Ronin influences the DNA damage response in pluripotent stem cells

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Early mammalian embryonic cells must maintain a particularly robust DNA repair system, as mutations at this developmental point have detrimental consequences for the organism. How the repair system can be tuned to fulfill such elevated requirements is largely unknown, but it may involve transcriptional regulation. Ronin (Thap11) is a transcriptional regulator responsible for vital programs in pluripotent cells. Here, we report that this protein also modulates the DNA damage response of such cells. We show that conditional Ronin knockout sensitizes embryonic stem cells (ESCs) to UV-C-induced DNA damage in association with Atr pathway activation and G2/M arrest. Ronin binds to and regulates the genes encoding several DNA repair factors, including Gtf2h4 and Rad18, providing a potential mechanism for this phenotype. Our results suggest that the unique DNA repair requirements of the early embryo are not met by a static system, but rather via highly regulated processes.

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1. Introduction

The pluripotent stem cell state is maintained by a core set of transcription factors (e.g., Oct4, Sox2, and Nanog) that activate self-renewal genes and suppress lineage-specific differentiation pathways (Dejosez and Zwaka, 2012; Ng and Surani, 2011). Although pluripotency-related transcription factors are known to bind upstream of several DNA repair genes (Marson et al., 2008), the exact links that connect genomic integrity, DNA repair, and pluripotency have not yet been clearly defined. A second class of transcription factors helps maintain pluripotency by controlling general cell-vital programs that are critical for the rapid growth of pluripotent stem cells (Smith et al., 2011; Dejosez et al., 2010; Dejosez and Zwaka, 2012). Ronin (Thap11) belongs to this second class and is hence a suitable candidate for altering the DNA repair capacity of pluripotent stem cells. Ronin is a DNA-binding protein that is essential for pluripotent stem cells and is known to regulate various genes that are important for the cellular homeostasis of highly proliferative cells (Dejosez et al., 2010, 2008). Here, we provide evidence that Ronin also influences the DNA repair machinery of embryonic stem cells (ESCs). We show that Ronin regulates genes involved in the response to UV-C irradiation, and that conditional Ronin knockout increases the sensitivity of ESCs to DNA damage and activates the Atr-mediated DNA damage response. Our findings suggest that, along with lineage-specific transcription factors like Oct4 and Sox2, Ronin helps to maintain the uniquely robust genomic integrity of pluripotent stem cells.

2. Materials and methods

2.1. Cell culture

Ronin−/−, Ronin+/−; Cre-ERT2, and Ronin+/−; Cre-ERT2 mouse ESCs were derived (Dejosez et al., 2008) and cultured on 0.1% gelatin (Sigma) in DMEM + GlutaMAX (Invitrogen) supplemented with 10% FBS (Gemini), 2 mML-glutamine (Invitrogen), 100 nM MEM non-essential amino acids (Invitrogen), 1000 U/ml LIF (Millipore), and 100 μM 3-mercaptoethanol (Sigma).

2.2. DNA damage dose response curves

Cells were plated at a density of 1000 cells/20 cm², and treated for four days with ethanol (Sigma) or 0.25 μM 4-Hydroxytamoxifen (4HT) (Sigma). For γ-irradiation (γIR), the cells were irradiated in ESC medium using a 137Cs source at rates indicated. To induce DNA damage by UV-C irradiation, cells were washed twice in PBS (Invitrogen) and irradiated in the second PBS wash using a UV Stratalinker 2400 (Stratagene). Cells were then fed for an additional three days with ES cell medium supplemented with ethanol or 0.25 μM 4HT.

2.3. Serum starvation

Cells were plated in triplicate at 25,000 cells/10 cm² and fed for four days with ethanol or 0.25 μM 4HT, washed with PBS and fed for 36 or 72 h with ESC medium containing 0.1% FBS and either 0.25 μM 4HT or ethanol.

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2.4. Colony and ViCell counting assays

For colony-counting assays, cells were washed in PBS, fixed in 2% PBS-buffered paraformaldehyde (Sigma), and stained with 3% Giemsa (Sigma). Colonies were counted and plotted relative to controls (mock-treated in PBS). For ViCell counting assays, cells were harvested by trypanization and counted with a ViCell XR Cell Viability Analyzer (Beckman Coulter). Viable cells were plotted relative to untreated controls. Data indicate the mean ± s.e.m. of at least three independent experiments. Statistics analysis included a two-way ANOVA.

2.5. Neutral comet assay

Cells were plated at a density of 250,000 cells/20 cm² and fed for four days with ethanol or 0.25 μM 4HT. The medium was then changed to ES cell medium without ethanol or 4HT and the cells were γ-irradiated with 2.3 Gy. Following 4 or 6 h of recovery time, the cells were harvested and kept on ice. For 0 h controls, the untreated controls were split and one set of cells was γ-irradiated in PBS and placed on ice immediately to inhibit repair. The neutral comet assay was then conducted according to manufacturer instructions (Trevigen, Inc.). Comet pictures were taken using an Axiosplatin 2 microscope (Carl Zeiss, Inc.) and a CoolSNAP HQ CCD camera (Photometrics) using Universal Imaging Metavue 6.1r1 software (Molecular Devices, LLC). Comets were analyzed with CometScore Freeware v1.5 (TriTek Corp.). Tail Moments were measured as the product of the DNA fluorescence in the tail and the distance between the means of the head and the means of the head and tail fluorescence (Olive and Banáth, 1993). Shown are the data from one representative experiment out of three independent experiments. Statistical analysis included a Kolmogorov-Smirnov test which measures the difference in distribution of all Tail Moments rather than the median Tail Moment. At least 150 comets were analyzed per condition in each experiment.

2.6. Western blots

To detect Cre-mediated Ronin knockout, cells were plated at a density of 250,000 cells/20 cm² and fed for four days with ES cell medium supplemented with ethanol or 0.25 μM 4HT. To detect protein expression after UV-C treatment, cells were plated at a density of 1.1 × 10⁶ cells/60 cm², fed as above for four days, and UV-C irradiated with 12 J/m². Cells were harvested by trypsinization, washed with PBS and whole-cell protein extracts were prepared and Western blots performed as described previously (Dejosez et al., 2010). Antibodies used were: Ronin/Thap11 (BD Biosciences), α-Tubulin (Sigma), p-Chk1 (Ser345, Cell Signaling Technology), Chk1 (Cell Signaling Technology), p-p53 (hSer15/mSer18, Abcam), p53 (Leica Biosystems), Anti-Rabbit IgG HRP Conjugate (Promega), and Anti-Mouse IgG HRP Conjugate (Promega). Signal intensities were quantified with the Image J software.

2.7. Flow cytometry of BrdU labeled cells

Cells were plated with a density of 300,000 cells/60 cm² and fed with ESC medium supplemented with ethanol or 0.25 μM 4HT for four days. The cells were washed twice with PBS and treated with 12 J/m² UV-C irradiation in the second PBS wash and allowed to recover in ESC medium for 9 h before they were pulse-labeled with BrdU as described by Savatier et al. (2002). 1–1.5 × 10⁶ cells were fixed in 70% ethanol and stained with an anti-BrdU antibody (BD Biosciences) and propidium iodide (Sigma) (Savatier et al., 2002). Cells were subjected to flow cytometry with an LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.). Values indicate the mean ± s.e.m. of three independent experiments. Statistical analysis included a three-way ANOVA.

2.8. Bioinformatics analysis

Ronin targets with a known biological function were filtered for those falling within the GO term: Response to DNA damage stimulus (GO: 0006974) using PERL scripts. Binomial distribution analysis was conducted in Excel (Microsoft, Inc.).

2.9. Microarray

RNA was isolated with the RNasy Mini Kit (Qiagen) according to the manufacturer’s instructions and subsequently hybridized to an Affymetrix array (Microarray Core Facility at Baylor College of Medicine). The array data were normalized and analyzed using Array Star software (DNA Star, Inc.).

2.10. ChIP-qPCR

Chromatin immunoprecipitation was conducted as described previously (Dejosez et al., 2010) with polyclonal rabbit anti-Ronin G4275 antiserum and rabbit G4275 preimmune serum. For verification of Ronin binding sites by qPCR, 2 μl of each ChIP product were used in triplicate reactions using Sybr Green PCR Master Mix (Applied Biosystems) in a final volume of 25 μl in the presence of 0.2 μM of each oligo (see Supplementary Table 1). Reactions were run on a 7900 Real-Time PCR system using standard reaction conditions (Applied Biosystems). qPCR was conducted on triplicate ChIP samples and normalized to input DNA. Values indicate the mean ± s.d.

2.11. qRT-PCR

RNA was isolated with the RNasy Mini Kit (Qiagen) and 1 μg was reverse-transcribed into cDNA using the iProm-II Reverse Transcription System (Promega). For each qRT-PCR reaction, 2 μl of cDNA were used in triplicate reactions using Sybr Green PCR Master Mix (Applied Biosystems) in a final volume of 25 μl in the presence of 0.2 μM of each oligo (see Supplementary Table 1). Reactions were run on a 7900 Real-Time PCR system using standard reaction conditions (Applied Biosystems). Data were analyzed using 2^−ΔΔCt for quantification of mRNA levels (i.e. normalization to β-Actin internal controls and subsequent normalization to EtOH-treated controls for each cell line). These normalized values for Ronin^fl/fl-, Cre-ERT2 ES cells were then plotted relative to Ronin^fl/fl- controls for each day of 4HT treatment. Values indicate the mean ± s.e.m. of three independent experiments. Statistical analysis included a two-way ANOVA followed by an unpaired Student’s t-test.

2.12. statistics

All statistics were conducted using SPSS software (IBM Corp.) or Excel (Microsoft Corp.)

3. Results

3.1. Ronin targets nucleotide excision repair and DNA damage genes

We examined Ronin (Dejosez et al., 2010) and Oct4 Chip-Seq data (Marson et al., 2008) from mouse ESCs to chart their DNA repair gene repertoires. We found that genes belonging to the gene ontology (GO) categories for “response to DNA damage stimulus” (GO:0006974) were enriched for the binding of Ronin (P = 0.0009) and Oct4 (P = 0.003) (Fig. 1A). Only four of these genes (Prpf19, Brc1, Rad51, and Rad18) are bound by both Ronin and Oct4, suggesting that the level of co-regulation between those two factors is low. Interestingly, the Ronin-bound genes fell into several broader DNA repair related categories, including homologous recombination repair (Brc1 and Rad51), nucleotide excision repair (Gtf2h4 and Erc1), post-replication repair (Rad18 and Wnnip1), and the DNA damage checkpoint (Claspin and Rad1). Genes in
Ronin and Hcf-1 at the promoter regions of DNA repair genes (P = 0.00086893) and Oct4 targets (P < 0.05), indicating enrichment for DNA repair genes among Ronin targets (Marinoni et al., 1997; Tateishi et al., 2003; Yoshimura et al., 2009). Therefore, all three genes lie in DNA repair pathways that respond to similar genotoxic stresses.

3.2. Ronin is critical for the expression of Gtf2h4 and Rad18

To test whether the binding of Ronin to DNA repair genes is associated with their transcriptional regulation, we used Roninfl/fl control and Ronin Cre-ERT2 ESCs to conditionally delete Ronin with 4-hydroxytamoxifen (4HT). Cells were treated for four days and complete knockout of the Ronin protein was confirmed by western blot (Supplementary Fig. S1A). Using the same knockout strategy, gene expression was profiled by microarray analyses on days 1.5, 3, and 6 by comparing 4HT-treated control cells with Ronin knockout cells (Fig. 2A). Our results indicated that, while overall gene expression changes were mild, some of the DNA repair genes that are bound by Ronin (Fig. 1B, C), including Gtf2h4 (P < 0.05), Rad18 and Wnmp1 were indeed downregulated upon Ronin knockout. qRT-PCR experiments validated reduced expression of Gtf2h4 and Rad18 in the absence of Ronin. In contrast, Wnmp1 was not significantly downregulated, suggesting that Ronin is not a major regulator of Wnmp1 expression (Fig. 2B). Notably, the level of Oct4 and other pluripotency related factors (Li and Belmonte, 2017) was not altered by Ronin knockout (Fig. 2B, Supplementary Table 2), indicating that the observed decrease in expression of these DNA repair genes was not a secondary consequence of differentiation.

3.3. Ronin knockout increases sensitivity to ionizing and UV-C irradiation

As Ronin knockout reduced the transcription of genes involved in nucleotide excision repair and replication fork stalling, we next investigated the effects ofRonin loss on the DNA damage response in ESCs. We used ionizing radiation (γIR) to induce double-strand breaks and UV-C irradiation to cause DNA helix-distorting lesions that arrest DNA replication (Eppink et al., 2011; Mladenov and Iliakis, 2011) (see Supplementary Fig. S1B for experimental outline). Interestingly, Ronin loss increased sensitivity to γIR and UV-C (Fig. 3A), while DNA damage-independent serum starvation-induced cellular stress (Brooks, 1976; Joza et al., 2001) and Cre recombinase-induced genotoxic stress (Loonstra et al., 2001), had no effect on Ronin-knockout cells (Supplementary Fig. S2). Consistent with this observation, the repair of the γIR induced double strand breaks measured in neutral comet assays (Banath et al., 2009) was impaired in Ronin-knockout cells compared with control cells 6 h after irradiation (Fig. 3B, C and Supplementary Fig. S3). Together, our results indicate that Ronin knockout increases the cellular sensitivity to DNA damage that induces helix-distorting lesions and double-strand breaks.

3.4. Ronin knockout increases Atr-dependent DNA damage checkpoint activation and G2/M arrest after DNA damage

Although failure of the nucleotide excision system may account for the increased DNA damage sensitivity of Ronin-knockout ESCs, we speculated that other systems could be responsible as well. It is known that stalled replication forks resulting from UV-C damage and other genotoxins activate a DNA damage checkpoint that involves Atr kinase and Chk1 (Eppink et al., 2011; Heffernan et al., 2002; Liu et al., 2000). Hence, Ronin-depleted cells could be unable to properly engage the DNA damage checkpoint, or they could fail to recover stalled replication forks (Eppink et al., 2011; Heffernan et al., 2002; Liu et al., 2000). Thus, we first assessed the extent of DNA damage checkpoint activation by examining Chk1 phosphorylation at serine 345 (Niida et al., 2007). We fabricated ChIP-Seq data (Fig. 1B, adapted from Dejosez et al., 2010) and validated Ronin binding to these genes by ChIP-PCR (Fig. 1C). Gtf2h4 is known to contribute to nucleotide excision repair by removing helix-distorting lesions that block DNA replication, whereas Rad18 and Wnmp1 physically interact with each other and respond to arrested replication forks (Marinoni et al., 1997; Tateishi et al., 2003; Yoshimura et al., 2009). Therefore, all three genes lie in DNA repair pathways that respond to similar genotoxic stresses.

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found that the levels of phosphorylated Chk1 were higher in Ronin-knockout cells than in control cells beginning at 1 h after UV-C damage (Fig. 4A, Supplementary Fig. S4). To further test the contribution of stalled replication forks, we looked at another Atr target, p53, which is phosphorylated in response to stalled replication forks (Tibbetts et al., 1999). As previously reported, both phosphorylated and basal p53 protein levels increased in response to UV-C damage (Chao et al., 2000; Corbet et al., 1999). However, we found that the levels of serine 18-phosphorylated p53 did not differ between Ronin-knockout and control ESCs (Fig. 4A, Supplementary Fig. S4), likely due to the increase in basal p53 levels in both conditions. Moreover, when we examined changes in the cell cycle, we observed that UV-C-exposed Ronin-knockout ESCs were more likely to be found in G2/M and less likely to be in S phase, compared to control cells (Fig. 4B, C). Together our results indicate that Ronin knockout was associated with a sustained and elevated DNA checkpoint activation as well as an increase of cells within G2/M after UV-C damage.

4. Discussion

We herein use Ronin-knockout ESCs to show that Ronin adjusts the cellular DNA damage response of pluripotent cells to the more stringent needs of the early embryo. We report evidence suggesting that Ronin may exert its effects on the DNA repair capacity by transcriptionally regulating DNA repair genes. The important role of Ronin during early embryogenesis and the lethal phenotype associated with its knockout fit this model (Dejosez et al., 2008). DNA maintenance and repair are costly in terms of their energy requirements, biomass needs, and the number of involved proteins, which must be provided in a manner appropriate to other stringent needs of the early embryo (Dejosez et al., 2010; Vander Heiden et al., 2009). Not all cells require their DNA integrity to be so robustly maintained. For example, non-cycling cells have relatively low needs for DNA repair, and DNA damage is not as consequential. On the other hand, highly proliferative cells, such as those of the embryo, have rapid and abbreviated cell cycles that increase their sensitivity to blocked DNA replication (Bielas and Heddle, 2004; Harfouche and Martin, 2010; Mandal et al., 2011; McKinnon, 2009). This weakness reflects a finely tuned balance of DNA repair, cell cycle arrest, and apoptosis (Corbet et al., 1999; de Waard et al., 2008; Savatier et al., 2002; White and Dalton, 2005). The results of our present study suggest that Ronin is part of this fine-tuning system, as its loss is specifically associated with particular aspects of DNA damage sensitivity. Indeed, as Ronin is expressed in oocytes (Dejosez et al., 2008), our observation might extend to germ cells that have been shown to share the high levels of genomic integrity seen in ES cells (Murphey et al., 2013).

Although our results suggest that Ronin directly impacts DNA repair through the transcriptional regulation of DNA repair genes, we further sought to determine how much the canonical pluripotency factors might contribute to the transcriptional regulation of DNA repair genes. Oct4 and Sox2 are known to bind upstream of the Gtf2h4 and Rad18 genes (Marson et al., 2008), yet the levels of Oct4 and Sox2 were not affected by Ronin knockout on transcriptional level in our microarray

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**Fig. 2.** Ronin loss causes misregulation of Ronin DNA repair target genes. (A) Microarray data from days 1.5, 3, and 6 of 4HT treatment for Roninfl/fl and Roninfl/fl; Cre-ERT2 ESCs. Green boxes indicate Ronin-bound DNA repair genes. (B) qRT-PCR of genes on days after induction of Ronin knockout as indicated. Shown are the relative expression changes between 4HT-treated Roninfl/fl and Roninfl/fl; Cre-ERT2 ESCs after normalization to ActB and respective normalization to ethanol-treated controls. Values indicate the mean ± s.e.m. (n = 3). ***P < 0.001 **P < 0.01. An unpaired Student’s t-test was conducted after determination of significance by two-way ANOVA.
analysis. This observation applies to other pluripotency related factors as well (Supplemental Table S2; Li and Belmonte, 2017). Additionally, Nanog, Suz12 and Tcf3 are also known not to bind to Gtf2h4 or Rad18 (Dejosez et al., 2010). While our results do not formally exclude the involvement of these or other factors, they support the notion that Ronin plays a decisive role in the transcriptional regulations of Gtf2h4 and Rad18. We previously proposed a model in which Ronin regulates its target genes by recruiting histone-modifying enzymes via an interaction with Hcf-1 (Dejosez et al., 2010, 2008). Because Gtf2h4 and Rad18 are bound by Ronin and downregulated upon Ronin knockout, transcriptional regulation of Gtf2h4 and Rad18 could be mediated in part through this mechanism.

Stalled replication forks resulting from UV-C damage and other genotoxins are known to activate a DNA damage checkpoint that involves Atr and Chk1 (Heffernan et al., 2002; Liu et al., 2000). The increase in phospho-Chk1 in Ronin-knockout cells after UV-C damage may be explained by an increase in unrepaired damage and/or the inability of DNA replication to proceed past UV-C damage (Coin et al., 2007; Marinoni et al., 1997; Tateishi et al., 2003). Furthermore, it is known that knockout of genes in ESCs that are involved in nucleotide excision repair (e.g. Xpc) leads to slowed S phase progression and G2/M arrest after UV-C damage (de Waard et al., 2008). Those observations are in line with our results in Ronin-knockout cells, suggesting that nucleotide excision repair defects contributed to the increased Chk1 phosphorylation and G2/M accumulation observed in our present study.

Ronin belongs to a unique protein family characterized by a highly conserved THAP DNA binding domain (Roussigne et al., 2003). The Thap family proteins arose through a process called “molecular domestication,” beginning from an ancient DNA transposon whose modern-day descendent is the P-element transposase (Hammer et al., 2005). As transposition of such elements involves DNA repair, we cannot exclude the possibility that Ronin in addition to its function as a transcriptional regulator (Dejosez et al., 2010; Sabogal et al., 2010) may play a more direct role in the DNA damage response (Weinert et al., 2005). Additionally, other Thap family members, including Thap5, have been suggested to play pro-apoptotic roles in the responses to UV-C irradiation and other sources of stress (Balakrishnan et al., 2011, 2009) and Thap9 was directly shown to have DNA nuclease activity, making this possibility even more likely (Majumdar et al., 2013).

5. Conclusion

In summary, we show Ronin-knockout ESCs to exhibit reduced expression of factors that respond to UV-C damage, as well as increased phospho-Chk1 and G2/M arrest. Future work is warranted to examine whether Ronin performs comparable functions in other cell types. Given the significant differences between ESCs and somatic cells in terms of their DNA repair capacities, spontaneous mutation rates, and cell cycle structures, the insights gained here may not apply universally (Tichy, 2011). However, we think that these findings could be relevant to other highly proliferative cell types, such as tumor cells.

Author contributions

BS and MD designed and performed experiments. TZ supervised all experiments and provided funding. BS, MD and TZ wrote the manuscript.

Conflict of interest

We report no conflict of interest in conducting the work within this manuscript.
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Appendix A. Supplementary data

Supplementary material

References


