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EXTRA VIEW

# Cohesin's role in pluripotency and reprogramming

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**ABSTRACT**
Cohesin is required for ES cell self-renewal and iPS-mediated reprogramming of somatic cells. This may indicate a special role for cohesin in the regulation of pluripotency genes, perhaps by mediating long-range chromosomal interactions between gene regulatory elements. However, cohesin is also essential for genome integrity, and its depletion from cycling cells induces DNA damage responses. Hence, the failure of cohesin-depleted cells to establish or maintain pluripotency gene expression could be explained by a loss of long-range interactions or by DNA damage responses that undermine pluripotency gene expression. In recent work we began to disentangle these possibilities by analyzing reprogramming in the absence of cell division. These experiments showed that cohesin was not specifically required for reprogramming, and that the expression of most pluripotency genes was maintained when ES cells were acutely depleted of cohesin. Here we take this analysis to its logical conclusion by demonstrating that deliberately inflicted DNA damage - and the DNA damage that results from proliferation in the absence of cohesin - can directly interfere with pluripotency and reprogramming. The role of cohesin in pluripotency and reprogramming may therefore be best explained by essential cohesin functions in the cell cycle.

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## Introduction

Several studies reported an essential role for cohesin in ES cell self-renewal and in the iPS-mediated reprogramming of somatic cells to pluripotency.[1-4] Given that in mammalian cells cohesin associates with CTCF, [5-8] NIPBL, Mediator and cell-type specific transcription factors[1,9,10] at gene regulatory elements and can mediate long-range chromosomal interactions,[1-4,11-16] these data suggested a special place for cohesin in the network of pluripotency where it enables the expression of pluripotency genes by forming connections between their regulatory elements.

However, cohesin has essential functions in preserving the integrity of the genome through the cell cycle. Cohesin consists of a heterodimer of SMC (structural maintenance of chromosomes) proteins - SMC1A and SMC3, and 2 non-SMC proteins - RAD21 and either STAG1 or STAG2 and forms a ring-like structure with a diameter of 40 nm. This is large enough to topologically entrap 2 strands of nucleosomal DNA.[17,18] Cohesin's association with chromatin is carefully regulated during the cell cycle to facilitates its cell cycle-dependent and cell cycle-independent functions[19] (Fig. 1A). In vertebrate cells, cohesin loading onto DNA is initiated in telophase[20,21] and requires the activity of the cohesin loading factor NIPBL and its partner MAU2.[22-24] During interphase, cohesin association with DNA is maintained in a dynamic equilibrium by the opposing unloading actions of the WAPL and PDS5 proteins.[25] Current models suggest that cohesin acts as a transcriptional regulator and genome organizer by forming chromatin interactions between distant DNA regions.[26,27] Locally, cohesin mediated enhancer-promoter interactions facilitate the rearrangement of the T cell receptor $\alpha$ chain locus *Tcra* in non-proliferating thymocytes.[12] On a global scale, cohesin associated with CTCF at the boundaries of topologically associating domains (TADs) is important for the structural organization of the genome. Loss of cohesin allows increased inter-domain interactions across TAD boundaries[28] and while architectural chromatin compartments are not affected, cohesin is required for specific interactions within the compartments.[29] In S phase, cohesin facilitates DNA replication.[30-32] The acetylation of SMC3 by ESCO1/2 establishes stable cohesin binding to DNA.[33,34] Once stably bound, cohesin holds the sister chromatids together until they segregate during mitosis. The proximity of replicated DNA strands provided by cohesin also enables homology-based repair of post-replicative DNA lesions.[35,36] After the onset of mitosis, most of the cohesin associated with chromosome arms is removed by the prophase pathway and the small fraction of cohesin retained at centromeres allows the continued alignment of chromosomes at the metaphase plate following spindle attachment. Cleavage of centromeric cohesin by separase at the onset of anaphase then facilitates the segregation of sister chromatids to daughter cells.[37]

RNAi-mediated knockdown has been widely used to probe cohesin's role in gene regulation, and RNAi screens identified cohesin as a factor required for the self-renewal of pluripotent

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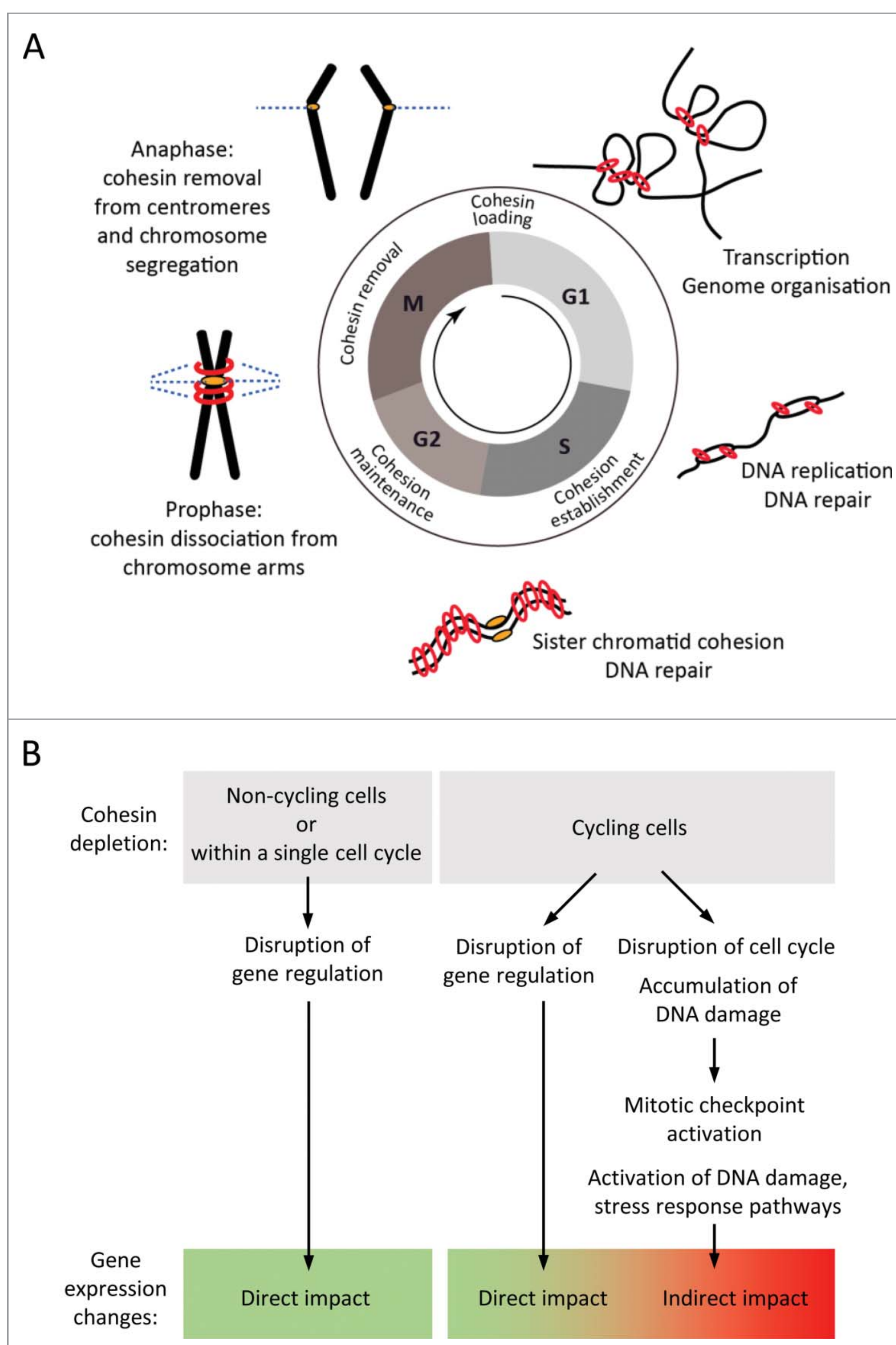


**Figure 1.** Cohesin functions during the cell cycle. (A) Cohesin dynamics during the cell cycle (see text for details). (B) Cohesin depletion in dividing cells can disrupt its cell cycle functions and indirectly impact gene expression due to the activation of mitotic checkpoints and cellular stress response pathways.

embryonic stem (ES) cells.[1,38-40] However, gene expression analysis in ES cells 5 d after cohesin knockdown [1] revealed a preferential deregulation of genes related to cell cycle and DNA damage.[41] Prolonged depletion of cohesin from rapidly dividing ES or iPS cells can therefore result in DNA damage, checkpoint activation, cell cycle arrest and the induction of p53 target gene expression (Fig. 1B). In turn, DNA damage responses abolish pluripotency gene expression[42-44] and reprogramming.[45-47] Hence, failure to establish or maintain pluripotency gene expression in cohesin-depleted cells does not necessarily implicate a loss of long-range interactions, but suggests the possibility that DNA damage responses could have interfered with pluripotency gene expression.

In a recent study we began to disentangle DNA damage responses from long-range interactions by conducting reprogramming experiments in the absence of cell division. Fusion of ES cells with somatic cells generates heterokaryons, which initiate reprogramming without cell division. In addition, nuclear transfer experiments eliminate the requirement for DNA replication. These experiments indicated that cohesin was not specifically required for reprogramming, and that ES cells maintained the expression of most pluripotency genes when

analyzed after cohesin depletion but before the onset of DNA damage responses.[41] Here we take this analysis to its logical conclusion by showing that deliberately inflicted DNA damage - or the DNA damage resulting from prolonged cohesin depletion in cycling ES cells - actively interferes with pluripotency and reprogramming. Our findings suggest that data concerning the role of cohesin in pluripotency and reprogramming derived from cells that cycle in the absence of cohesin should be reinterpreted in the context of essential cohesin functions in the cell cycle.

## Results

A simple but restrictive approach to dissociate cell cycle-related and gene regulatory functions of cohesin is the genetic deletion of cohesin from non-cycling cells.[12,28,48] Alternatively, cohesin can be acutely depleted from cycling cells at the gene level (by inducible deletion) or the protein level (by inducible cleavage or degradation), provided that depletion is sufficiently rapid to occur within a single cell cycle. Our experiments combined inducible ERt2Cre and conditional *Rad21* alleles [41] to efficiently deplete mRNA (Fig. 2A) and protein (Fig. 2B) in ES cells within 24h of ERt2Cre induction by 4-hydroxy tamoxifen. This was achieved without significant induction of DNA damage (as indicated by phosphorylation of histone H2AX, $\gamma$-H2AX, Fig 2C) and in the absence of p53-dependent stress responses (such as *Mdm2* induction, Fig. 2D) or cell cycle arrest (Fig. 2E).

Genome-wide transcriptional profiling showed that ~600 genes were deregulated. These genes were enriched for developmental functions but not for cell cycle or DNA damage responses. Deregulated expression was highly correlated with cohesin binding by ChIP-seq, indicating that many deregulated genes were direct targets of cohesin. [41] Quantitative reverse transcription PCR (RT-PCR) of selected pluripotency markers confirmed our array data indicating that the expression of *Nanog* remained unaffected, *Klf4* was downregulated and *Lefty1* was upregulated (Fig. 2F). Overall, 8% of deregulated genes were pluripotency-associated (Fig. 2G) and 12% of pluripotency genes were affected by cohesin depletion. Hence, acute cohesin depletion in ES cells did not cause a global collapse in pluripotency gene expression but had a selective and gene-specific impact where most pluripotency genes remained unaffected, whereas a minority were either up- or downregulated, most to a moderate extent. Many developmental genes in ES cells are marked by bivalent chromatin marks[49,50] and can be rapidly activated upon differentiation. Of 2902 bivalent genes, 125 were deregulated within 24 hours of *Rad21* deletion. Of these, only a minority (48) were upregulated, while 77 were downregulated (Fig. 2H). These data indicate that cohesin-depleted cells do not undergo wholesale differentiation and corroborate the conclusion that cohesin depletion does not result in a collapse of pluripotency gene expression.

As cohesin is thought to promote the expression of pluripotency genes by mediating enhancer-promoter interactions[1-4] we carefully assessed how acute cohesin depletion affected the binding of cohesin to gene regulatory elements and interaction between enhancers and promoters in ES cells. ChIP-PCR showed that RAD21 was indeed efficiently depleted from the promoters and enhancers of *Nanog*, *Lefty1*, and *Klf4* (red bars, Fig. 2i). RAD21 association in differentiating cells is shown for comparison (green bars, Fig. 2i). In contrast to RAD21, the cohesin loading protein NIPBL remained associated with the promoters and enhancers of *Nanog, Lefty1* and *Klf4* in *Rad21*-deleted ES cells (red bars, Fig. 2J). Unexpectedly, *Nanog, Lefty1* and *Klf4* enhancer-promoter interactions remained strong 24 hours after *Rad21* deletion as detected by chromatin conformation capture (3C) (red bars, Fig. 2K), despite reduced cohesin occupancy (red bars, Fig. 2i). As a control for the ability of our 3C assays to detect change, reduced enhancer-promoter interactions were readily detected in differentiating ES cells (green bars, Fig. 2K). Hence, in contrast to expectations based on ES cells suffering DNA damage,[1] enhancer-promoter interactions can be maintained at least at some pluripotency loci even after cohesin depletion. These interactions may be mediated by transcription factors, mediator or Nipbl (Fig. 2L).

When cohesin-depleted ES cells were allowed to proliferate, significant DNA damage occurred within 36 hours as indicated by $\gamma$-H2AX (Fig. 3A) and upregulation of the p53 target gene *Mdm2* to levels similar to those induced by causing deliberate DNA damage by exposure of ES cells to doxorubicin (Fig. 3B). After 48 hours of cohesin depletion ES cells were arrested in G2/M phase of the cell cycle (Fig. 3C). With the exception of *Lefty1* the expression of the pluripotency genes tested was downregulated (Fig. 3D, right) to levels that were comparable to those after deliberate DNA damage by exposure to Doxorubicin (Fig. 3D, center) or ES cell differentiation induced by withdrawal of 2i (Fig. 3D, right). These experiments show that (i) proliferation in the absence of cohesin causes DNA damage, and that (ii) deliberate DNA damage is sufficient to trigger a collapse of pluripotency gene expression in ES cells, reminiscent of what was reported in RNAi screens after prolonged depletion of cohesin in proliferating cells. These results are important because they question models where cohesin has a universal role in maintaining enhancer-promoter interactions.

The idea that cohesin has special functions in promoting the expression of pluripotency genes is not restricted to the maintenance of pluripotency gene expression in ES cells, but extends to the induction of pluripotency gene expression during the reprogramming of somatic cells to pluripotency by iPS. [2-4] Given that iPS reprogramming also requires multiple rounds of cell division and is sensitive to activation of stress responses [45-47] we wondered to what extent the requirement for cohesin in reprogramming reflects essential cohesin functions in the cell cycle. We addressed this question by examining early reprogramming events that occur when ES cells and somatic cells are fused to form heterokaryons because reprogramming in heterokaryons is initiated in the absence of proliferation.[51] Interestingly, acute cohesin depletion did not impair the ability of ES cells to initiate the expression of pluripotency genes in somatic nuclei.[41] On the contrary, acutely cohesin-depleted ES cells reprogrammed better than control ES cells (Table 1, top). This was explained by the expression of Myc, which was increased in cohesin-depleted ES cells in 2i

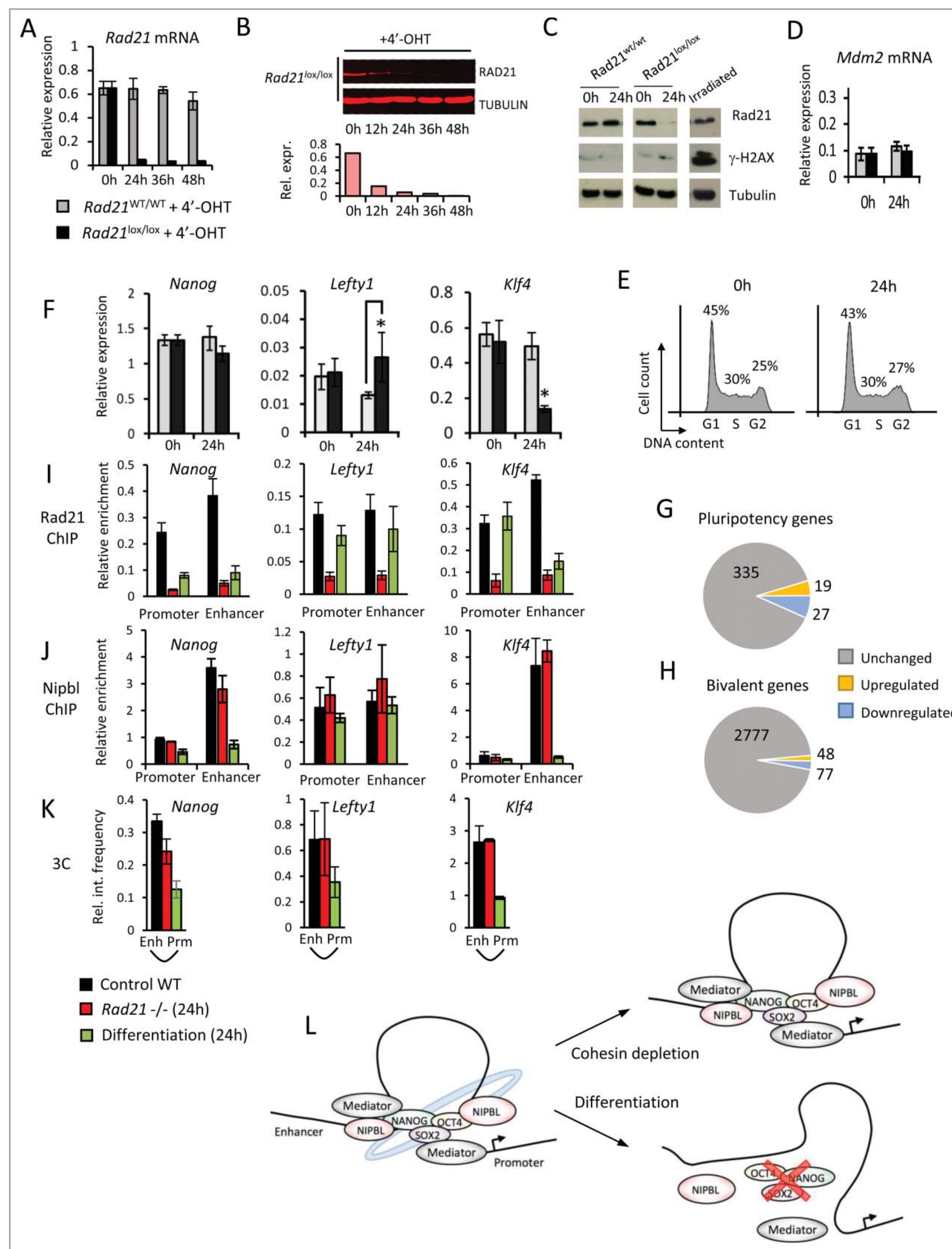


**Figure 2.** Acute cohesin depletion is compatible with pluripotency gene expression and enhancer-promoter interactions. (A-E) Time course of *Rad21* mRNA (A) and RAD21 protein depletion (B) induced by 4′-OHT-mediated activation of ERt2Cre in ERT2Cre-*Rad21*$^{lox/lox}$ ES cells (100nM 4′-OHT). Acute cohesin depletion did not result in significant DNA damage as indicated by phosphorylation of H2AX (γ-H2AX), irradiated ES cells were used as positive control (C); upregulation of the p53 target gene *Mdm2* (D) or cell cycle arrest (E). (F) Quantitative RT-PCR analysis of selected pluripotency genes in ES cells before (0h) and after acute cohesin depletion (24h). (G, H) Genome-wide expression analysis of pluripotency genes (G) and bivalent genes (H) in acutely cohesin-depleted ES cells at 24 hours. (I,J) ChIP for RAD21 (I) and NIPBL (J) at the promoters and enhancers of Nanog, Lefty1 and Klf4 in control ES cells (black), 24h Rad21-deleted ES cells (red) and differentiating ES cells (green). (K) Chromosome conformation capture (3C) assays for promoter-enhancer interactions at Nanog, Lefty1 and Klf4 in control ES cells (black), 24h Rad21-deleted ES cells (red) and differentiating ES cells (green) (L) Enhancer-promoter interactions at pluripotency loci in ES cells (left) are maintained after acute cohesin depletion (right, top) but lost during ES cell differentiation (right, bottom).

conditions.[41] Increased Myc expression drove increased DNA replication in somatic nuclei, which is known to promote reprogramming in ES cell heterokaryons.[52] Conversely, cohesin-depleted somatic cells showed reduced DNA replication and impaired reprogramming in ES cell heterokaryons, but reprogramming was rescued by nuclear transfer experiments in Xenopus oocytes, where reprogramming occurs in the absence of DNA replication.[53-55] Taken together, these experiments demonstrated that cohesin was not required for the re-expression of pluripotency genes in somatic nuclei.

To explore how prolonged cohesin depletion and DNA damage affect reprogramming we carried out cell fusions between somatic cells and ES cells that were *Rad21*-deleted 48 hours earlier and showed DNA damage, cell cycle arrest and reduced pluripotency gene expression. These fusions did not

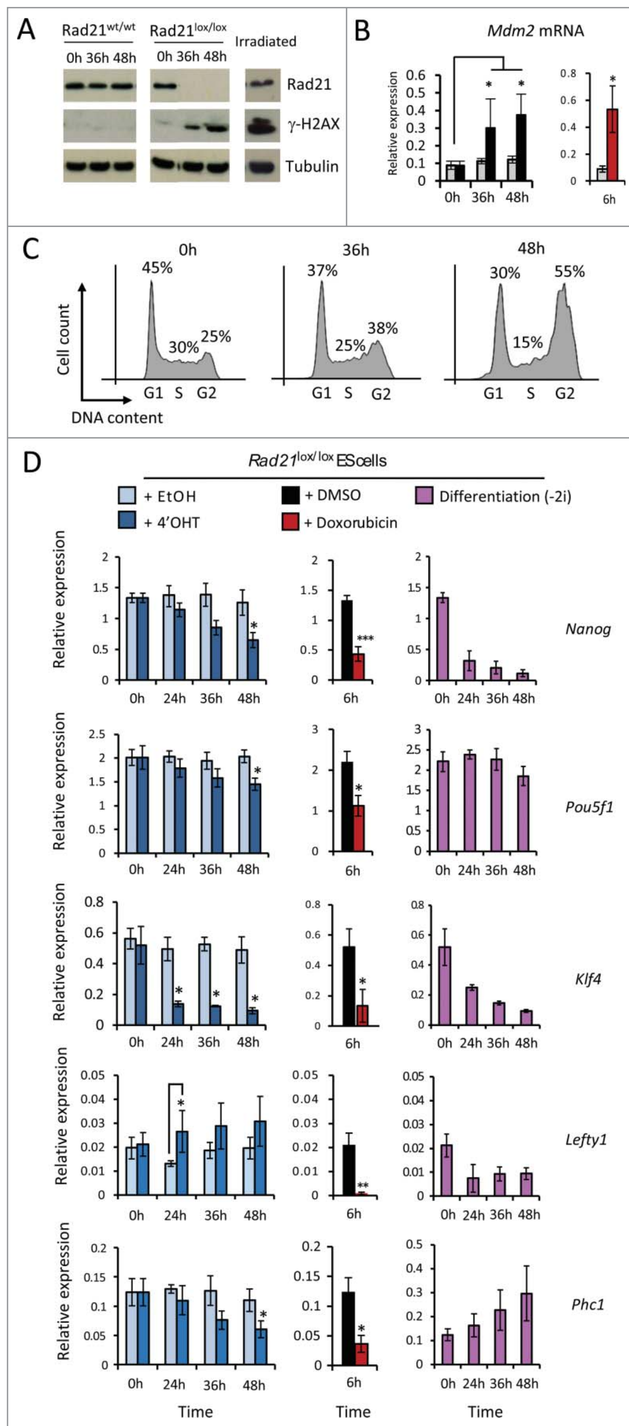


**Figure 3.** Incidental or deliberate DNA damage abolishes pluripotency gene expression. (A-C) Time course of DNA damage accumulation after cohesin depletion in proliferating ES cell indicated by phosphorylation of γ-H2AX (A), expression of the p53 target gene *Mdm2* (B) and cell cycle arrest (C). (D) Quantitative RT-PCR analysis of selected pluripotency genes in ES cells undergoing incidental DNA damage as a result of prolonged cohesin depletion (left), deliberate DNA damage inflicted by doxorubicin treatment (6h, middle) or induced differentiation (right).

result in viable heterokaryon formation or successful reprogramming (Table 1, bottom). To explore whether this failure could be ascribed to DNA damage we induced deliberate DNA damage by treating ES cells with doxorubicin for 6 hours prior to fusion with somatic cells. ES cells with DNA damage also failed to form viable heterokaryons and did not induce successful reprogramming (Table 1, bottom).

**Table 1.** Incidental or deliberate DNA damage abrogates ES cell reprogramming potential.

| ES cells: | Control | Rad21 ko (24h) | |
|---|---|---|---|
| DNA damage: | No | No | |
| ES cell # | $100\times10^6$ | $100\times10^6$ | |
| Somatic cell # | $100\times10^6$ | $100\times10^6$ | |
| Heterokaryon # | $\sim6\times10^{6*}$ | $\sim6\times10^{6*}$ | |
| Reprogramming | ++ | +++ | |
| **ES cells:** | **Control** | **Rad 21 ko (48h)** | **Doxorubicin** |
| **DNA damage:** | **No** | **Incidental** | **Deliberate** |
| ES cell # | $25\times10^6$ | $25\times10^6$ | $25\times10^6$ |
| Somatic cell # | $25\times10^6$ | $25\times10^6$ | $25\times10^6$ |
| Heterokaryon # | $\sim1.5\times10^{6*}$ | None | None |
| Reprogramming | ++ | N/A | N/A |

*Estimate based on 3% fusion efficiency determined by flow cytometry
Acutely cohesin-depleted ES cells not only retained the ability to reprogram somatic cells in heterokaryons but in addition showed an unexpected increase in their reprogramming potential (top, n = 3 biological replicates, $100 \times 10^6$ ES cells per fusion).[41] Fusion with Rad21 KO (48h) ES cells and doxorubicin-treated (6h) ES cells did not result in viable heterokaryon formation or reprogramming. Poor survival meant that lower ES cell numbers were available ($25 \times 10^6$), and control ES cell numbers were reduced accordingly (bottom, n=2 biological replicates per treatment condition).

## Discussion

We have addressed the role of cohesin in pluripotency and reprogramming. To this end we designed experimental systems with the power to separate the spectrum of cohesin functions in the cell cycle from cohesin functions in gene regulation. Unexpectedly, cohesin-depleted ES cells maintained pluripotency gene expression and the ability to reprogram somatic nuclei in heterokaryons, provided that ES cells did not incur DNA damage as a result of attempting cell division in the absence of cohesin.[41] Data presented in the current manuscript show that experimentally induced DNA damage was sufficient to erase pluripotency gene expression and to abolish heterokaryon formation and reprogramming. These findings affect the interpretation of data from previous studies that had linked cohesin with pluripotency and reprogramming where cohesin was depleted over the course of several cell divisions. We suggest that results obtained after protracted cohesin depletion should not be ascribed to long-range chromosomal interactions or other functions of cohesin in transcription. Rather, they should be re-interpreted in the context of essential cohesin functions in the maintenance of genome integrity during the cell cycle.

## Abbreviations

| | |
|---|---|
| 2i | combination of MEK and GSK3 inhibitors |
| ES | cells embryonic stem cells |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| TAD | Topologically associating domain |

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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