Chapter 5

Towards elucidating the role of ESCO1 in sister chromatid cohesion

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ABSTRACT

After a cell has replicated its DNA in S phase of the cell cycle, the two sister chromatids are held together until cell division. This process is mediated by the cohesin complex, which is already loaded onto the DNA before replication. During S phase, the cohesin complex becomes cohesive due to acetylation of the complex by the acetyltransferase Eco1. This enzyme is well-studied in yeast. In humans, two poorly explored orthologs exist: ESCO1 and ESCO2. Of these enzymes, ESCO2 is the best studied Eco1 ortholog, because of its involvement in Roberts syndrome (RBS), a cohesinopathy associated with centromeric cohesion defects. However, recent studies suggest that ESCO1 is the Eco1 ortholog responsible for the acetylation of the cohesin complex. To learn more about the role of ESCO1 in the human cell and compare this protein with ESCO2, we studied an mCherry-tagged ESCO1 protein ectopically expressed in RBS fibroblasts that were either ESCO2-deficient or functionally corrected by ectopic expression of GFP-ESCO2. The overexpressed ESCO1 protein was able to partially correct the cohesion defects observed in ESCO2-deficient fibroblasts, indicating an overlap in function with ESCO2. A difference in regulation between ESCO1 and ESCO2 was observed; ESCO1 was present during the entire cell cycle, and its expression and localization were not influenced by S phase blockage or proteasome inhibition. A distinct ESCO1 localization on the chromosomes was found during mitosis, which was not observed for ESCO2. These differences in localization and regulation suggest distinct functions of both proteins during the cell cycle.
Sister chromatid cohesion is needed to keep sister chromatids together from the duplication of DNA in S phase, until cell division. This process is carried out by the cohesin complex, which is thought to form a ring around the two sisters. The majority of knowledge about sister chromatid cohesion has been obtained from studies in budding yeast *Saccharomyces cerevisiae*. In yeast, the cohesin complex consists of four core subunits; Smc1, Smc3, Mcd1/Scc1 and Irr1/Scc3. Together with associated proteins Pds5 and Rad61/Wpl1 this complex is loaded onto the DNA in G1 by another complex consisting of Scc2 and Scc4. When DNA is duplicated in S phase, the cohesin complex becomes active by the establishment of sister chromatid cohesion. The precise mechanism of cohesion establishment is unknown, but an essential step is assigned to the acetyltransferase Eco1. By acetylating lysine K112 and K113 of the Smc3 subunit, Eco1 establishes sister chromatid cohesion at the replication fork, because this modification counteracts the antiestablishment function of Wapl/Pds5. Besides replication-coupled cohesion establishment during S phase, double strand breaks can induce sister chromatid cohesion in G2 phase of the cell cycle, which is also Eco1-dependent.

Although the cohesin complex and its associated proteins are highly conserved, in the human cell two Eco1 orthologs exist; ESCO1 and ESCO2. The reason for this dichotomy and the function of the two orthologs have not been elucidated so far. Both proteins are required for sister chromatid cohesion, and have a conserved C-terminal domain containing a zinc finger motif and an acetyltransferase domain. Although in both proteins the N-terminus is necessary for association with the chromosomes, no similarity can be found in this domain. Western blot analysis on extracts of synchronized cells revealed that the proteins are regulated in a different manner during the cell cycle: ESCO1 is present during the whole cell cycle and possibly regulated by phosphorylation, while ESCO2 acts specifically in S phase and is regulated by proteasomal degradation. Of the two proteins, ESCO2 is the best described because of its involvement in the etiology of the cohesinopathy Roberts syndrome (RBS). Patients with Roberts syndrome have biallelic mutations in the ESCO2 gene, leading to inactivation of the acetyltransferase activity of ESCO2 and defects in sister chromatid cohesion. So far, no cohesinopathy patients with mutations in the ESCO1 gene have been described. Interestingly, siRNA experiments recognized ESCO1 as the Eco1 ortholog responsible for the acetylation of lysine residues K105 and K106 on SMC3 in the sister chromatid cohesion establishment pathway of human cells. However, there are indications that ESCO2 is also involved in this process. The cellular phenotype of ESCO2-deficient cells is already described in detail: defective chromatid cohesion and an increased sensitivity to DNA-damaging agents acting in S phase, but less is known about ESCO1. In this report, we studied the ESCO1 protein in...
more detail by ectopic expression of epitope-tagged ESCO1 and time-lapse videos. Our study confirms the difference between ESCO1 and ESCO2 in their regulation during the cell cycle. In contrast to ESCO2, ESCO1 clearly associates with condensed chromosomes. The significance of this association remains to be determined, however.

RESULTS

ESCO1 overexpression can partially complement the cohesion defect in RBS cells

Recently, we showed that ectopic expression of ESCO2 corrected the cohesion defects in RBS fibroblasts. Because of the similarity between the acetyltransferase domains of ESCO1 and ESCO2 we wondered if ESCO1 would be able to complement ESCO2-deficient RBS cells. To test this, RBS fibroblasts were generated that stably overexpress mCherry-ESCO1 (Figure 1A, B). In these cells, mCherry-ESCO1 was localized in the nucleus (Figure 1B). Cohesion defects due to the absence of ESCO2 in this cell line were partially corrected by the overexpression of mCherry-ESCO1 (Figure 1C). These results show that despite the presence of endogenous ESCO1, overexpressed mCherry-tagged ESCO1 is active in these cells, and can partially correct for ESCO2 deficiency in RBS cells, which points to (partial) overlapping activities of these proteins.

![Figure 1. mCherry-ESCO1 overexpression can partially correct the cohesion defects in Roberts syndrome (RBS) fibroblasts.](image)

- (A) ESCO1 protein expression in immortal RBS fibroblasts stably transfected with cDNA encoding mCherry-ESCO1.
- (B) Ectopically expressed mCherry-ESCO1 is localized in the nucleus.
- (C) Cohesion defects in RBS fibroblasts are partially corrected by stable transfection of mCherry-ESCO1. LN9SV cells serve as a wild type control. The percentages of cells containing the indicated number of railroad chromosomes (RR) or total premature centromere separation (PCS) were determined, as summarized in the histograms.
Figure 2. ESCO1 and ESCO2 are differentially regulated throughout the cell cycle. An immortal RBS fibroblasts cell line was obtained that stably expressed mCherry-ESCO1 as well as GFP-ESCO2. Cells were treated for 4 hours with 100 nM bortezomib to determine ESCO1 and ESCO2 protein expression levels after proteasome inhibition by (A) Western blot analysis and (B) fluorescence microscopy. (C) Time-lapse microscopy showed differences in expression levels of GFP-ESCO2, but not of mCherry-ESCO1 throughout the cell cycle (see also Supplementary video 1). (D) Endogenous ESCO1 and ESCO2 expression after synchronization of T98G glioma cells by serum deprivation. After release, samples were collected at various time points for Western blot analysis and DNA content was determined by FACS analysis. (E) Localization of mCherry-ESCO1 and GFP-ESCO2 during cell division (see also Supplementary video 2). mCherry-ESCO1 is localized at the chromosomes from prophase until telophase, while GFP-ESCO2 intensity increases during telophase. After cell division, both proteins are dispersed throughout the nucleus.
ESCO1 and ESCO2 are differentially regulated

In order to study the mCherry-tagged ESCO1 protein next to ESCO2 in the cell, GFP-ESCO2-expressing RBS cell lines were transfected with cDNA encoding mCherry-tagged ESCO1 and stable clones were obtained (Figure 2A). In this cell line (VU1199-F SV40 + GFP-ESCO2 + mCherry-ESCO1), we were able to simultaneously visualize the ESCO1 protein next to the ESCO2 protein by fluorescence microscopy. A clear difference in localization was found: ESCO1 mainly showed a diffuse localization throughout the nucleus, while ESCO2 was also localized in the nucleus and an additional accumulation of protein in the nucleoli was observed (Figure 2B). As was described earlier, ESCO2 expression levels varied throughout the cell cycle, with increased intensities in S phase, leading to differences in expression levels between cells. This was not seen for the mCherry-ESCO1 protein. In contrast to ESCO2, ESCO1 levels were quite similar amongst the cells, and did not change drastically during progression of the cell cycle (Figure 2C, Supplementary Video 1). This difference in expression levels was confirmed by Western blot analysis on endogenous ESCO1 and ESCO2 protein in synchronized T98G glioma cells (Figure 2D). Live-cell-imaging also revealed that during cell division mCherry-ESCO1 was associated with the chromosomes, which was not seen for GFP-ESCO2 (Figure 2E, Supplementary Video 2).

ESCO2 is regulated throughout the cell cycle by proteasomal degradation. To test whether ESCO1 expression is also dependent on the proteasome, VU1199-F SV40 + GFP-ESCO2 + mCherry-ESCO1 cells were treated with the proteasome inhibitor bortezomib. After treatment, ESCO2 expression levels increased, which was not observed for ESCO1 (Figure 2A, B). Consistent with our previous work\(^9\), an increase in nucleolar ESCO2 staining was observed, which was not the case for ESCO1.

Hydroxyurea (HU) arrests cells in S phase. After HU treatment ESCO1 expression levels were not affected, while ESCO2 expression levels increased (Figure 3A, B and Supplementary Video 3). Together these results indicate a marked difference between ESCO1 and ESCO2 in their regulation during the cell cycle.

DISCUSSION

In this report, we studied the ESCO1 protein in more detail by investigating ectopically expressed epitope-tagged ESCO1 in immortal human fibroblasts. We found a diffuse and permanent localization in the nucleus. During cell division, the protein was strikingly associated with condensed chromosomes. These results point to a marked difference in regulation between ESCO1 and ESCO2.

Previous results from siRNA experiments indicated that both proteins are responsible for sister chromatid cohesion and are not functionally redundant\(^8\). Our
experiments, however, indicate that there must be some degree of redundancy between the ESCO1 and ESCO2 proteins based on the observation that overexpression of ESCO1 in ESCO2-deficient RBS cells partially corrects the railroad-like chromosomes observed in RBS cells.

Complementation of RBS cells by GFP-ESCO2 indicated that epitope-tagging at the N-terminus of the protein did not interfere with its function. Although the main difference between ESCO1 and ESCO2 can be found in the N-terminal part of the proteins, partial correction of the cohesion defects in RBS cells by ectopic ESCO1 expression indicates that the N-terminal mCherry-tagged ESCO1 protein is (at least partially) functional. However, one can not rule out the possibility that this complementation is only partial due to the epitope-tagging and that complementation could have been complete if the protein had been overexpressed in a non-tagged form. The functional activity of the mCherry-tagged ESCO1 might be testable in wild type cells by expressing short hairpins against ESCO1 simultaneously with an mCherry-tagged ESCO1 cDNA construct that is resistant to the short hairpin induced knockdown by introduction of a silent mutation in the cDNA. In such a set-up, endogenous ESCO1 protein could be inactivated, while allowing to test the complementing potency of mCherry-tagged ESCO1 by itself. Unfortunately, up till now we have failed to perform such an experiment, as we have not been able to properly knock down ESCO1. Still, such an experiment would also be valuable as it could provide information about the processes the ESCO1 protein is involved in and whether its deficiency would generate a similar cellular phenotype as that observed in ESCO2-deficient RBS cells.

So far, we generated immortalized fibroblast cell lines stably expressing short hairpins against ESCO1. Although knockdown of the protein could not be shown convincingly by Western blotting (data not shown), these cell lines did show severe cohesion defects, which were even more drastic than in RBS cells, and reminiscent of the cytogenetic features observed in fibroblasts from a patient with Warsaw breakage syndrome, a cohesinopathy associated with DDX11 deficiency15. The fact that until now there are no patients found with mutations in the ESCO1 gene may suggest that ESCO1 deficiency is not compatible with life13. This might also explain our failure to make cell lines with stable ESCO1 knockdown. Future attempts to knockdown the protein with e.g. an inducible system will give a definite answer about the cellular phenotype of ESCO1 insufficiency.

Our results indicate that there is a clear distinction between ESCO1 and ESCO2, because of differences in localization and regulation in the cell. ESCO2 seems to play a role in S phase and at specific chromosomal locations, in particular the centromere. In contrast, ESCO1 seems to have a more general role in sister chromatid cohesion and SMC3 acetylation during the entire cell cycle. The association of ESCO1 with the chromosomes in
mitosis suggests that ESCO1 might be important for the cohesion establishment after DNA damage in G2/M. The difference between ESCO1 and ESCO2 may be explained by the heterogeneity at their N-terminus. This region is involved in DNA binding\(^8\), but is completely different between the two ESCO molecules. The functional differences between the proteins remain to be clarified in more detail, in which the identification of substrates for both proteins might give more definite clues. Such data will bring us closer to understanding the role of the human Eco1 orthologs in sister chromatid cohesion.

**MATERIALS AND METHODS**

**Cell culture**
The SV40-immortalized fibroblast cell line VU1199-F, derived from a patient with Roberts syndrome, was cultured in Ham’s F10 medium (Gibco, Paisley, UK) supplemented with 10% FBS (Hyclone, Logan, USA). The VU1199-F SV40 cell line stably expressing GFP-tagged ESCO2 was cultured at 150 μg/ml G418 (Calbiochem, Nottingham, UK). Stable cell lines
were generated by transfection of PvuI-linearized expression vector pIRESpuro2 containing cDNA encoding mCherry-ESCO1 in the VU1199-F SV40 and VU1199-F SV40 + GFP-ESCO2 cell lines. These stable cell lines were cultured in complete medium containing puromycin at 0.5 μg/ml. ESCO1 and ESCO2 expression levels upon proteasome inhibition were measured 4 h after incubation with 100 nM bortezomib (VUmc pharmacy). The T98G human glioblastoma cells were maintained in Dulbecco’s modified Eagle medium containing 1 g/L D-Glucose (DMEM; Invitrogen) supplemented with 10% FBS (Gibco). For synchronization by serum deprivation, subconfluent T98G cells were grown for 72 h in DMEM with 0.2% FBS, and released by re-stimulation with 10% FBS, and cells were collected at various time points.

mCherry-ESCO1 plasmid construct
Full-length ESCO1 cDNA was PCR amplified from fibroblasts with a forward cDNA primer containing a PstI restriction site followed by a XhoI restriction site and a reverse primer containing a BamHI restriction site. This PCR product was subcloned in the PstI and BamHI restriction sites of mammalian expression vector pC1 (Clontech, Mountain View, USA), in which GFP cDNA was substituted by mCherry cDNA (kindly provided by Dr. R. Toonen, VUmc). The ESCO1 cDNA was then cut with XhoI and BamHI and subcloned in the pC1 vector where it was fused to the 3’ end of a mCherry-tag. This plasmid was isolated and digested with the enzymes NheI and BamHI. The isolated mCherry-tagged ESCO1 product was ultimately cloned in the NheI and BamHI restriction sites of the mammalian expression vector pIRESpuro2 (Clontech), after which the sequence was verified.

Western blot analysis
Whole-cell extracts were prepared in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl and 1% Triton X-100 supplemented with protease and phosphatase inhibitors). T98G cell lysates were made in Giordano buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5mM EDTA, and 0.1% Triton X-100) supplemented with protease inhibitors (Pefabloc, leupeptin, pepstatin, aprotinin) and phosphatase inhibitors (Na3VO4, and β-glycerophosphate). Proteins were separated on an 8%-16% Tris-glycine gradient gel (Invitrogen, Eugene, USA) and transferred to Immobilon-P membrane (Millipore, Billerica, USA). Membranes were blocked with 5% dry milk in TBST (10 mM Tris HCl pH 7.5, 150 mM NaCl, 0.05% Tween20) probed with a rabbit anti-ESCO1 (1:1000, Bethyl laboratories, Montgomery, USA), purified guinea pig antiserum against ESCO2217-359 (1:1000, described in 18) or a mouse anti-tubulin (1:20.000, Abcam, Cambridge, UK) antibody. After washing with TBST, the membranes were incubated with peroxidase-conjugated goat immunoglobulins (DAKO, Glostrup, Denmark). Proteins were visualized with the ECL Western blotting detection system (Amersham Biosciences, Piscataway, USA).
**Fluorescence microscopy**
To study the cellular localization of ESCO1 and ESCO2, cells were grown on sterile chamber slides (Nunc, Roskilde, Denmark). Cells were fixed with 4% methanol-free formaldehyde solution (Thermo Scientific, Waltham, USA). Nuclei were stained with DAPI diluted in PBS (1 μg/ml). The chamber slides were mounted on cover slides with Vectashield mounting medium (Vector Laboratories, Burlingame, USA), and cells were examined with a fluorescence microscope (Leica, Wetzlar, Germany).

**Time-lapse fluorescence microscopy**
Stable VU1199-F SV40 + GFP-ESCO2 + mCherry-ESCO1 cells were plated on 35 mm glass-bottom culture dishes (Willco-dish, Amsterdam, The Netherlands) and transferred to a heated culture chamber (37°C, 5% CO₂) on a Zeiss Axiovert 200M microscope, equipped with a 0.55 numeric aperture condenser and a 40X Plan-Neo DIC objective (N.A. 1.3). A Photometrics Coolsnap HQ CCD camera (Scientific, Tucson, USA) with a GFP/DsRed dual band pass filter set (Chroma Technology Corp. Rockingham, USA) was used to visualize specific fluorescence. Images were processed using MetaMorph software (Universal Imaging, Downington, USA). To arrest cells in S phase, cells were treated with 1 mM hydroxyurea.
REFERENCES


