 , 0.5% NP-40 , 1 mM EDTA , 0.25% sodium deoxycholate) supplemented with Protease Inhibitor Cocktail Complete(tm) (Roche), and sonicated (10 pulses at 30% duty cycle) using a Branson Sonifier 450 (Branson Ultrasonics , Danbury, CT). Western blot analysis was performed using the Odyssey system following the manufacturer's protocol. Histone H3K9 di-methylation was detected using the mouse monoclonal H3K9me2 primary antibody (Sigma; 1:2000 dilution) and secondary goat anti-rabbit IgG (H+L) Alexa-Fluor 680

antibody (Molecular Probes; 1:10000 dilution). (*b*-actin (control) was detected using a mouse monoclonal (-actin primary antibody (Santa Cruz; 1:2000) and IR-Dye 800-conjugated goat anti-mouse IgG (H+L) secondary antibody (Rockland; 1:10000 dilution).

Immunoprecipitation and mass spectrometry

D3 cells, plated 24 hours previously on gelatin in 10-cm dishes, were transfected with p*EF1a-Luc-Ires-Neo* and p*EF1a-hRonin-FLAG-Ires-Neo*. Six 10-cm dishes were used for each plasmid and the medium was changed 3 hours before transfection. Sixteen hours after transfection, cells were resuspended in lysis buffer (10 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5% Triton X-100, 0.25% sodium dodeoxycholate) containing Protease Inhibitor Complete (Roche)), incubated on ice for 15 minutes and sonicated. The lysate was centrifuged and the supernatant was pre-cleared by incubating with a mixture containing 2 mg normal mouse IgG and 20 *m*l of washed protein A/G beads (Santa Cruz) for 1.5 hours with continuous shaking. FLAG-tagged Ronin was immunoprecipitated from pre-cleared lysates by incubating with 20 *m*l of protein A/G beads and 2 *m*g of anti-FLAG antibody (Clone M2, Sigma) for 2 hours at 4°C. Immunoprecipitated proteins were eluted with 5 *m*l of FLAG peptide (Sigma) at 4°C for 1 hour and subjected to mass spectroscopic analysis.

Ronin complex purification from nuclear extracts by glycerol gradient centrifugation

Nuclear extracts, prepared from 2.5 x 10^9 *EF1a-Ronin* ES cells (25 x 15-cm dishes) as described for HeLa cells by Dignam et. al. (1997), were used to isolate and purify Ronin complexes, as described for the purification of the HCF-1 complex from HeLa cells by Wysocka et al. (2006). Briefly, the nuclear extract was further purified using wheat-germ agglutinin (WGA; Vector Laboratories) agarose; WGA-bound proteins were eluted with N-acetyl glucosamine (vector Laboratories) and concentrated using Microcon Y-10 filters (Millipore). Protein complexes were separated on a 25-50% glycerol gradient by ultra-centrifugation for 10 hours at 32,000 rpm. Collected fractions were separated by SDS-PAGE, followed by Western blot analysis using an anti-FLAG primary antibody (M2, Sigma; 1:1000 dilution) and HRP-conjugated goat anti-mouse secondary antibody (GE Healthcare; 1:2000 dilution). The secondary antibody was detected using ECL Plus solution following the manufacturer's recommendations (GE Healthcare).

Chromatin immunoprecipitation

A172 dells were plated at 2x105 cells per 15 cm culture dish. 16 hours after plating, differentiation was induced by addition of 1 µM Retinoic acid in the absence of LIF. After 7 days chromatin immunoprecipitation (ChIP) was essentially performed as described by Cui et al. (2005). Briefly, cells were treated with 1% formaldehyde for 10 minutes a room temperature to crosslink DNA with bound protein. The crosslinking reaction was stopped with 0.125 M glycine and the cells were harvested by scraping in PBS. The cells were washed twice with PBS, swelled on ice for 10 minutes in LB1 (5 mM Hepes pH 8, 85 mM KCl, 0.05% NP40) and disrupted by douncing five times with a loose pestle. The nuclei were spun down and resuspended in 50 µl LB2 (50 mM Tris pH 7.5, 10 mM EDTA, 1% SDS). Following incubation on ice for 10 minutes, LB3 (167 mM NaCl, 16.7 mM Tris pH 7.5, 1.2 mM EDTA, 1.1% Triton-X-100, 0.01% SDS) was added to a final volume of 1 ml. Samples were sonicated on ice with five pulses for 10 seconds on maximum setting with a Fisher Scientific Model 100 Sonic Dismembrator. 200 µl of the sonicated lysate were mixed with 300 µl LB3 and samples were precleared with 4 µl rabbit IgG (Santa Cruz) or 6 µl pre-immune serum by rolling for 1 hour at 4°C. 45 µl of unblocked protein A bead slurry (GE Healthcare) were added and samples were incubated for an additional hour. After centrifugation, 6 µl of the corresponding antibody were added to the supernatant and immunoprecipitation was performed over night rolling at 4C. The antibodies were Histone 3 (Abcam, #1791), Ronin anti-serum (as described), Histone 3 (di-methyl K9) (Active Motif, #39239) and Rabbit IgG (Santa Cruz) as control. 50 µl of Protein A bead slurry that had been blocked with Salmon Sperm DNA were added and samples were incubated for 2 additional hours. The samples were washed twice with Buffer B (0.05% SDS, 1% Triton-X-100, 20mM Tris pH 7.5, 2mM EDTA, 150mM NaCl), once with Buffer D (0.05% SDS, 1% Triton-X-100, 20mM Tris pH 7.5, 2mM EDTA, 500mM NaCl), once with Buffer 3 (250mM LiCl, 1% NP40, 10mM Tris pH 7.5, 1mM EDTA, 1% Sodium Deoxycholate), once with Buffer C (0.1% Triton-X-100, 20mM Tris pH 7.5, 2mM EDTA, 150mM NaCl), and once with TE plus 30mM NaCl. Protease inhibitor (Complete, Roche) was added to LB1, 2, 3 and all wash buffers. The bead-chromatin complexes were resuspended in 300 µl TE plus 30 mM NaCl and treated with 500 µg/ml RNase (Sigma) and 500 µg/ml Proteinase K (Sigma) for 30 minutes at 37°C. To reverse the crosslinking of DNA bound protein, the samples were incubated for 20 hours at 65°C. The DNA was recovered by phenol/chloroform extraction. Following primers were used in subsequent PCR reactions to amplify promoter regions of GATA4 and GATA6 containing the Ronin binding motif: (GATA4-f) 5'-GCA GAC CAG ATG CTG GAA GT-3' and (GATA4-r) 5'-TTT TCT CCG GTC CTG ATG TC-3'; (GATA6-f) 5'-GCC ACA CAC ACA CCC TTG T-3' and (GATA6)-R 5'-AAG GCA AGG CAT CCT GAC TA-3'. (Oct4-f) 5'-GGA TGG CAT ACT GTG GAC CT-3' and (Oct4-r) 5'-AGT TGC TTT CCA CTC GTG CT-3'; (Nanog-f) 5'-CCA GTG GAG TAT CCC AGC AT-3' and (Nanog-r) 5'-GAA GTT ATG GAG CGG AGC AG-3

Stat3 Western blot

For detection of STAT-3 and Phosho STAT-3, R1 and EF1a-Ronin cells (stably expressing Ronin) were plated at a density of 1.5x106 cells/10 cm dish in the presence of Lif, in the absence of Lif and in presence of 0.1 *m*g/ml *a*Lif (BD Biosciences). Cells were lysed after 24 hours in the presence of PhosStop (Roche) phosphatase inhibitor. Equal amounts of protein were analysed by Western blotting. The Stat3 antibody (BD Biosciences, #610189) was used in a 1:2500 dilution with the secondary anti-mouse-HRP antibody (Promega) diluted 1:2000, while Phospho-Stat3 antibody (Cell Signaling, #9131) was used in a 1:2000 dilution with the secondary anti-rabbit-HRP antibody (Abcam) diluted 1:2000.

Doxycycline induced Oct4 knockdown during transient expression of Ronin

ZBHTc4.1 cells (Niwa, 2000) were plated at a density of 80000 cells per 10 cm dish coated with gelatin. Six hours after plating the cells were transfected with 24 *m*g of a 1:10 mixture of pCMV-GFP-N1 (Clontech) and pEF1*a*-loxP-Ronin-Ires-Neo or the pEF1-C control vector (Invitrogen) using Lipofectamine2000 (Invitrogen) following the manufacturers instructions. 16 hours after transfection GFP positive cells were sorted and plated at 100000, 50000, 25000, 125000 and 6250 cells per 6 well. 20 hours after plating oct-4 knockdown was induced by treatment with 1 µg/ml doxycycline for 12 hours. Cells were washed twice with medium and then incubated for further 72 hours. LIF was included in the medium during the course of the experiment while no genetecin was added (the oct-4 transgene in ZBHTc4.1 is linked by an IRES to neomycin). Differentiation status was assessed after alkaline phosphatase staining as described.

Establishment of ZBHTc4 (EF1*a*) and ZBHTc4.1 (EF1*a*-Ronin) cell lines

To establish a ZBHTC4.1 cell line stably expressing Ronin, a EF1*a*-Ronin-IRES-Puro containing vector was constructed and linearized with SspI. ZBHTc4.1 (Niwa, 2000) cells were grown in the presence of 200 *m*g/ml genetecin and $2x10^7$ cells were electoporated with 40 *m*g of linearized plasmid with a 950 *m*F and 220 V pulse. Cells were incubated on ice for 10 minutes and plated on three 10 cm dishes in medium without antibiotics. Puromycin selection was started 24 hours after plating at a concentration of 2.5 *m*g/ml Puromycin, which was reduced to 1 *m*g/ml after 4 days. Puromycin resistant colonies were picked after 11 days and expanded. The corresponding control vector pEF1*a*-IRES-PURO was used to generate the control cell line in parallel. 200 *m*g/ml genetecin were included in the medium to maintain oct4 expression.

Doxycycline induced oct-4 knockdown under stable expression of Ronin

ZBHTc4 [EF1*a*] and ZBHTc4.1 [EF1*a*-Ronin] cells were plated at a density of 15.000 cells per 6 well. 24 h later, oct-4 knockdown was induced by addition of 1 µg/ml doxycycline for 8, 24 or 72 hours. LIF was included in the medium during the course of the experiment while genetecin was excluded (the oct-4 transgene in ZBHTc4.1 is linked by an IRES to neomycin). Differentiation status was assessed after alkaline phosphatase staining as described.

Cell Proliferation Assay

For the cell proliferation assay 2000 cells per 6 well were plated and Ronin siRNA knockdown was performed as described. 18 hours after transfection Ronin expression was assessed by qPCR as described. Cell proliferation was measured 1,2,3 and 4 days after siRNA treatment with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)(Promega) following the manufacturers protocol

Teratoma formation

To induce teratoma formation, 1×10^6 cells from each cell population (see Figure 6) were injected into the quadriceps muscle of the hind legs of immunocompromised Fox Chase SCID beige mice (The Jackson Laboratories). Teratomas were dissected after 16 days. A small piece of each tumor was used in PCR analyses to confirm genotypes. Half of the remaining tissue was fixed in 0.4% paraformaldehyde and embedded in optimal cutting temperature (OCT) medium (Sakura Finetek Inc) after incubating overnight in 30% sucrose; the remaining half was fixed in 10% Formalin overnight, transferred to 70% ethanol and paraffin embedded. Paraffin-embedded sections were analyzed by hematoxylin and eosin (H&E) staining. OCT-embedded tissue was used for X-Gal staining, as described in Supplementary Experimental Procedures.

Directional yeast two-hybrid screen for potential Ronin-interacting proteins

A total of 32 cDNA sequences corresponding to proteins identified by mass spectrometry were amplified by PCR from corresponding human cDNA clones (Open Biosystems). Sequence-specific oligonucleotides with attB overhangs were used, generating PCR products that were subsequently cloned into pDONR221 (Invitrogen) using the Gateway technology, according to the manufacturer's protocol (Invitrogen). Ronin, an N-terminal fragment corresponding to amino acids (aa) 1-103, and a C-terminal fragment corresponding to aa 132-315 were amplified from p*EF1-hRonin-FLAG* and introduced into pDONR221 using the same strategy. The specific primers used were: 1) Ronin, MAD110 (5'-GGG GAC AAC TTT GTA CAA AAA AGT TGG CAT GCC TGG CTT TAC GTG CTG CG -3') and MAD111 (5'-GGG GAC AAC TTT GTA CAA GAA AGT TGG TCA CAT TCC GTG CTT CTT GCG G-3'); 2) aa1-103, MAD110 and MAD207 (5'-GGG GAC AAC TTT GTAC AAGA AAGT TGGT TAC CTG CGG CGG GCG GCC GCG GCC CCA GC-3'); aa132-315, MAD208 (5'-GGG ACA ACT TTG TAC AAA AAA GTT GGC TCC TCA CCC TCT GCC TCC ACT GCC -3') and MAD111. The HCF-1 cDNA clone represents aa 1 - 429 and the Sin3a sequence corresponds to aa 1 - 123 of the mature protein; all others code for full-length proteins. cDNAs were subsequently shuttled into the yeast two-hybrid destination vectors, pDEST-DB and pDEST-AD. The resulting vectors expressed fusion proteins with an N-terminal activation domain or a DNA binding domain (reflecting the corresponding functional requirements of the directional screen) and screened using the Proquest yeast two-hybrid system (Invitrogen) following the manufacturer's protocol. Briefly, vectors expressing Ronin or the truncated versions of Ronin were co-transfected into the yeast strain, MAV103, with the corresponding vectors expressing the potential Ronin-interacting protein. Transfected yeast were then selected on -Trp-Leu plates and evaluated for growth phenotype, as described by the

manufacturer (Invitrogen). All potential interacting proteins were tested as activation-domain fusions and DNA-binding-domain fusions to overcome functional repression associated with either fusion context.

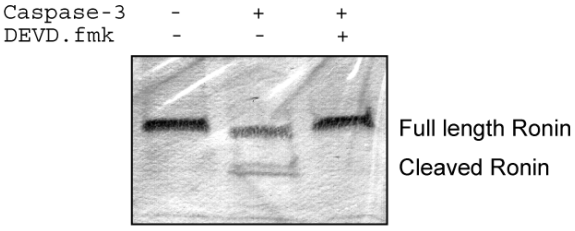
Immunoprecipitation with GST-bound agarose beads

cDNAs for Ronin, and N- and C-terminal Ronin deletion mutants (see above), were shuttled into the pMyc-DEST vector (a kind gift of Dr. Huda Zhogbi) and the pGST-DEST vector (Invitrogen) using the gateway technology to generate fusion proteins carrying an N-terminal Myc tag or GST domain, respectively. Co-transfection of HEK293 cells, GST-immunoprecipitation and detection of precipitated myc-tagged proteins were performed essentially as described by Zhogbi et al. Briefly, HEK293 cells were plated at 1 x 10^6 cells/well in a six well plate and were co-transfected after 24 hours with expression vectors for two potential interaction partners (one in pMyc vector, the other in pGST vector; 0.8 *m*g each). Forty-eight hours later, cells were lysed in lysis buffer (20 mM Tris pH 8, 180 mM NaCl, 1 mM EDTA, 0.5% NP-40, Protease Inhibitor Complete (Roche)) and cell lysates were immunoprecipitated using glutathione sepharose 4B (GE Healthcare). After washing, beads were resuspended in 12.5 *m*l SDS loading buffer, heated at 70ºC for 10 minutes, separated on 4-15% Tris-HCl gradient gels (Biorad) and analyzed by Western blotting using standard procedures. The following antibodies (dilutions) were used: rabbit anti-glutathion-S-transferase (Sigma, 1:2000), mouse anti-c-myc (Sigma, clone 9E10, 1:2000), HRP-conjugated sheep anti-mouse IgG (GE Healthcare, 1:4000) and HRP-conjugated donkey anti-rabbit IgG (GE Healthcare, 1:10000). Signals were developed using the ECL system (GE Healthcare) following the manufacturer's protocol. An aliquot of the cell lysate (input) was tested to verify expression of the corresponding GST and myc proteins.

Figure S1.

Ronin is a conserved protein containing a THAP domain and is cleaved by Caspase-3. (A) In vitro Caspase-3 cleavage assay. Recombinant Ronin was exposed to recombinant Caspase-3 alone or in combination with the Caspase blocker DEVD.fmk. The reduction of the molecular size of the full length Ronin and the appearance of an additional smaller band are indicative for Caspase-3 mediated cleavage of Ronin. (B) Sequence alignment of Ronin shows that is has an exceptionally conserved N- and C-terminus, even among more distant species. (C) Phylogenetic diagram documenting a close clustering of Ronin among mammals and the presence of Ronin throughout the animal kingdom. (D) Gel-mobility shift experiments with ohter oligonucleotides than *3x* were not able to abolish the gel shift.

A



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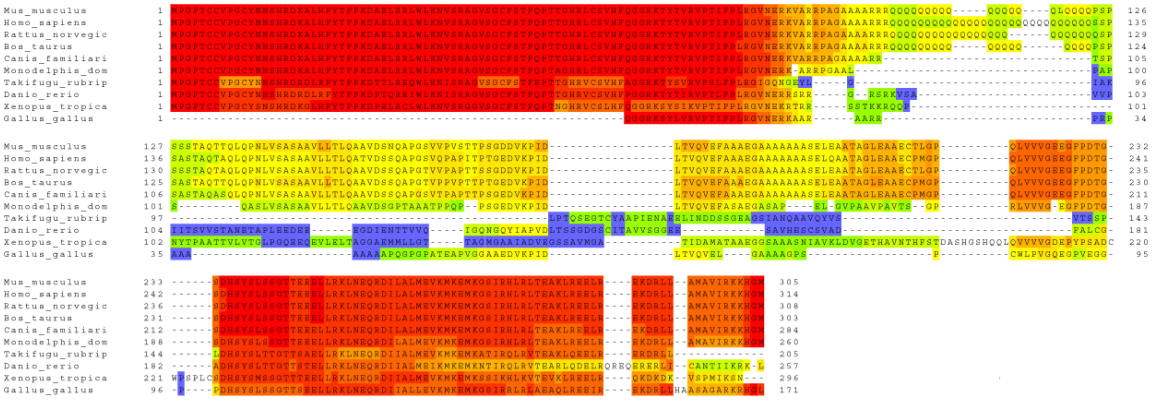

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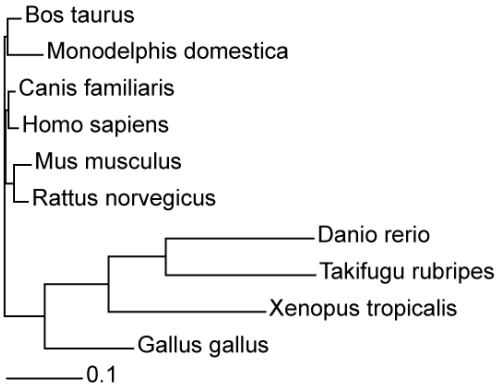

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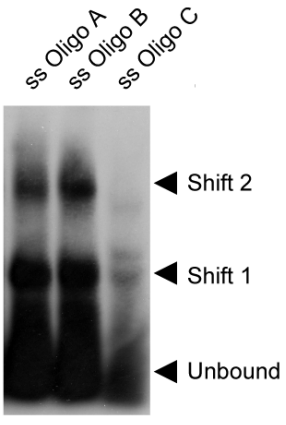
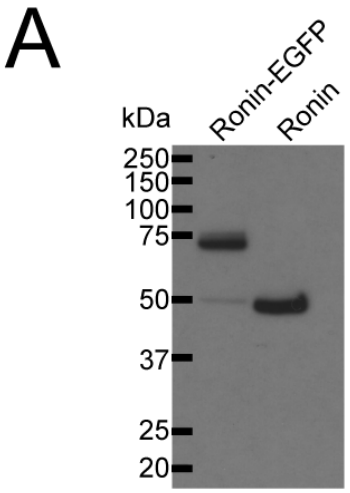
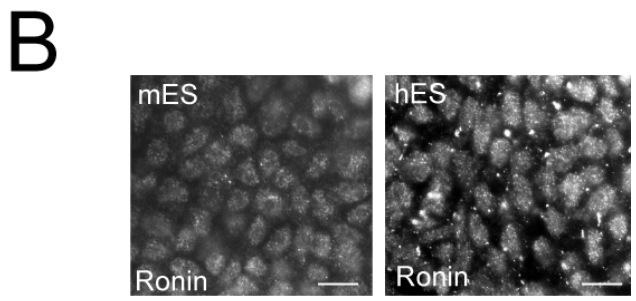


Suppl. Figure 1 *Dejosez et al.*

Figure S2.

Ronin antibody is specific for Ronin. (A) HEK293 cells were transfected with a FLAG-tagged Ronin-EGFP fusion protein and with a FLAG-tagged Ronin construct. The Ronin antiserum was used at a dilution of 1:20,000 and resulted in a very specific band that in both cases coincides with the band that was detected in an independent experiment with a FLAG antibody. (B) Immunostaining of endogenous Ronin in mouse and human ES cells (bar = 10 μm).

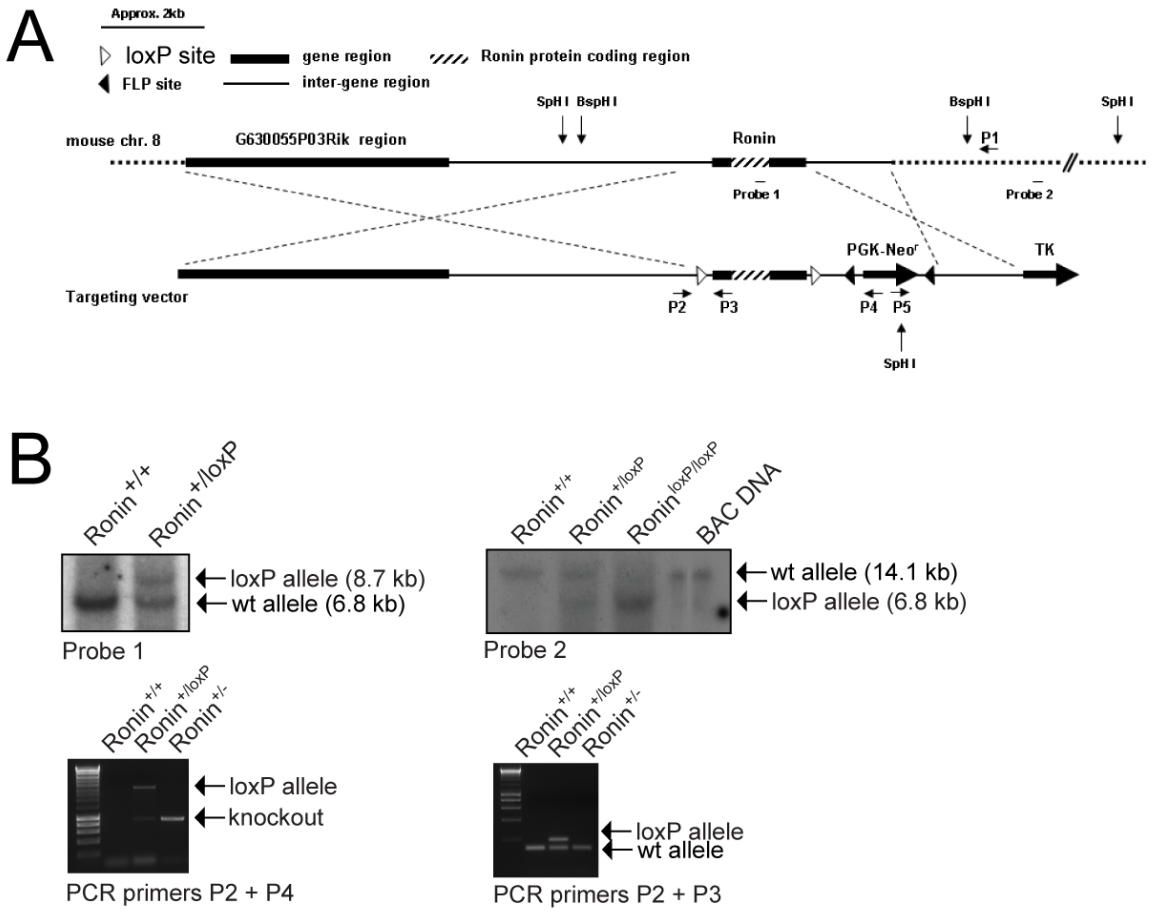




Supp. Figure 2 *Dejosez et al.*

Figure S3.

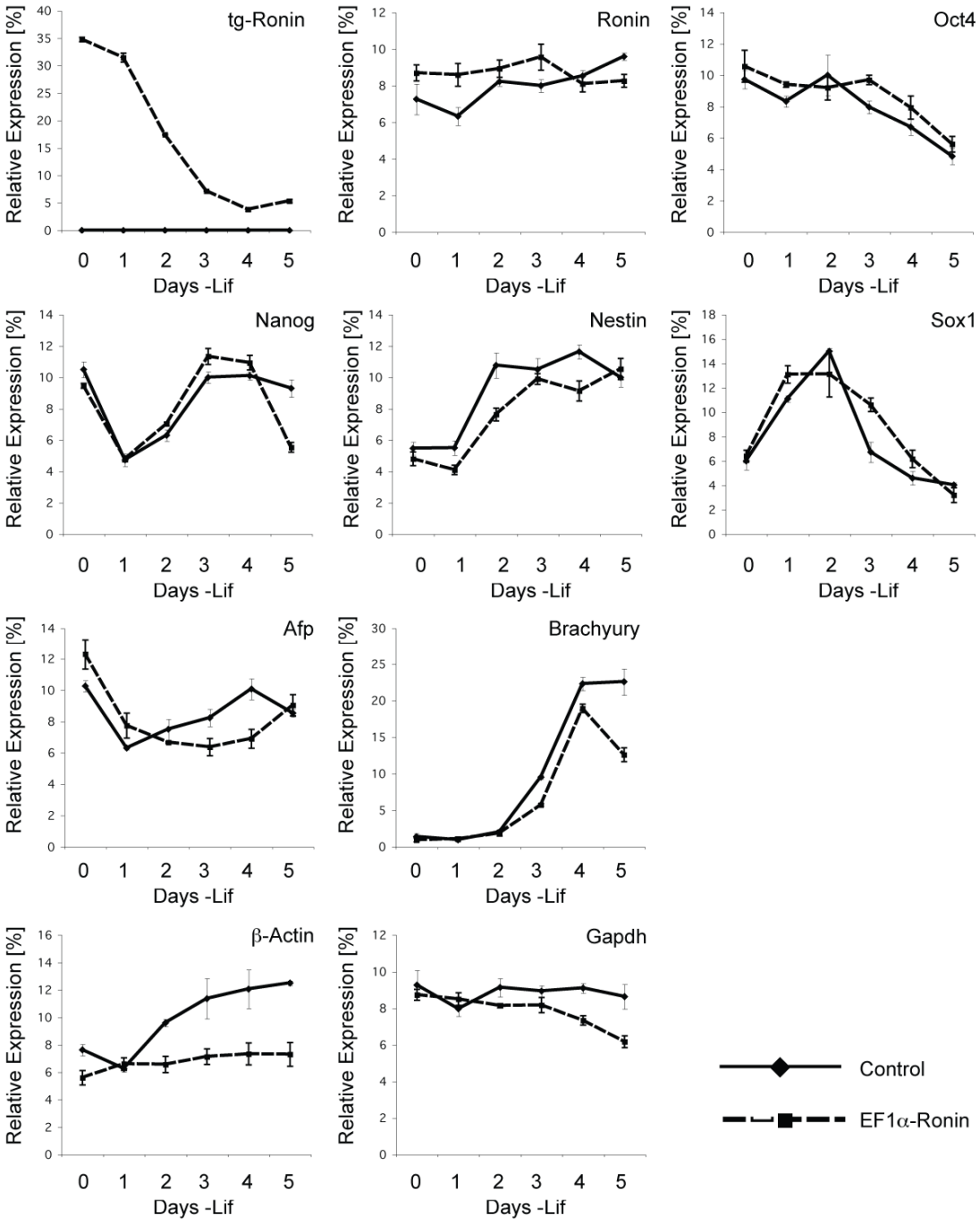
Knockout of Ronin is periimplantational lethal. (A) Illustration of the gene targeting strategy to create a conditional knockout mouse model. *loxP* flanked *Ronin* is located between two regions of low homology. (B) Southern blot (upper panel) and PCR (lower panel) - based genotyping. Correct genotype of the conditional knockout animals was confirmed by Southern blotting, using an inside probe (1) and an outside probe (2) as well as PCR with two oligo combinations, P2/P4 and P2/P3. (C) Crystal violet staining of *Cre/GFP* transfected R1 ES cells after sorting of GFP positive cells. Viability of Cre/GFP cotransfected Ronin$^{flox flox}$ ES cells is significantly reduced after plating (right) compared to control/GPF transfected cells (left).



Supp. Figure 3 *Dejosez et al.*

Figure S4.

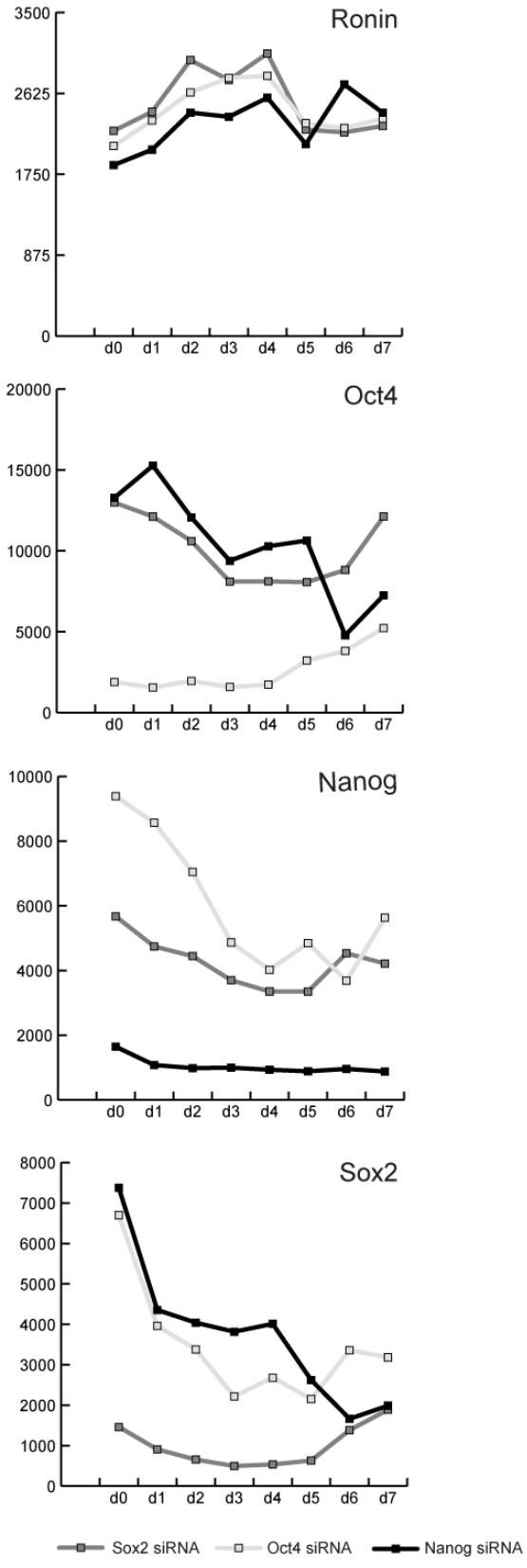
Quantification of RT-PCRs for house keeping genes in control ES cells and EF1a-Ronin ES cells. The relative (%) value is the cumulative intensity of individual bands in comparison to the entire gel image analyzed. Three independent experiments were analyzed.



Suppl. Figure 4 *Dejosez et al.*

Figure S5.

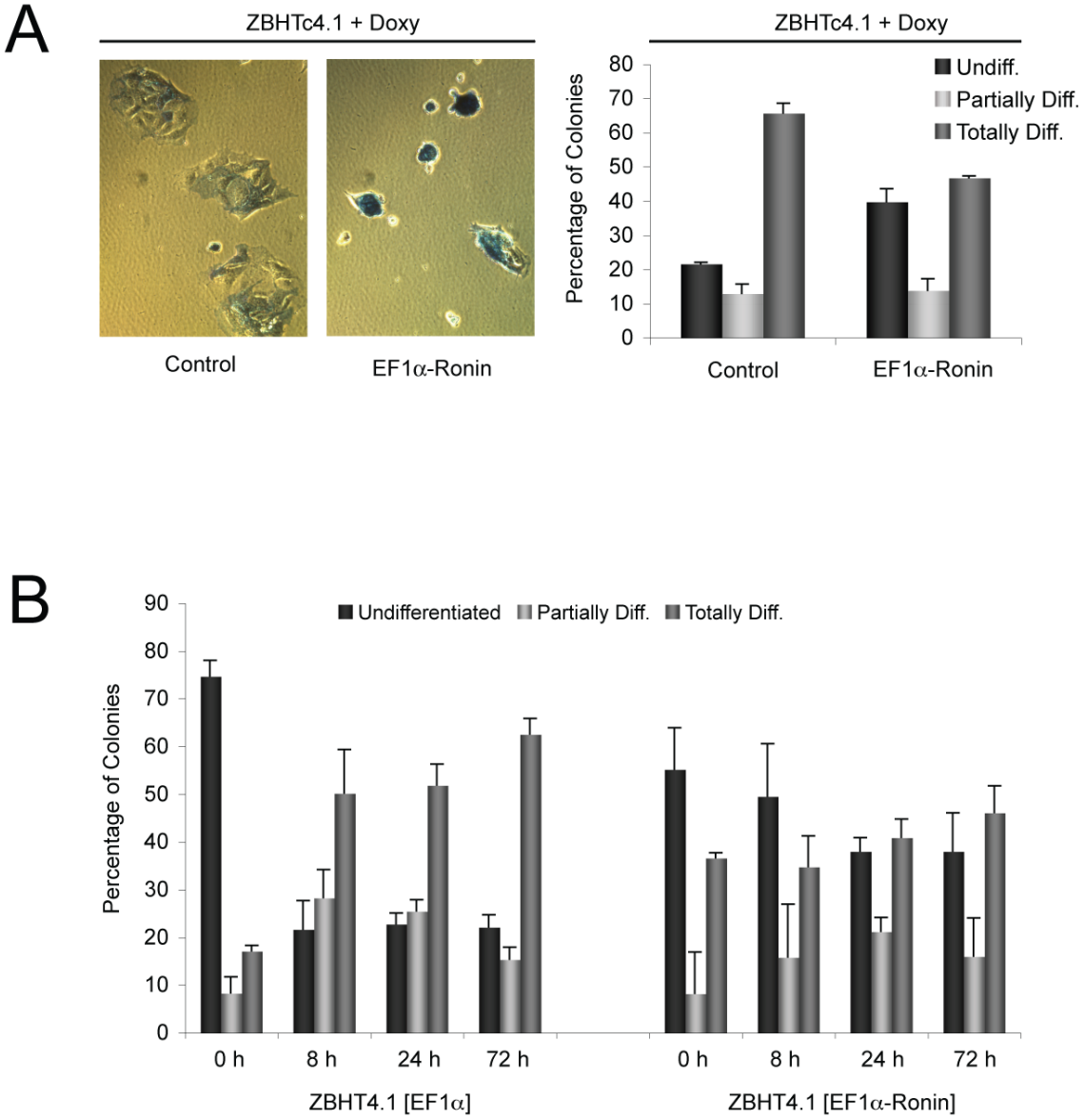
Microarray analysis of *Ronin*, *Oct4*, *Nanog* and *Sox2* expression after shRNA knockdown of *Oct4*, *Nanog* and *Sox2*. The data were restored from Ivanova et al. (Ivanova et al. 2005). Ronin was not affected by knockdown of all factors, while *Oct4*, *Nanog* and *Sox2* were downregulated.



Supp. Figure 5 *Dejosez et al.*

Figure S6.

(A) Transient transfected ZBHTC4.1 cells with EF1*a*-C (control) or pEF1*a*-Ronin and induction of Oct4 knockdown by 1 μg/ml doxycyclin for 12h after sorting of positively transfected ES cells. (B) Quantification of experiment described in Figure 4 after induction of Oct4 knockdown for 8, 24 and 72 h in ZBHTc4 [EF1*a*] control or [EF1*a*-Ronin] stably expressing cells.



Suppl. Figure 6 *Dejosez et al.*

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