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RESEARCH COMMUNICATION

Ronin/Hcf-1 binds to a hyperconserved enhancer element and regulates genes involved in the growth of embryonic stem cells

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Self-renewing embryonic stem (ES) cells have an exceptional need for timely biomass production, yet the transcriptional control mechanisms responsible for meeting this requirement are largely unknown. We report here that Ronin (Thap11), which is essential for the self-renewal of ES cells, binds with its transcriptional coregulator, Hcf-1, to a highly conserved enhancer element that previously lacked a recognized binding factor. The subset of genes bound by Ronin/Hcf-1 function primarily in transcription initiation, mRNA splicing, and cell metabolism; genes involved in cell signaling and cell development are conspicuously underrepresented in this target gene repertoire. Although Ronin/Hcf-1 represses the expression of some target genes, its activity at promoter sites more often leads to the up-regulation of genes essential to protein biosynthesis and energy production. We propose that Ronin/Hcf-1 controls a genetic program that contributes to the unimpeded growth of ES cells.

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In contrast to all other mammalian cells, embryonic stem (ES) cells are characterized by a truncated cell cycle, relative autonomy from extracellular signal-regulated kinase (Erk) signaling, and an unusually rapid growth rate, analogous to that of cancer cells and primitive unicellular organisms, including bacteria (Orford and Scadden 2008; Ying et al. 2008; Wang et al. 2009). Thus, the task of replicating the cellular biomass, including nucleotides and amino acids, to support the prolific growth of ES cells imposes stringent metabolic demands. Emerging evidence indicates that such requirements are met not by a self-correcting, homeostatic system of house-keeping enzymes, but by a precisely regulated genetic network (Vander Heiden et al. 2009). Although much has been learned about the factors governing the pluripotency of ES cells (Boyer et al. 2005; Bernstein et al. 2006; Loh et al. 2006; Chen et al. 2008; Cole et al. 2008; Kim et al. 2008), relatively little is known about the transcriptional control of ES cell growth. A better understanding of the links between ES cell growth and pluripotency may be key to realizing the full potential of these cells in genetic engineering and regenerative medicine.

We recently discovered a novel zinc finger transcriptional regulator, Ronin (also Thap11), that is essential for the self-renewal of ES cells (Dejosez et al. 2008). Conditional knockout of the *Ronin* gene induces ES cell death, while its forced expression enables the cells to proliferate transiently without differentiation under conditions that normally do not promote self-renewal. While studying *Ronin*-overexpressing mouse ES cells, we noticed that they possess a strikingly enlarged nucleolus (Fig. 1A; Supplemental Fig. S1A), the prime site of ribosome synthesis and assembly, suggesting that *Ronin* overexpression may alter the production of a key growth-related body in ES cells. This observation, coupled with our previous finding that Ronin can interact with the cell growth factor Hcf-1 (Julien and Herr 2003; Wysocka and Herr 2003; Dejosez et al. 2008), which lacks its own DNA-binding domain, led us to predict that Ronin/Hcf-1 may play a pivotal role in meeting the biosynthetic needs of mouse ES cells. Our results support this hypothesis, and provide a new paradigm for understanding the regulation of ES cell growth and its relationship to pluripotency.

Results and Discussion

Ronin binds to a hyperconserved enhancer element in mouse ES cells that is shared with Hcf-1

We first sought to identify the DNA-binding sites of Ronin using chromatin immunoprecipitation (ChIP) and DNA sequencing (ChIP-seq). Our mapping results revealed 866 Ronin-bound regions (Supplemental Table S1), most of which were located at or immediately upstream of transcription start sites (TSSs) (Fig. 1B,C), suggesting that Ronin participates in transcription initiation. Alignment analysis identified a Ronin-binding motif (CTGGGARWTGTAGTY, designated here as RBM) in 844 of the target gene promoters (Fig. 1D). This functional element was highly enriched compared with random sequences ($P \ll 10^{-100}$), but correlated poorly with the Ronin-binding sequence ("3X") we determined previously by the SELEX method ($E = 6.395 \times 10^{-1}$,

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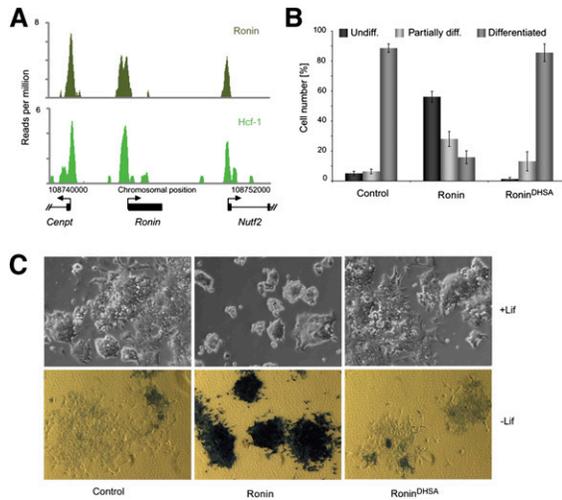


Figure 2. Ronin and Hcf-1 bind together to specific genes. (A) ChIP-seq results obtained in mouse ES cells after immunoprecipitation with Ronin or Hcf-1 antibodies. A representative region containing three Ronin-bound genes shows substantial overlap between Ronin- and Hcf-1-binding peaks in the promoter regions of all three genes. (B) Quantification of the experiment shown in the bottom panel of C. Values are means \pm SD of triplicate experiments. (C, top panel) Morphology of control, *Ronin*-overexpressing, and *Ronin*^{DHSA}-overexpressing mouse ES cells after 3 d of culture in the presence of Lif (10 \times magnification). (Bottom panel) Cells were stained for alkaline phosphatase activity after 4 d of culture in the absence of Lif (20 \times magnification).

those promoters. Because expression of Ronin effectively supports the self-renewal of ES cells in leukemia inhibitory factor (Lif)-free medium (Dejosez et al. 2008), we tested the effect of Hcf-1 on this property by plating control, *EF1 α -Ronin*, and *EF1 α -Ronin*^{DHSA} ES cells at clonal densities in medium without Lif. As reported previously (Dejosez et al. 2008), Ronin overexpression robustly made Lif nonessential for ES cells, while *EF1 α -Ronin*^{DHSA} ES cells still differentiated (Fig. 2B,C), suggesting that Ronin must interact with Hcf-1 to produce its anti-differentiation effect. Rescue experiments using *Ronin* knockout ES cells revealed that transient overexpression of wild-type Ronin has a positive effect on self-renewal, while expression of the Ronin mutant incapable of binding to Hcf-1 did not show this effect (Supplemental Fig. S4A). Finally, careful analysis of *Ronin*^{loxP/loxP} MEFs for changes in morphology, proliferative capacity, and cell cycle phase distribution did not reveal any obvious phenotype after Cre-mediated excision of *Ronin* that could be linked to loss of *Ronin* expression (Supplemental Fig. S4B,C), suggesting that Ronin function is restricted to certain cell types.

Approximately 40% of the 866 promoters bound by Ronin were also occupied by one or more of the transcription factors Oct4, Sox2, and Nanog, which have central roles in pluripotency control (Boyer et al. 2005; Bernstein et al. 2006; Loh et al. 2006; Chen et al. 2008; Cole et al. 2008; Kim et al. 2008; Marson et al. 2008). We find it interesting that Ronin consistently occupied sites within promoter regions that were only 50–100 base pairs (bp) upstream of TSSs, in contrast to the more distant sites occupied by Oct4 (Fig. 3A) and other core transcription factors (data not shown). To assess the genome-wide binding preferences of Ronin versus those of other regu-

latory factors in ES cells, we calculated target similarity scores for genomic regions identified as highly enriched in ChIP-seq experiments (Chen et al. 2008; Ku et al. 2008; Marson et al. 2008; Seila et al. 2008), and subjected the matrix of scores to hierarchical clustering analysis. Ronin and Hcf-1 clustered together rather than with canonical pluripotency factors (Fig. 3B).

Genes targeted by Ronin/Hcf-1 in mouse ES cells function in protein biosynthesis and energy production

The prominent nucleolus of Ronin-overexpressing cells (Fig. 1A; Supplemental Fig. S1A) and the co-occupancy of a hyperconserved DNA-binding motif by Ronin and Hcf-1 led us to consider that these factors may be involved in the regulation of biomass production supporting ES cell growth. To test this hypothesis, we focused on the subset of genes whose promoters were bound solely by Ronin/Hcf-1. Using the PANTHER tool, we determined the functional categories of all genes that met this stringent requirement. Transcription initiation, mRNA splicing, and metabolism were among the most overrepresented categories, while cell signaling and cell development were underrepresented (Fig. 4A,B; also Supplemental Tables S4,S5). Close inspection of the individual genes within these categories yielded a more informative functional portrait (Fig. 4C). Ronin/Hcf-1 recognized as many as 30% of genes encoding ribosomal proteins and two key subunits of RNA polymerase I, *Rpo1-2* and *Rpo1-4* (protein biosynthesis); *Ctd*, *Cnot4/8*, and *Med4* (transcription initiation); *Rab1b*, *Nup133*, and *Timm22* (protein trafficking); and *Frap1* (mTor), *Eif4a1*, *Eif4ebp1*, *Eef2*, *Tsc2*, *Rps6kb2*, and *Rps6* (mTor signaling pathway). These results are important because alterations in ribosomal biosynthesis, transcription initiation, protein transport, and overall control of growth and metabolism can have profound effects on the metabolome (Warner 1999; Moss and Stefanovsky 2002; Tsai and McKay 2002). Moreover, identification of key constituents of the mTor signaling pathway in this analysis, including the mTor protein

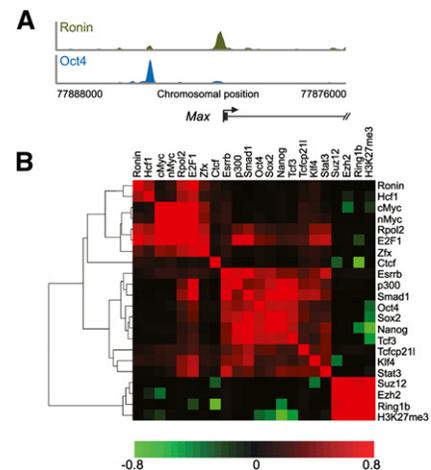


Figure 3. Comparison of binding characteristics of Ronin and canonical pluripotency factors. (A) ChIP-seq results obtained by precipitation with antibodies against Ronin or Oct4. (B) Hierarchical clustering analysis of 22 prominent transcriptional regulators in mouse ES cells, based on target similarity scores calculated with a Pearson correlation similarity metric.

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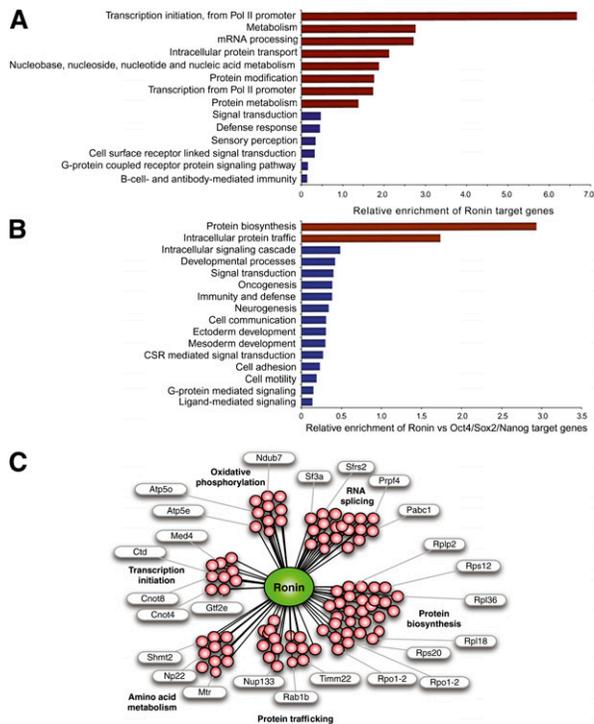


Figure 4. Cellular functions of genes targeted by Ronin/Hcf-1 in mouse ES cells. (A) PANTHER analysis of target genes. Categories with enrichment values >1 are significantly overrepresented (red), while those with lower values are significantly underrepresented (blue). (B) PANTHER analysis of Ronin versus Oct4/Sox2/Nanog targets, as in A. (C) Summary of Ronin/Hcf-1 target genes by major functional categories.

itself, provides a mechanism by which Ronin/Hcf-1 could exert a profound effect on cell growth and metabolism through regulation of a relatively limited number of target genes. Ronin/Hcf-1 also bound specifically to genes encoding mitochondrial ribosomal proteins (*Mrpl19*, *Mrpl32*, *Mrpl50*, and *Mrpl54*), mitochondrial translation factors (*Tufm*), and rate-limiting members of the oxidative phosphorylation cascade (*Atp5e*, *NADH dehydrogenase*, and *Atp5e*), suggesting involvement in the control of energy production in ES cells. Finally, some of the target genes (e.g., *Mtr*) encode threonine catabolic enzymes, whose increased expression in ES cells facilitates a high-flux metabolic state characterized by enhanced threonine catabolism (Wang et al. 2009). Thus, Ronin/Hcf-1 appears to transcriptionally regulate a subset of genes with specific functions in protein biosynthesis and energy production, but not cell development (Fig. 4).

Ronin can either activate or repress its transcriptional targets in mouse ES cells

To explore the different dimensions of target gene regulation by Ronin/Hcf-1, we conducted gene set enrichment analysis (GSEA) of RNA from Ronin targeted genes in wild-type ES cells compared with those in differentiated, *Ronin*-overexpressing or *Ronin* knockout cells (Fig. 5A). The results show that genes occupied by these factors are generally highly transcribed, and that the transcripts are significantly overrepresented in ES cells. Thus, Ronin/Hcf-1 up-regulates the expression of many

(although not all) of its target genes, consistent with the ability of Hcf-1 to either positively or negatively affect transcription, depending on the cellular context (Wysocka and Herr 2003). To test the reverse prediction, we transfected *Ronin*^{loxP/-} ES cells with the gene encoding Cre recombinase, sorted Cre-positive cells at 18 h post-transfection, extracted the RNA, and performed microarray analysis of gene expression. Interestingly, the entire subset of 133 genes found to be up-regulated in *Ronin* knockout cells (Fig. 5B, right) were down-regulated in our *Ronin*-overexpressing clones, whereas, in the converse situation, only 43 of 99 genes found to be down-regulated after knockout (Fig. 5B, left) were down-regulated in the *Ronin*-overexpressing clones. Additional evidence for direct transcriptional control of Ronin targets was obtained in experiments in which we cloned a set of the Ronin targeted promoters and performed luciferase reporter assays. As shown in Figure 5C, Ronin gain of function had a positive effect on gene expression, while loss of the RBM diminished or abolished the results of Ronin overexpression. To track the expression of

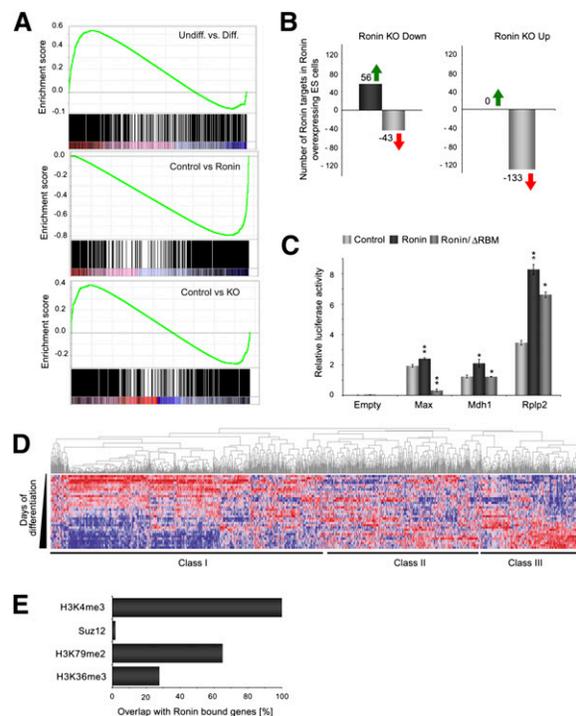


Figure 5. Ronin/Hcf-1 can either activate or repress its transcriptional targets in mouse ES cells. (A) GSEA analyses of Ronin/Hcf-1 target genes, showing enrichment of bound genes in undifferentiated versus differentiated mouse ES cells (top panel), in *Ronin*-overexpressing versus control ES cells (middle panel), and in control compared with *Ronin* knockout ES cells (bottom panel). (B) Overlap between the numbers of Ronin-bound genes that are up-regulated or down-regulated in *Ronin*-overexpressing ES cells in relation to their status in *Ronin* knockout ES cells. (C) Luciferase reporter assays with pGL3-based promoter constructs, showing effects on the expression of selected target genes. (*) $P < 0.05$; (**) $P < 0.01$. (ΔRBM) Mutant reporter construct with deleted RBM. (D) Relative expression levels of Ronin target genes during the differentiation of mouse ES cells. (Red) Up-regulated genes; (blue) down-regulated genes. (E) Prevalence of H3K4me3-, Suz12-, H3K79me2-, or H3K36me3-enriched regions at Ronin-binding sites.

Ronin/Hcf-1-controlled genes more closely, we analyzed the results of DNA microarrays over 14 d of ES cell differentiation (Fig. 5D). As expected, the largest class of Ronin target genes (Class I) showed rapid down-regulation after induction of differentiation, while the two remaining classes either were up-regulated (Class III) or demonstrated complex regulation (Class II).

Considering that Ronin's transcriptional activity depends on its interaction with Hcf-1, we performed Western blot analysis to detect Hcf-1 in wild-type control and Ronin-overexpressing ES cells. Hcf-1 protein was clearly up-regulated in the Ronin-overexpressing cells. The Ronin Hcf-1-binding mutant, on the other hand, appeared to disrupt the Ronin–Hcf-1 complex, leading to Hcf-1 overflow that was consequently counteracted by down-regulation of Hcf-1 expression in the Ronin mutant cell line (Supplemental Fig. S3E). Although the *Hcf-1* gene was not directly targeted by Ronin, its only known regulator, *Hpip*, was (Supplemental Table S1), supporting the notion that Ronin and Hcf-1 form a single functional unit under the control of an autoregulatory loop.

Results of the present analysis confirm the dependence of Ronin on interaction with Hcf-1, and broaden our understanding of how this transcriptional modulation influences Ronin action. Most critical, perhaps, is the demonstration that Ronin must bind to Hcf-1 in order to be functionally active. Although Ronin retained some activity in the absence of Hcf-1, its interaction with this coregulator clearly amplified its induced effects. Finally, we would stress that recruitment of an Hcf-1-containing complex enables Ronin to either up-regulate or repress target genes, thus increasing the versatility of its regulatory action. However, Hcf-1 has many more binding targets than Ronin does, suggesting that it mediates other activities in ES cells, possibly through interaction with site-specific DNA-binding factors such as E2F and Luman (Lu et al. 1997, 1998; Tyagi et al. 2007). Further analysis of the Ronin-occupied promoter regions (Fig. 5E) showed that Ronin is closely associated with promoters containing histone H3K4me3-modified nucleosomes, a mark of genes that undergo transcription initiation (100% overlap), and with H3K36me3 (28%) and H3K79me2 (80%), both marks of genes that are fully transcribed. In contrast, there was essentially no overlap with Suz12 (0.02%), a component of Polycomb-Repressive Complex 2, which catalyzes the H3K27me3 mark associated with transcriptionally repressed bivalent domains (Bernstein et al. 2006), indicative of a very strong negative correlation (binomial $P < 10^{-9}$). These results agree with the well-documented ability of Hcf-1 to recruit Ash2/Set1 to target gene promoters (Wysocka and Herr 2003).

Concluding remarks

Our results indicate that Ronin/Hcf-1 contributes to ES cell pluripotency by binding to a hyperconserved enhancer element and regulating the transcription of genes involved in key metabolic processes that sustain the growth of self-renewing ES cells until they exit the undifferentiated state. The highly conserved nature of this DNA sequence and its tissue specificity (Xie et al. 2005) suggest that genes controlled through Ronin binding are apt to perform essential functions in ES cells. Moreover, as the only transcriptional regulator known to bind to this conserved motif in ES cells, Ronin separates itself from the canonical pluripotency factors. We ac-

knowledge that Oct4, Sox2, Nanog, and Tcf3 co-occupy the promoters of certain genes with Ronin, but the sites they recognize are entirely different from the RBM (Fig. 3A). Although these canonical factors can interact among themselves to regulate target gene expression (Kim et al. 2008), there is no evidence to suggest that they cooperate with Ronin in transcriptional regulation. This dissociation is underscored by (1) the apparent lack of Ronin binding to Suz12-enriched genomic regions (Fig. 5E), which are often targeted by Oct4 and other canonical factors (Boyer et al. 2006); (2) the difference in global genomic binding pattern between Ronin and Oct4, Sox2, Nanog, and Tcf3 (Fig. 3A); (3) the absence of canonical factors at approximately half of the gene promoters bound by Ronin; and (4) the marginal overlap between Ronin targets identified in this study and those down-regulated upon Oct4 knockdown in a previous report (Ivanova et al. 2006). The observation that Ronin/Hcf-1 binding to target sites more commonly leads to gene activation than repression revises our earlier suggestion (Dejosez et al. 2008) that Ronin is primarily a global repressor, based on the assumption that acute up-regulation of Ronin under otherwise steady-state conditions exerts a dominant-negative effect on Ronin function, similar to observations for other proteins that harbor the Thap domain (Cayrol et al. 2007). We interpret the reported increase in H3K9 methylation (Dejosez et al. 2008) as a secondary or indirect effect of Ronin. Gene activation by Ronin/Hcf-1 is most likely mediated through an epigenetic mechanism involving Hcf-1/Ash2/Set1.

Recently, the life cycle of ES cells was compared with that of yeast cells and other unicellular metazoans, in the sense that it follows a relatively primitive set of behavioral rules that differ from those of more mature cells (Silva and Smith 2008; Ying et al. 2008; Wang et al. 2009). We suggest that the growth and metabolic capacity of ES cells may represent a previously unrecognized level of pluripotency control. Indeed, the genes bound by Ronin could well account for most of the protein and metabolic budget expended by ES cells engaged in self-renewal, a process that may consume as much as 50% of the total energy produced by a cell (Moss and Stefanovsky 2002). Thus, any shortfall in energy could cause ES cells to lose their full self-renewal capacity, leading to apoptotic death or perhaps a rapid transition to differentiation. Hence, it will be important to determine if the transcriptional activity of Ronin/Hcf-1 is intrinsically self-maintaining, or is modulated by signaling from upstream molecules.

Materials and methods

Cell culture, differentiation, and alkaline phosphatase staining

Mouse ES cells (line R1 and derivatives), were cultured in DMEM + GlutaMax I (Invitrogen) supplemented with 1000 U/mL Lif (Millipore).

Establishment of cell lines with stable integration of control, Ronin, or Ronin^{DHSA} vectors

R1 mouse ES cell lines overexpressing control (EF1a-Neo), Ronin (EF1a-Ronin-Flag-Ires-Neo), or Ronin^{DHSA} (EF1a-Ronin^{DHSA}-Flag-Ires-Neo) constructs were established as described previously (Dejosez et al. 2008).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed essentially as described (Dejosez et al. 2008) using the Lightshift Chemiluminescent EMSA kit (Pierce).

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ChIP bioinformatic analysis and ChIP-PCR

Ronin and HCF-1 ChIP-derived reads were aligned to the mouse genome (NCBI build 36, University of California at Santa Cruz build mm8) using an iterative version of ELAND to improve the read count, and all mapped reads were analyzed as described previously (Marson et al. 2008).

Directional yeast two-hybrid analysis

Yeast two-hybrid analysis was performed as described previously (Dejosez et al. 2008) using the ProQuest Two-Hybrid system (Invitrogen) and gateway technology, according to the manufacturer's recommendations.

See the Supplemental Material for full materials and methods.

Acknowledgments

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