

The non-redundant function of cohesin acetyltransferase Esco2

Some answers and new questions

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Cohesin and cohesin regulatory proteins function in an essential pathway enabling proper cohesion and segregation of sister chromatids. Additionally, these proteins are involved in double-strand break (DSB) repair and transcriptional regulation. Mutations in Establishment of cohesion 1 homolog 2 (*Esco2*), an evolutionary conserved cohesin acetyltransferase, are the cause of Roberts syndrome (RBS), a human congenital disorder. To explore the mechanism by which the deficiency in *Esco2* affects cohesin's functions, we generated a mouse harboring a conditional *Esco2* allele. To our surprise and in marked contrast to RBS, mouse *Esco2* turns out to be a cell viability factor, the absence of which results in severe chromosome segregation defects and apoptosis. We found that the acetylation of the cohesin subunit Smc3 is significantly reduced in *Esco2*-deficient cells resulting in a marked reduction of Sororin recruitment to several, but not all cohesin bound loci. Here, we provide evidence that *Esco2* is also required for DSB repair, which is consistent with previous studies in RBS cells.

Accurate segregation of mitotic chromosomes in eukaryotic cells hinges on cohesion between sister chromatids. Cohesion is established in S phase at which time sister chromatids are generated by DNA replication and remain linked until their separation in anaphase. Sister chromatid cohesion, is mediated by the ring-like protein complex, cohesin, composed of Smc1, Smc3, Rad21 and SA1 or SA2 subunits.^{1,2} The establishment of cohesion is

thought to be associated with the acetylation of the Smc3 subunit³⁻⁶ during S phase. In higher organisms Smc3 acetylation has been linked to the recruitment of Sororin⁷ which increases of stability of cohesin's association with chromatin.⁸ In yeast, Smc3 acetylation is essential for viability and depends on an evolutionary conserved acetyltransferase, *Eco1*.^{3,4} Mammalian genomes encode two *Eco1* orthologs, *Esco1* and *Esco2*, characterized by a highly divergent N-terminus followed by a C2H2 zinc finger and an acetyltransferase domain.⁹ Both enzymes have been shown to acetylate Smc3.⁷ A deficiency in *ESCO2* in humans causes Roberts syndrome (RBS),^{10,11} characterized by craniofacial and limb dysmorphologies. RBS patients can survive to adulthood, even though many of them harbor *ESCO2* loss-of-function mutations.¹⁰ Typically, prometaphase chromosomes from RBS cells show loss of cohesion at and near the centromere, but the sister chromatids retain their parallel alignment and show a "railroad track" appearance.¹²

In view of the RBS findings, it was surprising that our recent work in the mouse showed that *Esco2* deficiency leads to a loss-of-viability in early preimplantation embryos, which failed to develop beyond the second cell division. We had established a mouse genetic model containing a floxed *Esco2* allele, which allowed us the deletion of *Esco2* in a tissue-specific manner. Deletion of *Esco2* in cortical progenitors led to cortical agenesis since *Esco2*-deficient neural progenitors underwent cell death.

Reminiscent of RBS cells, mouse embryonic fibroblasts deficient for *Esco2*

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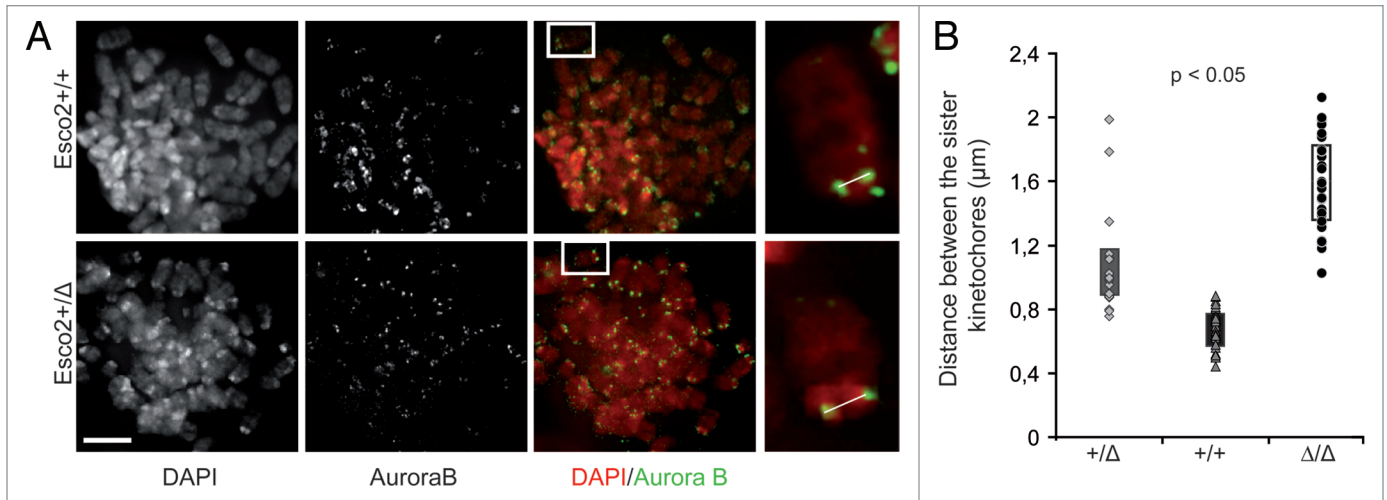


Figure 1. Deletion in single *Esco2* allele leads to measurable increase in the interkinetochore distance. (A) Prometaphase chromosomes from wild type (*Esco2*^{+/+}) and heterozygous (*Esco2*^{+/Δ}) MEFs were labeled with anti-Aurora B antibody to visualize sister kinetochores. Boxed areas depict a single chromosome in high magnification. The distance between the sisters kinetochores (white line), the position of which was determined by the peak Aurora B fluorescence, was measured in deconvoluted samples using Leica Advance Fluorescence software. The mean values from individual cells were plotted in graph (B). Scale bars-5 μm and 1 μm, n > 200, p value was obtained using Mann-Whitney U-test.

(MEFs^{*Esco2*Δ/Δ}) are characterized by parallel alignment of sister chromatids in prometaphase chromosomes, but in contrast to RBS cells, MEFs^{*Esco2*Δ/Δ} show severe chromosome segregation defects resulting in highly abnormal nuclear morphology.¹³ It is likely that the observed chromosome segregation defects in MEFs^{*Esco2*Δ/Δ} primarily result from their inability to properly establish cohesion between the sister chromatids. Support for this view comes from our finding that in the absence of *Esco2*, the acetylation of Smc3 and the recruitment of Sororin, which is essential for sister chromatid cohesion, was > 50% diminished from mid S phase until prometaphase, with the most striking reduction in Sororin binding at pericentric heterochromatin (PCH). Previous studies, using knock-down techniques in HeLa cells, suggested a partial redundancy of ESCO1 and ESCO2, since in such cells detectable changes in acetylation of SMC3 required depletion of both acetyltransferases.⁷ This discrepancy with our studies underlines the usefulness of mouse genetic models in the analysis of cohesion mechanisms. Taken together, our results thus suggest that *Esco2* has an essential function in the establishment of sister chromatid cohesion, which cannot be substituted by *Esco1*.

We noticed that the deletion of single copy of the *Esco2* gene as in MEFs^{*Esco2*+/Δ}

leads to a small, but measurable increase in inter-kinetochore distance in prometaphase chromosomes ($0.696 \pm 0.132 \mu\text{m}$ in MEFs^{*Esco2*+/+} vs. $0.988 \pm 0.212 \mu\text{m}$ in MEFs^{*Esco2*+/Δ} and $1.66 \pm 0.273 \mu\text{m}$ in MEFs^{*Esco2*Δ/Δ}, Fig. 1A and 1B). Despite the increase in distance between the sister kinetochores, heterozygosity in *Esco2* does neither significantly impact chromosome segregation nor affect embryonic development. Heterozygous animals are born at expected frequency and do not show any developmental malformations.¹³

Additionally, our recent study provides first insights toward the molecular mechanism leading to the railroad track appearance of the chromosomes in MEFs^{*Esco2*Δ/Δ}. This cytological phenotype might involve diminished phosphorylation of histone H2A-threonine 120 at the kinetochore in MEFs^{*Esco2*Δ/Δ}. This would prevent proper targeting of the cohesin protector Shugoshin (Sgo1) to the centromere.¹⁴ Indeed, we found that prometaphase chromosomes in MEFs^{*Esco2*Δ/Δ} show a reduction in Sgo1 immunofluorescence at the centromere. Interestingly, Sgo1 became localized to the chromosome arms. This change in Sgo1 localization may de-protect centromeres from the cohesin-removing prophase pathway and instead protect of cohesin at the chromosome arms. This view is consistent with the finding that the distribution of

cohesin along the prometaphase chromosomes in MEFs^{*Esco2*Δ/Δ} is rather uniform, explaining also the parallel alignment of sister chromatids.¹³

It is possible that the defect in Sgo1 recruitment to the kinetochores is an indirect consequence of deficiency in PCH cohesion in MEFs^{*Esco2*Δ/Δ}. We speculate that PCH cohesin lacking the stabilizing factor Sororin dissociates in prophase. This could affect the establishment of chromatin marks at the kinetochore, which are necessary for proper targeting of chromosomal passenger complex proteins and shugoshin to the centromere.¹⁵⁻¹⁷ The existence of a direct link between chromatin bound cohesin and histone modification was demonstrated for Haspin-mediated phosphorylation of Histone 3 at threonine 3.¹⁷ This however needs to be analyzed in more detail in *Esco2*-deficient cells. Taken together, we envisage two mechanisms, which are mutually not exclusive and which may both contribute to the cytogenetic phenotype of cells lacking *Esco2*. One is the failure to properly establish PCH cohesion in S phase. The other may be based on defects in cohesin protection at the kinetochore.

Although Smc3 acetylation and Sororin binding are markedly reduced in MEFs^{*Esco2*Δ/Δ}, both are still detectable by immunological methods. The residual Smc3 acetylation may be performed by

Esco1. We envisage two hypotheses explaining the non-redundant function of Esco2. The “dosage hypothesis” presumes that the amount of enzymatic activity provided by Esco1 might be insufficient to adequately acetylate Smc3 and hence, cohesion at critical chromosomal regions is impaired. Support for this hypothesis comes from our finding that PCH and a locus at the arm of chromosome 11, both of which are characteristically bound by high amounts of cohesin, show marked reduction in Sororin recruitment in Esco2-deficient cells.¹³ One would predict that overexpression of Esco1 should rescue or at least ameliorate the Esco2-deficiency.

The “regional specificity hypothesis” is based on the finding that in somatic vertebrate cells there are at least two distinct cohesin populations which differ from each other by the presence of either the SA1 or the SA2 subunit.^{18,19} While SA1-cohesin is specifically required for telomere cohesion,^{20,21} depletion of SA2²⁰—similar to the depletion of Esco2¹³—abolishes the cohesion at the centromeres. SA2-cohesin and Esco2 thus show overlapping “regional” specificity, which could result from higher affinity of Esco2 to a SA2-cohesin substrate. To substantiate the regional specificity hypothesis, it will be necessary to examine the levels of Smc3 acetylation in MEFs^{Esco2Δ/Δ} in SA1- and SA2-cohesins separately.

Additionally, future work should directly determine sites and levels of Smc3 acetylation at cohesin-bound loci on a genome-wide scale. At the present time, we used Sororin ChIP as surrogate readout for Smc3 acetylation. There is an agreement between our acetylation data and findings based on Sororin immunofluorescence and ChIP. While Smc3 acetylation was described to occur from yeast to mammals,^{3,4,22} Sororin or a functional ortholog has not been identified in the yeast genome yet.⁷ This raises the question of whether Smc3 acetylation in mammalian cells by Esco1 and Esco2 serves purposes additional to Sororin recruitment. For example, it

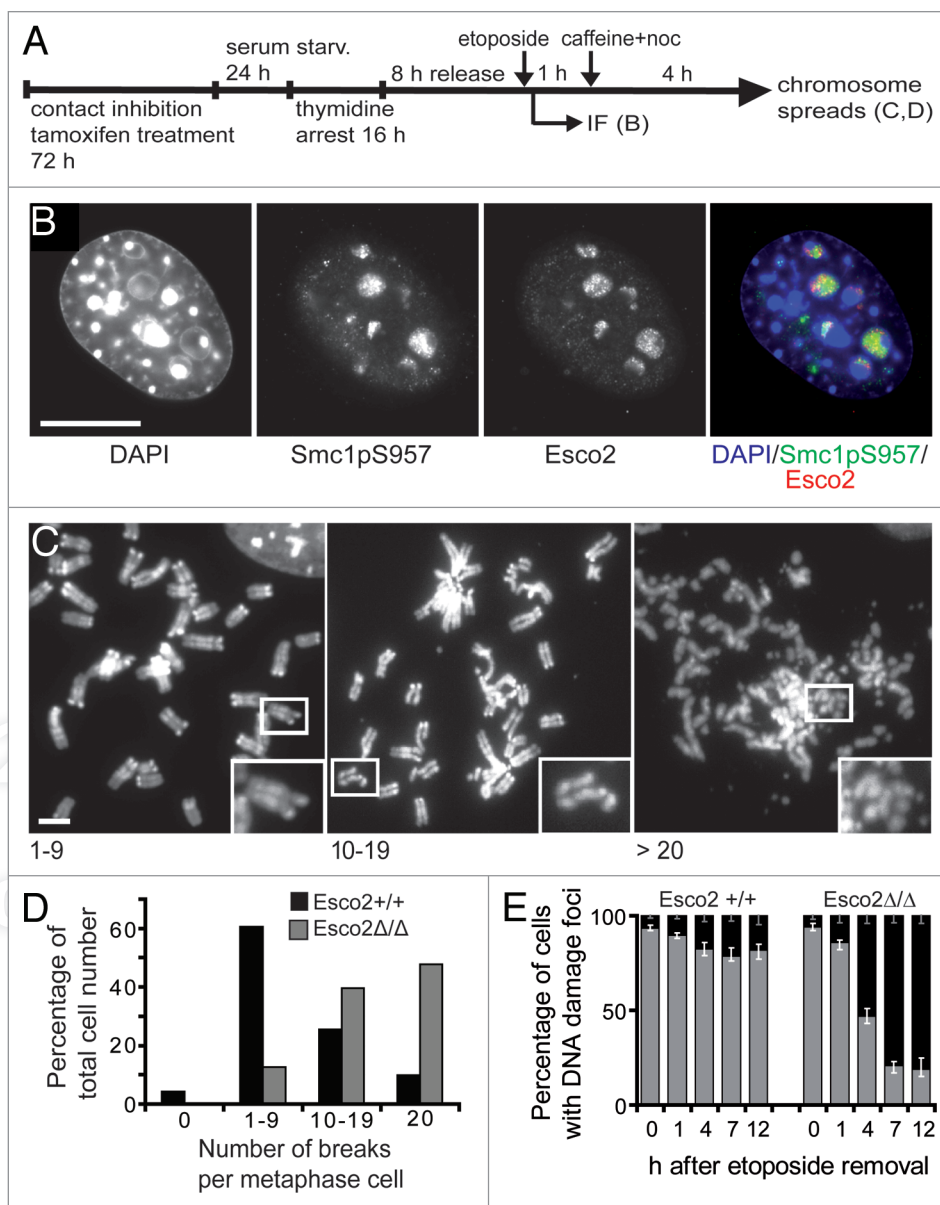


Figure 2. Esco2 localizes to the sites of DNA double strand breaks and is required for the efficient DNA DSB repair. (A) Experimental scheme. Esco2 was depleted in contact inhibited/serum starved MEFs by tamoxifen treatment. Cells were then arrested at the G1/S boundary using thymidine block. Eight h after release into S phase, double strand breaks were induced using etoposide (5 μM) or bleomycin (20 μg/ml) treatment. DNA damage checkpoint was blocked by caffeine (2 mM). (B) Efficiency of DSB induction was monitored by immunofluorescence (IF) using an antibody raised against Smc1 phosphorylated at S957 (Rockland, clone 5D11G5, dilution 1:50). In wild type MEFs, Esco2 localized to the sites of DSBs marked with Smc1pS957 IF. Scale bar 8 μm. (C) Prometaphase chromosomes isolated from wild type and Esco2-deficient MEFs were divided into four groups according to the number of breaks: chromosomes without the breaks, chromosome spreads containing between 1–9 breaks, chromosome spreads containing between 10–19 breaks and chromosome spreads containing more than 20 breaks. Examples of prometaphase chromosomes with 1–9, 10–19 and more than 20 breaks are depicted. Boxed areas are magnified in insets. Scale bar 3 μm. (D) Esco2 is required for efficient DSB repair. The frequencies of prometaphase chromosomes with 0, 1–9, 10–19 and more than 20 breaks were plotted into the bar graph (n > 500). (E) Esco2-deficient MEFs are characterized by the persistence of DNA damage foci. DSB were induced 8 h after thymidine block release and the presence of DNA damage foci was monitored on the basis H2A.XpS139 IF (Abcam, ab22551, dilution 1:200) 1, 4, 7 and 12 h after the etoposide withdrawal. The frequency of H2A.XpS139 positive (black) and H2A.XpS139 negative (gray) cells was plotted (n > 500, per group and time point).

will be interesting to compare MEFs from Sororin-deficient mice with *Esco1/Esco2* double mutant mice with regard to cohesion and gene regulation which has been shown to be regulated in part by cohesin's association with CTCF²³ or Mediator.²⁴ One reason for addressing the gene regulation issue is our observation that *Smc3* acetylation was already detected in the G₁ phase of the cell cycle, at which time there is no Sororin⁸ but gene expression takes place.

Similar to RBS cells,²⁵ yeast strains with Roberts syndrome mimicking mutation in *Eco1*'s acetyltransferase domain (*eco1-W216G*) show only mild cohesion defects, but have a severe deficiency in DNA damage repair.²⁶ During the double strand break (DSB) repair, new cohesion has to be formed in order to ensure close proximity between the broken chromatid and the repair template.²⁷ Moreover, even a limited number of DSBs will induce genome wide, postreplicative reinforcement of sister chromatid cohesion in *Eco1*-dependent manner.^{28,29} Consistent with a role of *Eco1* in DSB repair and DSB-induced cohesion enforcement, we found that upon the

induction of DSBs by etoposide or bleomycin treatment in late S/G₂ (Fig. 2A) the bulk of *Esco2* protein localizes to the sites of the DNA lesion (Fig. 2B). Our data further shows that MEFs^{*Esco2ΔΔ*} are characterized by a high frequency of chromosome fragmentation (Fig. 2C and 2D) and the persistence of histone H2AX gamma positive foci (Fig. 2E), indicating that these cells are deficient in the repair of damaged DNA. Taken together these data suggest that *Esco2* is required for DNA repair and this may be so for two reasons. In the absence of *Esco2* cohesion defects could prevent homologous recombination and DSB repair. Second, *Esco2* could be required for acetylation of *Smc3* or another substrate³⁰ directly at the site of DSB, where we observed high amount of *Esco2* protein accumulation.

Collectively, our recent study reveals that the cohesin acetyltransferases *Eco1* and *Esco2* have non-redundant functions. The paradox why human mutations in this gene do not cause lethality remains to be resolved. Alternative transcripts or suppressor mutations could contribute to generally milder cytological phenotype

and survival of RBS patients and their presence in the patient samples needs to be examined. Future work should be also directed at genome-wide changes in *Smc3* acetylation and/or Sororin binding in cells lacking *Esco1* and/or *Esco2*. Mouse lines enabling conditional inactivation of *Esco1* and *Esco2* genes will be indispensable tools to address this issue. They would also provide an entry way in the question of whether *Esco1/2* proteins contribute to the non-canonical function of cohesin.

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