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CAF-1 Is Essential for Heterochromatin Organization in Pluripotent Embryonic Cells

Martin Houlard¹,²,²*, Soizik Berlivet¹, Aline V. Probst², Jean-Pierre Quivy², Patrick Héré¹, Geneviève Almouzni², Matthieu Gérard¹

Introduction

During mammalian development, chromatin dynamics and epigenetic marking are important for genome reprogramming. Recent data suggest an important role for the chromatin assembly machinery in this process. To analyze the role of chromatin assembly factor 1 (CAF-1) during pre-implantation development, we generated a mouse line carrying a targeted mutation in the gene encoding its large subunit, p150CAF-1. Loss of p150CAF-1 leads to developmental arrest at the 16-cell stage. Absence of p150CAF-1 in these embryos results in severe alterations in the nuclear organization of constitutive heterochromatin. We provide evidence that in wild-type embryos, heterochromatin domains are extensively reorganized between the two-cell and blastocyst stages. In p150CAF-1 mutant 16-cell stage embryos, the altered organization of heterochromatin displays similarities to the structure of heterochromatin in two- to four-cell stage wild-type embryos, suggesting that CAF-1 is required for the maturation of heterochromatin during preimplantation development. In embryonic stem cells, depletion of p150CAF-1 using RNA interference results in the mislocalization, loss of clustering, and decondensation of pericentric heterochromatin domains. Furthermore, loss of CAF-1 in these cells results in the alteration of epigenetic histone methylation marks at the level of pericentric heterochromatin. These alternations of heterochromatin are not found in p150CAF-1-depleted mouse embryonic fibroblasts, which are cells that are already lineage committed, suggesting that CAF-1 is specifically required for heterochromatin organization in pluripotent embryonic cells. Our findings underline the role of the chromatin assembly machinery in controlling the spatial organization and epigenetic marking of the genome in early embryos and embryonic stem cells.
Synopsis

Chromatin is the support of our genetic information. It is composed of numerous repeated units called nucleosomes, in which DNA wraps around a core of histone proteins. Modifications in the composition and biochemical properties of nucleosomes play major roles in the regulation of genome function. Such modifications are termed “epigenetic” when they are inherited across cell divisions and convey new information to chromatin, in addition to the genetic information provided by DNA. It is usually believed that during genome replication, the basic chromatin assembly machinery builds up “naive” nucleosomes, and in a subsequent step, nucleosomes are selectively modified by a series of enzymes to acquire epigenetic information. Here, the authors studied the role of a basic chromatin assembly factor (CAF-1) in mouse embryonic stem cells and early embryos. Surprisingly, they show that CAF-1 confers epigenetic information to specific genomic regions. In addition, this study revealed that CAF-1 is required for the correct spatial organization of chromosomes in the nucleus. This new knowledge may contribute to better understanding the role of chromatin in the maintenance of embryonic stem cell identity and plasticity.

Results

We used gene targeting in ES cells to delete exon 3 in the Chaf1a gene, which encodes p150CAF-1 (Figure 1A). Chaf1a+/− mice were born at a Mendelian frequency, were of normal size and weight, displayed no obvious abnormalities, and were fertile. Crossing heterozygous mice failed to generate viable newborn Chaf1a−/− mice. Furthermore, no homozygous Chaf1a−/− embryos were detected at any of the post-implantation stages. However, we could detect Chaf1a−/− embryos at embryonic day 4 (E4) using a PCR strategy (Figure 1B). They represent only 10% of E4 embryos obtained from Chaf1a+/− mice intercrosses, suggesting that more than half of the homozygous mutant embryos had degenerated before this stage. Moreover, mutant embryos contained only eight to 16 cells at the E4 stage, instead of 32 cells observed for wild-type or heterozygous blastocysts (Figure 1C and 1D). Further analysis by light microscopy using immunofluorescence (IF) allowed us to compare wild-type and p150CAF-1-depleted embryos. A loss of p150CAF-1 staining could not be detected before the eight-cell stage (unpublished data) suggesting that maternally contributed protein is present in the embryo up to the four-cell stage. Thus, depletion of p150CAF-1 protein from this stage allows a maximum of two additional cell divisions before developmental arrest.

Strikingly, we found that the nuclear organization of heterochromatin appeared abnormal in the nuclei of Chaf1a−/− embryos. Pericentric heterochromatin, the major component of constitutive heterochromatin, can easily be visualized in interphase nuclei by the fluorochrome DAPI and by immunostaining with HP1α [16,17–19]. In wild-type blastocysts and in mouse somatic cells, pericentric heterochromatin domains cluster together and form higher-order chromatin structures called chromocenters [20], which are visualized as DAPI-dense foci (Figure 1C). These structures were not detected in Chaf1a−/− embryos (Figure 1D). Since chromatin architecture and higher-order structures are important for genome function [21], we decided to characterize this phenotype in more detail. For this purpose, we examined the status of heterochromatin organization between the two-cell and blastocyst stages. In two-cell stage wild-type embryos, DAPI staining is diffuse and fibrillar, with regions of higher density around the nucleolar precursor bodies (Figure 1E). Heterochromatin domains are progressively assembled into DAPI-dense foci between the four-cell and 32-cell blastocyst stages (Figure 1F). Localization of HP1α confirmed that the structures visualized by DAPI staining correspond to constitutive heterochromatin (Figure 1E). Thus, the nuclear organization of heterochromatin is dramatically modified during pre-implantation development, between the two-cell and blastocyst stages. In Chaf1a−/− E4 embryos, which are arrested between the eight- and 16-cell stage, DAPI-dense foci were barely detectable (Figure 1D). Instead, DAPI staining was diffuse within the nuclei, with regions of higher density around the nucleoli and at the periphery of the nuclei (Figure 1D). Localization of HP1α showed a diffuse pattern similar to the DAPI, with some enrichment at the nuclear periphery, and around the nucleoli (Figure 1D). This abnormal organization of heterochromatin in Chaf1a−/− embryos is reminiscent of the heterochromatin organization found in two- to four-cell stage wild-type embryos (compare Figure 1D and 1F). These data evidence a key role for p150CAF-1 during pre-implantation development, and reveal that this protein is required for the proper 3-D organization of heterochromatin within embryonic cell nuclei.

Next, we wondered whether a similar requirement for CAF-1 could also be observed in ES cells, which are derived from the blastocyst inner cell mass. Given the early developmental arrest observed in Chaf1a−/− embryos, such cells could not be derived directly from null embryos, and we thus used an RNAi strategy (Figure 2A). p150CAF-1 knockdown in ES cells was quantified by Western blot analysis and IF. (Figure S1). p150CAF-1 RNAi-depleted ES cells displayed a phenotype very similar to the cells of Chaf1a−/− E4 mice. DAPI-dense foci were lost, and we observed diffuse HP1α and DAPI staining around the nucleoli and at the periphery of the nucleus (Figure 2B). This result indicates that p150CAF-1 is essential for nuclear organization of heterochromatin in ES cells in a similar way to early pre-implantation embryos. Surprisingly, we did not observe this severe alteration in heterochromatin organization in primary mouse embryonic fibroblasts (MEFs) following p150CAF-1 depletion by RNAi. p150CAF-1 depletion in MEFs resulted in a strong inhibition of cell proliferation (unpublished data), but did not alter the clustering of heterochromatin domains (Figures 2C and S2). Similarly, p150CAF-1 depletion in 3T3 cells did not affect heterochromatin organization [16]. These observations reveal a specific function for CAF-1 in the early embryo and ES cells. We also wondered whether loss of p150CAF-1 in ES cells specifically affects the heterochromatin nuclear subcompartment, or whether it might result in a more global loss of
nuclear organization and architecture. We used a specific antibody against the promyelocytic leukemia (PML) protein to study the fate of the PML nuclear bodies, which are well-characterized subnuclear structures [22]. We could not detect any significant difference in the distribution and aspect of PML nuclear bodies between control and p150CAF-1-depleted ES cells (Figure 2D). These data show that nuclear architecture is not globally altered following CAF-1 loss-of-function. In addition, Western blot analysis revealed that CAF-1 depletion did not result in altered levels of chromatin architectural proteins such as histone H3 and HP1α (Figure S1). Thus, CAF-1 is specifically required in early embryos and in ES cells for the proper organization of the heterochromatin subnuclear compartment.

Figure 2. Depletion of p150CAF-1 in ES Cells Results in a Severe Alteration of Heterochromatin Organization
(A) Strategy used to deplete p150CAF-1 by RNAi in ES cells. The siRNA expression vector includes a puromycin selection cassette (Puro), the mouse H1 promoter, and the siRNA encoding sequence. ES cells were kept under puromycin selection during 48 h following transfection.
(B) Abnormal heterochromatin organization in p150CAF-1-depleted ES cells. Immunodetection of p150CAF-1 (green) and HP1α (red) in ES cells transfected with control (cont) and p150CAF-1 siRNA. siRNA expression results in efficient p150CAF-1 depletion. The right-hand image shows the merge between HP1α fluorescence and DAPI-stained DNA in blue. Pink color indicates the association of HP1 with DAPI-dense material. The bottom of each panel shows the magnification of a nucleus selected from the above embryo (white square). The arrowhead indicates the typical heterochromatin foci revealed by DAPI and HP1α staining in wild-type embryos. These foci are not visible in p150CAF-1-depleted embryos. DAPI and HP1α staining are diffuse within the nucleus of p150CAF-1-depleted embryos, with enrichment at the nuclear periphery, revealing abnormal heterochromatin organization. Scale bars represent 10 μm.
(C) Wild-type embryos isolated at embryonic day 2 (E2, two cells), E2.5 (four cells), E3 (eight cells), E3.5 (16 cells), and E4 (32 cells) are shown. Heterochromatin was monitored by DAPI staining (upper panel) and HP1immunolabeling (in red, lower panel).
(D) Magnification of a nucleus (DAPI-stained) representative of each stage. The right-hand panel shows the nucleus of an ES cell. Scale bar represents 10 μm.

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Previous studies performed in mammalian cell lines showed that CAF-1 activity is required for S phase progression [16,23,24]. We show here that 3 d after transfection of the RNAi vector, p150CAF-1-depleted cells (identified by a complete absence of CAF-1 IF signal) appear still active for replication, as revealed by incorporation of the thymidine analog bromodeoxyuridine (BrdU) (Figure 3A), the PCNA (proliferating cell nuclear antigen) pattern (Figure 3B), and flow cytometry analysis (Figure 3C). Hence, in these cells, DNA replication persists despite the drastic changes in heterochromatin organization. However, 24 h later, ES cells cease to proliferate and die, revealing that p150CAF-1 is required for an essential cellular process, as in embryos. Altogether, our data demonstrate that loss of p150CAF-1 function in ES cells and early embryos alters first the organization of heterochromatin in the nucleus and, in a subsequent step, cell cycle and viability.

In order to better characterize the defects in heterochromatin organization, we performed immunofluorescence staining for BrdU and PCNA in control and p150CAF-1-depleted ES cells. Figure 3A shows representative images of BrdU and p150CAF-1 localization, with a merged view in Figure 3B. Figure 3C presents a flow cytometry analysis of cell cycle distribution in control and p150CAF-1-depleted ES cells. These results indicate that loss of p150CAF-1 function affects the organization of heterochromatin and cell cycle progression in ES cells.

Figure 3. p150CAF-1 Depletion and Loss of Heterochromatin Organization Are Compatible with Active DNA Replication in ES Cells
(A) ES cells were transfected with p150CAF-1 or control siRNA vectors. After 3 d under puromycin selection, cells were pulse-labeled for 10 min with BrdU and immediately analyzed by IF. No significant difference in BrdU incorporation could be detected between control and p150CAF-1-depleted cells. The right-hand image shows the merge between the p150CAF-1 and BrdU fluorescence. Scale bar = 10 μm.
(B) Immunodetection of PCNA (red) revealed no significant difference in the formation of replication foci between control and p150CAF-1-depleted ES cells. Immunodetection of p150CAF-1 is shown in green and the merging of p150CAF-1 and PCNA appears in yellow.
(C) Flow cytometry analysis showed a similar cell-cycle profile for control and p150CAF-1-depleted ES cells. Results are presented as the percentage of cells in each phase of the cell cycle (G1, S, G2/M), as defined by BrdU incorporation and DNA content. Data presented are the mean of three independent experiments; error bars indicate the standard deviation. All experiments were performed 3 d after transfection of the siRNA vectors.

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matin organization, we performed two-color DNA fluorescence in situ hybridization (FISH) experiments to reveal the spatial distribution of pericentric and centric chromosomal regions, which in mouse are mainly composed of large blocks of major and minor satellite repeats, respectively [20]. Pericentric domains from different chromosomes form clusters, which are revealed by FISH as large spots that coincide with DAPI-dense foci in the interphase nucleus (Figure 4A). At the periphery of each pericentric domain, centric regions form individual entities (Figure 4A). In p150CAF-1 RNAi-depleted cells, we observed a disruption of pericentric heterochromatin clusters that coincides with the disappearance of DAPI-dense foci (Figure 4B). Individual pericentric domains from single chromosomes are now found either isolated or aggregated in a pattern less dense than the regular clusters observed in control cells (Figure 4B). Such aggregates, which are often found at the nuclear periphery, are also revealed by DAPI staining as a typical signature of p150CAF-1 loss-of-function. Quantification of fluorescence along a line randomly drawn across the nucleus revealed lower fluorescence intensity and a broader distribution of DAPI and major satellite hybridization signals in p150CAF-1-depleted cells (Figure 4C and 4D), indicating decondensation of pericentric heterochromatin domains. In contrast, centric domains showed an intensity and shape similar to control cells, suggesting that they remain unaffected by p150CAF-1 depletion (Figure 4C and 4D). In conclusion, these results show that p150CAF-1 is required for the proper condensation and clustering of pericentric heterochromatin domains.

Given the known role of CAF-1 in the deposition of histone H3.1 and H4 associated with DNA synthesis [11,12], we wondered whether this defect in higher-order chromatin organization could reflect an aberrant nucleosomal organization. Using DNase I and micrococcal nuclease (MNase) assays, we could not observe any significant difference between control and p150CAF-1-depleted ES cells at the level of bulk genome chromatin or at pericentric repeats (Figure 5). In a second series of experiments, we compared the association of histone H3 with chromatin in nuclei isolated from cells transfected with control and p150CAF-1 RNAi plasmid vectors. Nuclei were incubated in buffers with...
different salt concentrations ranging from 100 mM NaCl to 1 M NaCl. In all conditions, the amount of histone H3 remaining associated with chromatin was indistinguishable in control and p150CAF-1-depleted ES cells (unpublished data). Therefore, the loss of clustering and decondensation of pericentric heterochromatin are unlikely to be the consequence of severe defects in nucleosomal organization.

Our findings showed that the nuclear localization of HP1α is severely altered in Chaf1α−/− embryos and p150CAF-1-depleted ES cells. We therefore examined the status of other epigenetic marks previously shown to characterize pericentric heterochromatin. We first tested DNA CpG methylation [25] at major satellite repeats and found no significant difference between control and p150CAF-1-depleted cells (Figure 6). As the LTM7 ES cell line that we used in our RNAi experiments is a female (XX) cell line, DNA methylation is globally reduced in these cells [26]. To rule out the possibility that hypomethylation of pericentric repeats might contribute to the destabilization of heterochromatin domains in p150CAF-1-depleted ES cells, we tested our RNAi plasmid vector in a XY ES cell line, and confirmed that loss of p150CAF-1 function mainly affects H4K20me3 at pericentric heterochromatin. Moreover, no significant difference in immunoprecipitation efficiency was detected at intracisternal A particle (IAP) elements (Figure 7B), which are noncentromeric repeated DNA elements also enriched in H4K20me3 [31]. This result shows that p150CAF-1 contributes to normal levels of H4K20me3 at pericentric heterochromatin, but not necessarily at other loci. The H3K9me3 mark [32] was also significantly reduced in p150CAF-1-depleted cells, though to a lesser extent than H4K20me3 (Figure 7A and 7B).

In wild-type cells, most of H4K20me3 and H3K9me3 signals are present at the level of DAPI-dense domains when visualized by IF [29] (Figures 7C and 7D). H3K9me3 was severely perturbed in p150CAF-1-depleted cells, displaying a diffuse pattern similar to HP1α (Figure 7C). The H4K20me3 pattern was even more severely altered, showing both a loss of the typical dots revealed in control cells and a reduction in signal intensity (Figure 7D). Consistent with our ChIP experiments, residual H4K20me3 epitopes were detected at the level of relocated DAPI-dense material. Altogether, our results show that depletion of CAF-1 in ES cells leads to a simultaneous disruption of heterochromatin 3-D organization and an alteration in epigenetic marking.

Discussion

Here, we provide evidence that p150CAF-1 has a crucial function during mouse early embryogenesis and in ES cells.
Previous studies showed that CAF-1 is essential for the progression of DNA replication in vertebrates. In *Xenopus laevis*, p150CAF-1 function is required for the rapid cell divisions that occur during early development [33]. Similarly, CAF-1 is essential in mammalian cell lines for the progression of DNA replication [16,23,24,34]. Our findings reveal a role for CAF-1 in the nuclear organization of heterochromatin domains during early development and in ES cells. We show that in p150CAF-1-depleted ES cells, while DNA replication initially persists without significant perturbation in nucleosomal organization, severe heterochromatin organization defects can be observed. Loss of p150CAF-1 did not affect PML bodies in ES cell nuclei, showing that nuclear architecture is not generally altered. Ultimately, p150CAF-1 depletion results in the arrest of ES cell division and cell death. Given the major defects observed in heterochromatin organization, it is tempting to speculate that these defects cause the developmental arrest in the embryos and death in ES cells. The role of CAF-1 in the nuclear organization of heterochromatin includes spatial localization, condensation, and clustering of pericentric domains. This important function of CAF-1 was not revealed in primary MEFs, 3T3 cells, or human cell lines, suggesting that it is specific to pluripotent embryonic cells. Alternatively, early embryos and ES cells, whose genome is more plastic than somatic cells, might represent a particularly sensitive context in which the importance of CAF-1 in heterochromatin organization is exacerbated. In ES cells, major architectural chromatin proteins are hyperdynamic and bind relatively loosely to chromatin [35]. In this cellular context, our findings show that proper heterochromatin nuclear architecture relies on CAF-1 function. In contrast, in undifferentiated cells that are already lineage committed (such as MEFs) genome architecture might be more stable than in ES cells and thus cannot be disrupted by CAF-1 depletion. This important function of CAF-1 in pluripotent embryonic cells could be partially conserved during evolution. Indeed, in *Arabidopsis thaliana*, in which CAF-1 is not essential for viability [36], loss of CAF-1 is associated with a mild alteration in chromocenter size [37]. This phenotype is somehow reminiscent of loss of CAF-1-mediated defects in heterochromatin organization in early mouse embryos. In mouse ES cells, we show that CAF-1 is also required for regular levels of H4K20me3 at pericentric repeats, but not at IAP elements, which are repeated sequences spread all over the mouse genome. These data suggest that proper nuclear organization and epigenetic marking of pericentric heterochromatin are coupled in ES cells. Alternatively, 3-D organization of heterochromatin and epigenetic marking might represent two independent functions of CAF-1.

The variable requirement for CAF-1 across species might reflect the acquisition of new properties affecting the function of histone H3 variants during evolution of eukaryotes. CAF-1 is essential in mammals but dispensable in *Saccharomyces cerevisiae* [38]. Interestingly, the budding yeast genome does not contain heterochromatin regions equivalent to those of higher eukaryotes, and several hallmark features of heterochromatin such as di- and trimethylation at H3K9, H3K27, and H4K20 are not observed in the silent chromatin of *S. cerevisiae*. In mammals, H3K9 dimethylation is predominantly associated with H3.1, but not with H3.3, which carries modifications typically found in transcribed chromatin regions [39]. Outside of the centromeric H3 variant, *S. cerevisiae* possesses only H3.3, which is expressed and incorporated into chromatin in a replication-independent fashion in higher eukaryotes [12]. These data suggest a specific role for H3.3 in heterochromatin regions. We show here that CAF-1, which is a H3.1 replication-dependent loading complex [11], is specifically required for the nuclear organization of pericentric heterochromatin in embryos and ES cells. One possibility would be a scenario in which incorporation of H3.1 into chromatin is required for the proper nuclear organization of constitutive heterochromatin in an embryonic context. While this has to be addressed experimentally, it is worthwhile to mention an alternate possible mechanism that might explain the disruption of heterochromatin domains in p150CAF-1 null embryos and ES cells. CAF-1 could be required for the loading, into heterochromatin, of an interacting partner required for the clustering of pericentric domains. Following one or two rounds of DNA replication in the absence of p150CAF-1, heterochromatin would become deprived of this molecule, which would cause the disruption of its 3-D organization. A potential candidate is the HP1 protein, which interacts with CAF-1 [13,16].

In agreement with a recent report [40], our analysis of heterochromatin in pre-implantation embryos has revealed drastic changes in nuclear organization between the two-cell and blastocyst stages. Cloning experiments in mouse revealed that the nuclear organization of heterochromatin in ES cell nuclei was quickly reverted into the one-cell stage-specific form after nuclear transfer [40]. Hence, remodeling of heterochromatin 3-D organization parallels nuclear reprogr-
ramming toward an early embryonic status in cloned embryos. This drastic reorganization of heterochromatin organization is similar to that observed in p150CAF-1-depleted ES cells, and in developmentally arrested 16-cell stage Chaf1a−/−/embryos. These data show that CAF-1 is involved in setting up proper heterochromatin architecture during the first cell divisions of embryonic life. Given the importance of nuclear organization in the control of gene expression [21,41], this finding suggests that CAF-1 contributes to the coordinated programs of gene expression during early embryogenesis. Our findings open new perspectives in the understanding of chromatin dynamics during early development and in pluripotent embryonic cells. While most studies focused on the role of DNA methylation and histone
modifying enzymes [3], our data point to the importance of the chromatin assembly machinery. We show that in addition to assembling nucleosomes, CAF-1 provides spatial and epigenetic information to heterochromatin domains in early embryos and ES cells.

**Materials and Methods**

**Generation of Chaf1a mutant mice and embryos.** Using PCR, we amplified two genomic fragments (about 3 kb each) flanking Chaf1a exon 3. These DNA fragments were assembled by conventional cloning with the neomycin and DT (diptheria toxin) cassettes, as described in Figure 1A. The construct was transfected in ES cells by electroporation. We identified recombinant ES cells carrying the mutant allele by recombination. PCR reactions were run onto an agarose gel, which revealed the presence of the wild-type (150 bp) and recombinant (200 bp) alleles.

**P150CAF-1 depletion by RNAi.** The RNAi plasmid vector that we used in this study contains the mouse H1 promoter and a puromycin selection gene (SB and MG, unpublished data). We characterized in detail the properties of this new vector using a GFP target gene and FACS. Extraction of target genes in ES cells is highly efficient in 70% of the cells, of intermediate efficiency in 15%, and inefficient in the remaining 15% of cells analysis. The sequence of p150CAF-1 siRNA (short interfering RNA) duplex [16] was cloned into this plasmid. The control vector expressed a siRNA that targets GFP RNA degradation. (short interfering RNA) duplex [16] was cloned into this plasmid. The used in this study contains the mouse H1 promoter and a puromycin

**ChIP.** We prepared native chromatin fragments of two to six nucleosomes in length as described [28]. 5 μg of chromatin were incubated overnight with 10 μl of commercial antibodies. After incubation with protein-G sepharose and three washes, immunoprecipitated DNA was purified on an agarose gel, and analyzed by Southern blot. Hybridization signals were quantified using an Instant Imager (PerkinElmer, Wellesley, California, United States). Antibodies specific for H4K20me3 were purchased from Abcam. Antibodies for H3K9me3 were purchased from both Abcam and Upstate Biotechnology and gave similar results.

**Supporting Information**

**Figure S1.** Analysis of p150CAF-1 Depletion by RNAi with Western Blot and IF

(A) Western blot analysis of ES cells transfected with control (cont) or p150CAF-1 (150) RNAi plasmid vectors. β-actin was used as a loading control. Amount of cells used in each lane is indicated. p150CAF-1 Knockdown does not significantly affect HP1α, histone H3, or H3K9me3 levels.

(B) IF analysis. ES cells transfected with control (cont) or p150CAF-1 (150) siRNA vector were immunostained with p150CAF-1 antibody (green) and DAPI (blue). Scale bar = 5 μm. Fluorescence was quantified along a line drawn across the nucleus and data were plotted, revealing efficient p150CAF-1 depletion by RNAi.

The apparent difference in the efficiency of CAF-1 knockdown revealed by Western blot and IF likely reflects the observation that the RNAi plasmid vector used in this study is efficient in 85% of the transfected ES cell population, whereas 15% of cells display no inhibition of the target gene (see Materials and Methods).

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Author contributions. MH, SB, AVP, JPQ, GA, and MG conceived and designed the experiments. MH, SB, AVP, and PH performed the experiments. MH, SB, AVP, and JPQ analyzed the data. MG wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.


