The molecular mechanism underlying Roberts syndrome involves loss of ESCO2 acetyltransferase activity

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Roberts syndrome/SC phocomelia (RBS) is an autosomal recessive disorder with growth retardation, craniofacial abnormalities and limb reduction. Cellular alterations in RBS include lack of cohesion at the heterochromatic regions around centromeres and the long arm of the Y chromosome, reduced growth capacity, and hypersensitivity to DNA damaging agents. RBS is caused by mutations in ESCO2, which encodes a protein belonging to the highly conserved Eco1/Ctf7 family of acetyltransferases that is involved in regulating sister chromatid cohesion. We identified 10 new mutations expanding the number to 26 known ESCO2 mutations. We observed that these mutations result in complete or partial loss of the acetyltransferase domain except for the only missense mutation that occurs in this domain (c.1615T>G, W539G). To investigate the mechanism underlying RBS, we analyzed ESCO2 mutations for their effect on enzymatic activity and cellular phenotype. We found that ESCO2 W539G results in loss of autoacetyltransferase activity. The cellular

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phenotype produced by this mutation causes cohesion defects, proliferation capacity reduction and mitomycin C sensitivity equivalent to those produced by frameshift and nonsense mutations associated with decreased levels of mRNA and absence of protein. We found decreased proliferation capacity in RBS cell lines associated with cell death, but not with increased cell cycle duration, which could be a factor in the development of phocomelia and cleft palate in RBS. In summary, we provide the first evidence that loss of acetyltransferase activity contributes to the pathogenesis of RBS, underscoring the essential role of the enzymatic activity of the Eco1p family of proteins.

INTRODUCTION

Roberts syndrome (RBS, MIM #268300) is an autosomal recessive developmental disorder described initially by John Roberts in affected siblings from a consanguineous Italian couple (1). Later, in four individuals from two families of European descent, Herrmann et al. (2) reported similar, but milder malformations which were referred to as SC phocomelia (MIM #269000). These two conditions are considered the same syndrome with varying phenotypic expression and hereafter, will be referred to as RBS (3). This syndrome is rare with approximately 100 cases described in the literature. Typical clinical features of RBS are pre-natal and post-natal growth retardation, bilateral symmetric limb reduction and craniofacial abnormalities (3,4). Survival is poor beyond the neonatal period. Of interest, four RBS patients have been reported with neoplastic processes including one malignant melanoma, one rhabdomyosarcoma and two ocular motor nerve cavernous angiomas (5–8).

At the cytogenetic level, RBS chromosomes present with a rod-like morphology resulting in a ‘railroad-track’ appearance due to the absence of the primary constriction at the centromeric regions and with a ‘puffing’ or ‘repulsion’ localized at their heterochromatin especially of chromosomes 1, 9 and 16, the acrocentrics, and the distal segment of the long arm of the Y chromosome (9–12). This phenomenon known as premature centromere separation (PCS) or heterochromatin repulsion (HR) constitutes the major diagnostic marker for RBS. Associated with PCS/HR is aneuploidy most likely due to outlying, lagging or prematurely advancing chromosomes during mitosis (11,13). RBS cells also exhibit hypersensitivity to clastogens such as mitomycin C (MMC), cisplatin, and gamma and ultraviolet radiation (14,15). In RBS fibroblasts, abnormalities in cell division include reduced cell growth, prolonged metaphase duration, abnormal cytokinesis and failure of cells to divide in mitosis (16).

Cohesion of sister chromatids is essential for accurate chromosome segregation and genomic stability. Cohesion is mediated by a multimeric complex known as cohesin (17,18). Cohesin is loaded onto chromatin from telophase (in mammalian cells and fission yeast) or G1 (in budding yeast) until the next mitosis (18–20). Binding of cohesin to chromatin is not sufficient for functional cohesion. In Saccharomyces cerevisiae, cohesion is established during S phase in a process dependent on Eco1/Ctf7, the founding member of a family of highly conserved acetyltransferases (21–23). Cohesion can also be induced by DNA damage in a process that is also mediated by Eco1/Ctf7 (24,25).

The causative gene for RBS is Establishment of Cohesion 1 Homolog 2 (ESCO2) with 16 different mutations reported to date (26–29). ESCO2 codes for a 601 amino acid protein belonging to the Eco1/Ctf7 family. Characteristic of members of this family, including S. cerevisiae Eco1/Ctf7, human ESCO1 (a second human homolog of Eco1/Ctf7) and ESCO2 proteins, are C2H2 zinc finger and acetyltransferase domains at the C-terminus. In some of the members of the family, this conserved C-terminal domain is fused to an N-terminal extension that is neither conserved among species nor in paralogs in the same organism (21–23,26,30–33). The ESCO2 N-terminal extension has no similarity to any other known proteins to date. Eco1/Ctf7, ESCO1 and ESCO2 exhibit in vitro autoacetyltransferase activity and it has been proposed that establishment of cohesion might be regulated directly or indirectly by the acetylation activity of these proteins (23,32,33). Based on studies of viability and fidelity of chromosome transmission in some Eco1/Ctf7 alleles defective in acetyltransferase activity in yeast, it was suggested that this enzymatic activity might not be relevant to the essential function of the protein (34). However, recent evidence indicates that reduction of Eco1/Ctf7 in vitro enzymatic activity is correlated with defects in cohesion establishment and DNA damage-induced cohesion (24,25,35). The relevance of the enzymatic activity in vertebrates is unknown.

Here, analysis of ESCO2 in RBS pedigrees revealed 10 novel mutations. Most ESCO2 mutations are predicted to cause premature stop codons that may result in truncated proteins or mRNA instability due to nonsense-mediated mRNA decay (NMD). We demonstrated protein loss for three different frameshift mutations and one nonsense mutation. In contrast, the missense mutation ESCO2 W539G did not affect mRNA or protein instability, but resulted in the disruption of the in vitro autoacetyltransferase activity of the protein. Furthermore, we found that the cytogenetic and cellular phenotypes of cells with the missense mutation were indistinguishable from the phenotypes associated with nonsense and frameshift mutations. Taken together, our results indicate the importance of ESCO2 acetyltransferase activity in human cells and strongly suggest that loss of this activity is implicated in the pathogenesis of RBS.

RESULTS

ESCO2 mutations

Mutation analysis of the DNA sequence of the 11 exons of ESCO2 in 16 pedigrees with 17 individuals who were clinically and cytogenetically diagnosed with RBS revealed 15
different mutations, 10 of which had not been reported previously. The mutations in our cohort are detailed in Table 1, Fig. 1, and Supplementary Material, Fig. S1. A total of 13 cases were apparently homozygous; for 9 of these, homozygosity was confirmed by analysis of parental DNA. Four individuals were identified as compound heterozygous. Among the 10 novel mutations we found four small deletions and two small insertions in exon sequences that lead to frameshifts predicted to produce either truncated proteins or to result in nonsense-mediated decay. In intron sequences we detected three single-nucleotide substitutions and one small deletion that putatively results in aberrant splicing of the primary transcription product. For the latter mutation, c.1132-7A>G (W539G) missense mutation leads to reduced ESCO2 acetyltransferase activity. All but one ESCO2 mutation associated with RBS cause premature stop codons prior or within the acetyltransferase domain. The one exception is a missense mutation, c.1615T>G, resulting in the substitution of a highly conserved tryptophan at position 539 by a glycine, which is located in the acetyltransferase domain (26) (Fig. 1). In order to investigate the impact of this missense mutation on the acetyltransferase activity of ESCO2 we evaluated the in vitro autoacetyltransferase activity of a C-terminal portion, including the acetyltransferase domain, of mutant and wild-type ESCO2 as previously described (33). The proteins were expressed in *E. coli* and after purification were located in the ESCO2 genomic sequence in six individuals, including members of three families previously reported, showed different haplotypes for families from the three different countries (Supplementary Material, Table S1). Mutations c.1132-7A>G and c.760_761insA, also previously reported (29), were found each in two families from different countries.

### Table 1. ESCO2 mutations identified in RBS individuals

<table>
<thead>
<tr>
<th>Family</th>
<th>Case</th>
<th>Origin</th>
<th>Mutation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location</th>
<th>Predicted effect on protein&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G197</td>
<td>USA</td>
<td>c.294_297delGAG</td>
<td>Exon 3</td>
<td>p.E98fsX2</td>
</tr>
<tr>
<td>2</td>
<td>G208&lt;sup&gt;f&lt;/sup&gt;</td>
<td>USA</td>
<td>c.308_309delAA</td>
<td>Intron 6</td>
<td>p.I377_378insLX&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>3275</td>
<td>Mexico</td>
<td>c.760delTA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Exon 3</td>
<td>p.K103fsX3</td>
</tr>
<tr>
<td>4</td>
<td>3349</td>
<td>Italy</td>
<td>c.760_761insA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Intron 3</td>
<td>p.T254fsX12&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>VU1366</td>
<td>Tunisia</td>
<td>c.1132-7A&gt;G&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Exon 3</td>
<td>p.I377_378insLX&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>6</td>
<td>3309&lt;sup&gt;f&lt;/sup&gt;</td>
<td>France</td>
<td>c.875_878delACAG</td>
<td>Intron 6</td>
<td>p.D292fsX47</td>
</tr>
<tr>
<td>7</td>
<td>VU1400</td>
<td>The Netherlands</td>
<td>c.879_880delAG&lt;sup&gt;de,f&lt;/sup&gt;</td>
<td>Exon 4</td>
<td>p.R293fsX7&lt;sup&gt;de,f&lt;/sup&gt;</td>
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<tr>
<td>8</td>
<td>3406</td>
<td>Turkey</td>
<td>c.879_880delAG&lt;sup&gt;de,f&lt;/sup&gt;</td>
<td>Exon 4</td>
<td>p.R293fsX7&lt;sup&gt;de,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>KAT</td>
<td>Turkey</td>
<td>c.879_880delAG&lt;sup&gt;de,f&lt;/sup&gt;</td>
<td>Exon 4</td>
<td>p.R293fsX7&lt;sup&gt;de,f&lt;/sup&gt;</td>
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<tr>
<td>10</td>
<td>SRSyM1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Sudan</td>
<td>c.879_880delAG&lt;sup&gt;de,f&lt;/sup&gt;</td>
<td>Exon 4</td>
<td>p.R293fsX7&lt;sup&gt;de,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>60749&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Tunisia</td>
<td>c.955+<em>2</em>+5delTAAG</td>
<td>Intron 4</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>3325</td>
<td>Denmark</td>
<td>c.1111_1112insG</td>
<td>Exon 6</td>
<td>p.T371fsX22</td>
</tr>
<tr>
<td>13</td>
<td>3303&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Turkey</td>
<td>c.1263+1G&gt;C</td>
<td>Intron 7</td>
<td>ND</td>
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<tr>
<td>14</td>
<td>3314</td>
<td>Finland</td>
<td>c.1354-18G&gt;A</td>
<td>Intron 8</td>
<td>p.V452_Q499del</td>
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<tr>
<td>15</td>
<td>UIM</td>
<td>Italy</td>
<td>c.1597_1598insT</td>
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<td>p.L533fsX5</td>
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<tr>
<td>16</td>
<td>UIA</td>
<td>Italy</td>
<td>c.1597_1598insT</td>
<td>Exon 10</td>
<td>p.L533fsX5</td>
</tr>
<tr>
<td>17</td>
<td>RIS</td>
<td>Israel</td>
<td>c.1674-2A&gt;G</td>
<td>Intron 10</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reference ESCO2 cDNA sequence used was GenBank accession NM_001017420.2.
<sup>b</sup>Reference ESCO2 protein sequence used was GenBank accession NP_001017420.1.
<sup>c</sup>Mutation previously reported (27).
<sup>d</sup>Mutation previously reported (26).
<sup>e</sup>Mutation previously reported (29).
<sup>f</sup>Using the above reference sequences the designation of these mutations has been changed from the original publications according to the standard nomenclature (52).
<sup>g</sup>Parental DNAs were not available. In other cases when a single mutation is listed, homozygosity was confirmed.

ND, not determined because RNA source not available.
in the LCL R22 bearing the c.1615T>G (W539G) mutation. We found that ESCO2 mRNA level was not reduced in this mutant with respect to wild-type controls (Fig. 2B). In contrast, human cell lines bearing the c.505C>T nonsense mutation and the c.750_751insG, c.760_761insA and c.879_880delAG frameshift mutations resulted in ESCO2 mRNA levels significantly reduced to <7% of wild type (ANOVA P < 0.0001, Dunnett’s post hoc test) most likely because of NMD. In addition, no evidence of alternative splicing was found in RT–PCR reactions (Fig. 2C). Concordantly with the mRNA analysis, when protein levels were evaluated, the c.1615T>G (W539G) mutation was detected at levels comparable to wild-type controls, while no full-length or truncated ESCO2 was detected in cell lines bearing the three frameshift mutations (Fig. 2D).

The RBS cellular phenotype produced by the c.1615T>G (W539G) missense mutation is equivalent to that produced by nonsense and frameshift mutations

To investigate if reduced acetyltransferase activity is sufficient to cause cellular defects similar to those found with other types of ESCO2 mutations, we evaluated PCS/HR, sensitivity to DNA damage and proliferation capacity in RBS LCLs. The percentage of cells with PCS/HR and the number of chromosomes affected per metaphase were analyzed in at least 75 metaphases spreads of control and RBS cells bearing the missense c.1615T>G (W539G) as well as the nonsense c.505C>T, and frameshift c.750_751insG, c.760_761insA, and c.879_880delAG mutations. We found that all five RBS cell lines presented the characteristic cytogenetic defect in 100% of the cells analyzed. In addition, there were no differences in the number of chromosomes affected per cell (Table 2). Sensitivity to DNA damaging agents was evaluated by treating RBS cells each bearing a different type of mutation, missense c.1615T>G (W539G), nonsense c.505C>T or frameshift c.750_751insG, with 0.8 μM MMC. We found that in contrast to controls and irrespective of the type of ESCO2 mutation cell viability was significantly decreased in all RBS cell lines after 48 h and continued to decrease during the 96 h of culture (Fig. 3).

To study proliferation capacity, we performed cell growth curves in RBS cell lines with the c.1615T>G (W539G), c.505C>T and c.750_751insG mutations. We found that cell growth was significantly decreased in RBS cells compared with normal cells, whereas no significant differences were found among the three RBS cell lines (Fig. 3). To determine if the reduction in cell proliferation in RBS could be due to increased cell death or to a slower progression of individual cells through the cell cycle, we analyzed cell viability and cell cycle duration in RBS and control cell lines. All RBS cell lines presented lower viability and therefore, higher cell death than control cells during the 96-h culture (Fig. 3). In contrast, no differences in cell cycle duration among RBS cell lines or between RBS and control cells were detected (Fig. 3). In particular, we investigated G2/M phase in RBS cells with the missense mutation because it was reported previously that the duration of G2/M is lengthened in RBS fibroblasts when compared with controls (16). For this purpose, we examined the mitotic index after Giemsa staining and the percentage of cells in G2/M by PI staining and flow cytometry. We found no differences among all cell lines tested suggesting that there is no evidence for a greater proportion of RBS LCLs at G2/M (Fig. 3).

DISCUSSION
ESCO2 mutations in RBS

In this study, we identified 15 mutations in 16 families affected with RBS. Of these mutations, 10 have not been reported previously increasing the number of known ESCO2 mutations to 26. These mutations include small deletions and insertions leading to frameshifts (61.5%), splice-site (23.1%), nonsense (11.6%) or missense (3.8%) mutations (26–29). At least 88% of ESCO2 mutations lead to premature stop codons prior or within the acetyltransferase domain (Fig. 1). Only two mutations are known to affect directly the acetyltransferase domain. These mutations correspond to the c.1615T>G missense mutation resulting in the substitution of a highly conserved tryptophan at the 539 position by a glycine and the c.1354-18G>A resulting in the deletion of 48 amino acids or approximately a third of the domain. No mutations have yet been described in the C2H2 zinc finger-like domain. Forty-six percent of the mutations (12/26) are found within the largest exon of ESCO2, exon 3 which consists of 808 bp representing 44.7% of the coding region. Two-thirds or eight of the mutations in this exon occur in two small regions that comprise 5.4% of the exon sequence suggesting that they are mutational hot-spots. Three of these eight mutations occur in a span of 23 nucleotides with inverted and direct repeats (normal sequence with repeats underlined GAGAGAAAGCTGATAAAAGAGAG; abnormal sequence showing a composite of the locations of the three deletion mutations c.294_297delGAGA, c.307_311delAAAGA or c.308_309delAA in lower case, GA gaga AACGTGATA aaaga/AaAGA GAG). The other five mutations occur within a
span of 21 nucleotides containing nine adenines (normal sequence GTCAGTGAAAAAAAAACTTTT; abnormal sequence showing a composite of the locations of the five mutations, c.745_746delGT, c.760_761delA or c.764_765delTT in lower case and c.750_751insG or c.760_761insA in uppercase, gt CAGT G GAAAAAAA a/A CTT tt). The adenine insertion or adenine deletion mutation, each was found in three families, presumed to be unrelated (27, present report). This area may be prone to mutations because of potential misalignment or slippage of these polyadenines during meiotic pairing or DNA replication, respectively.

Similarly, mutations c.1111_1112insA and c.1111_1112insG occur within a polyadenine-rich region in exon 6 (normal sequence TAAAAAAAACAAAAGAC, abnormal sequence showing a composite of the locations of the two mutations TAAAAAAA A/G CAAAAGAC). In exon 4, mutation c.879_880delAG was found in unrelated families and is recurrent as suggested by haplotype analysis. The occurrence of this mutation and mutation c.875_878delACAG might be favored because of the presence of direct repeats in the area where they are located (normal sequence with direct repeats underlined CAGATG A CAGAGTTTCT; abnormal sequence showing a composite of the location of the two adjacent mutations, deleted nucleotides acag and ag, CAGATG acag AG/ACAG AGTTTCT).

ESCO2 belongs to a family of proteins with in vitro autoacyltransferase activity (23,32,33). In addition, yeast Eco1/Ctf7 was found to be able to acetylate the cohesin subunits Scc1 and Scc3, and Pds5 (23). Missense mutations generated in the acetyltransferase domain of Eco1/Ctf7 and ESCO1 abolished or largely reduced the in vitro enzymatic activity of each protein (23,33). Here we show that a substitution of a highly conserved tryptophan at the 539 position by a glycine in the ESCO2 acetyltransferase domain also results in the reduction of enzymatic activity in vitro. In addition, we discard a possible effect of the W539G mutation on mRNA processing or protein stability resulting in reduction of

Table 2. PCS/HR in RBS and control lymphoblastoid cell lines

<table>
<thead>
<tr>
<th>Mutation</th>
<th>PCS/HR (%)</th>
<th>Chromosomes with PCS/Cell (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1615T&gt;G (W539G)</td>
<td>100</td>
<td>42–50</td>
</tr>
<tr>
<td>c.505C&gt;T</td>
<td>100</td>
<td>40–47</td>
</tr>
<tr>
<td>c.750_751insG</td>
<td>100</td>
<td>42–47</td>
</tr>
<tr>
<td>c.760_761insA</td>
<td>100</td>
<td>47–29</td>
</tr>
<tr>
<td>c.879_880delAG</td>
<td>100</td>
<td>30–47</td>
</tr>
<tr>
<td>WT</td>
<td>16</td>
<td>1–9</td>
</tr>
</tbody>
</table>

Metaphases with at least on chromosome affected.

Number of chromosomes with PCS/HR per cell.

Figure 2. Effect of ESCO2 mutations on mRNA levels and protein function. (A) Autoacetylation activity of W539G mutant and wild-type ESCO2. A C-terminal domain fragment (amino acids 215–601, containing a portion of amino terminal, C2H2 zinc finger and acetyltransferase domains) was tested. In the upper panel, the 14C labeling shows the acetylation activity of each protein. In lower panel loaded proteins are visualized by Coomassie blue staining. (B) mRNA levels of ESCO2 were determined using qRT–PCR analysis of mRNA isolates from RBS and control cell lines. Analysis was performed in cell lines bearing missense (c.1615T>G), nonsense (c.505C>T) and frameshift (c.750_751insG, c.760_761insA, c.879_880delAG) ESCO2 mutations. Two different cell lines with the mutation c.879_880delAG obtained from unrelated individuals were used in the analysis. Values are expressed as mean ± SEM (n = 3) of ESCO2 expression relative to β-actin expression. There was significant difference between RBS lymphoblastoid cell lines (LCLs) and control lines (ANOVA P < 0.0001, Dunnett’s post hoc test). (C) RT–PCR analysis of ESCO2 in a cell line homozygous for the missense c.1615T>G mutation and wild type. The amplified segment includes exons 5–11 (897 bp). (D) Western blot analysis of endogenous ESCO2 from RBS and control LCLs. Equal protein loading was assayed by evaluating total β-actin protein as a control. Portions of the gel shown would include proteins of the size of the full length and truncated ESCO2.
ESCO2 mRNA or protein levels in vivo. This indicates that the most likely mechanism for the c.1615T>G (W539G) mutation is loss of the enzymatic activity. Interestingly, ESCO2 mRNA was found to be elevated and western blot analysis showed no correlation between mRNA and protein amount as full-length ESCO2 mutant protein was found to be equivalent to that in controls (Fig. 2). Although we do not know the significance of these findings, lysine acetylation has been associated with protein stabilization because it prevents ubiquitination of the same residue (37,38). Possibly, transcriptional upregulation of ESCO2 may be the result of compensation for the increased instability of the non-acetylated protein.

Based on the analysis of different mutant alleles of yeast Eco1/Ctf7 with reduced in vitro autoacetyltransferase activity, the importance of the acetyltransferase domain in chromosome transmission has been questioned (34). However, recent evidence showed that in addition to being necessary for sister chromatid cohesion and chromosome transmission the acetyltransferase activity of Eco1/Ctf7 is required for cohesion induced by DNA double-strand breaks (DSB), post-replicative DSB repair and Eco1/Ctf7 genetic interaction with other proteins involved in cohesion (24,25,35). Here we also show evidence to support the importance of the acetyltransferase activity in vivo for ESCO2 in humans. We found that at the cellular level, the ESCO2 W539G mutation produces cytogenetic and cellular phenotypes equivalent to those produced by nonsense and frameshift mutations resulting in the absence of mRNA and protein. According to the model that proposes the sequence and structural similarity of Eco1/Ctf7 to the GCN5-related N-acetyltransferase superfamily (23), the tryptophan 539 residue is predicted to localize in the putative motif A of the ESCO2 acetyltransferase domain. This motif is the longest and most highly conserved among members of the superfamily and comprises the \( \beta_4 \) strand, which plays a crucial role in acetylCoA binding, and the \( \alpha_3 \) helix that together form the core of the acetylCoA binding site (39).

In RBS LCLs bearing this as well as other ESCO2 mutations that cohesion was lost in most chromosomes in 100% of cells, cell growth and viability was impaired and all cell lines were hypersensitive to cell death induced by MMC. In addition, the clinical phenotype associated with the

**Figure 3.** Comparison of the cellular phenotype in RBS LCLs with nonsense (c.505C>T), frameshift (c.750_751insG) and missense (c.1615T>G) ESCO2 mutations and controls. (A) Cell viability was studied after exposure to MMC. Viability was analyzed by FDA staining and flow cytometry every 24 h. Data were normalized to an untreated control of each cell line by determining the ratio of viable cells in exposed versus unexposed cells (n = 4, P < 0.01 between RBS and controls at 48, 72 and 96 h by ANOVA with Tukey’s post hoc tests). (B) Cell viability was determined every 24 h by FDA staining followed by flow cytometry analysis (n > 6, P < 0.01 between RBS and controls at all times tested by ANOVA with Tukey’s post hoc tests). The percent of dead cells can be interpreted from these data as it is equal to the percent of viable cells subtracted from 100%. (C) Proliferation capacity was evaluated by growth curves. Cells were counted each day with a hemocytometer (n > 4, P < 0.01 between RBS and controls at 72 and 96 h by ANOVA with Tukey’s post hoc tests). (D) Cell cycle duration was determined by incorporation of BrdU and analysis of cell cycle kinetics by the AGT method. Data correspond to the mean and error bars represent the minimum and maximum values obtained in four independent experiments. (E) Mitotic index was evaluated in cell preparations stained with Giemsa after methanol:acetic acid fixation (n > 25). (F) Fraction of cells in the G2/M cell cycle phase as measured by DNA content analysis by flow cytometry (n > 7). Values in A–C, E and F are expressed as mean ± SEM.
W539G mutation includes growth retardation, tetraphocomelia and craniofacial anomalies (11), which are indistinguishable from the phenotypic features described in RBS patients with nonsense, frameshift and splicing mutations (26,27). All together, our evidence indicates that the loss of ESCO2 acetyltransferase activity contributes significantly to the molecular mechanism underlying RBS.

Based on the abnormalities in cell growth and proliferation in RBS cells it was proposed that variations in cell cycle time together with cell loss due to defective mitosis might ultimately contribute to the reduced growth and developmental phenotype in RBS (13,16). Our evidence suggests that decreased proliferation capacity in RBS cells is related to decreased cell viability and increased cell death rather than to overall cell cycle delay. Increase in cell death is a likely result of cell cycle checkpoints activation because of the cohesin defect in RBS. Checkpoints are mechanisms that arrest the cell division cycle in response to adverse intracellular conditions, and cell death after a transient delay is one of the possible outcomes of checkpoints activation. The fact that we did not find accumulation of cells in G2/M, as previously described in RBS fibroblasts, might be related to the variable apoptotic response in different cell types. A similar difference in other cell characteristics was reported in liver and lung tissues in a fetus with RBS (40). In contrast to observations in RBS fibroblasts in culture, liver and lung cells were smaller, more regular and rounder than control nuclei and no nuclei lobulation or blebbing was detected probably due to rapid removal of abnormal micronuclei in vivo (40). In this way, activation of different checkpoints in RBS followed by rapid elimination of arrested cells might disrupt the fine balance of cell division, cell survival, cell competition and cell death required for normal embryo development. Recently, it was proposed that in addition to proliferation problems, gene expression could be critical to the etiology of the RBS (41). This proposal was based on the role of cohesin in gene silencing in yeast (42,43) and the reduced binding of cohesin to centromeric regions during mitosis in mutants of deco, the homolog of Eco1/Ctf7 in Drosophila (31). However, there is evidence that suggests the contrary as deco mutations do not affect the expression of cut in the developing wing margin (44), and the distribution and levels of chromatin-bound cohesin were unchanged in the ecol-1 mutant (45). Future work, including analysis in mice and other animal models, should allow substantiation of the hypotheses related to ESCO2 function in cohesion and embryo development.

**Materials and Methods**

**Human subjects**

Thirty-nine individuals from 16 unrelated families with RBS/SC phocomelia were ascertained. Seventeen individuals were affected and 22 were unaffected parents. All affected individuals had each of the following phenotypic features of RBS: growth retardation, craniofacial anomalies, limb reduction and PCS on cytogenetic analysis. DNA was extracted from peripheral blood lymphocytes or LCLs by a standard non-organic extraction procedure.

**Mutation analysis**

Twelve pairs of ESCO2 specific primers were used for PCR amplification of the 11 exons and 20 intron–exon boundaries (Supplementary Material, Table S2). PCR was performed in 50 μl final volume with 100 ng of genomic DNA, 0.8 mM dNTPs (0.2 mM each), MgSO4 2 mM, 0.2 μM each primer and 1 U Platinum® Taq DNA polymerase High Fidelity (Invitrogen). Purified PCR products (QiAgen) were sequenced on an ABI 3730 DNA analyzer (Applied Biosystems).

**Cell culture**

Established LCLs were cultured in RPMI 1640 medium supplemented with penicillin, streptomycin and amphotericin B (Gibco) and 10% fetal bovine serum (HyClone) and maintained at 37°C in a 5% CO2 incubator. For cell synchronization, cells were arrested in early S phase by incubation with 2 mM hydroxyurea (Wako). After 16 h, cells were washed twice and released in fresh medium.

**Autoacetylation activity**

The acetyltransferase activity assay was performed as described previously (33). Briefly, recombinant ESCO2 C-terminal domain (amino acids 215–601) wild-type and W539G fragments were expressed in BL21(DE3) E. coli cells using pET28 vectors and, later, purified over TALON metal affinity resin (BD Bioscience Clontech). For each autoacetylation reaction, 0.5 μg of the recombinant protein was added to reactions containing 50 mM HEPES (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 1 μl of [3H]-acetyl-CoA (55 mCi/mmol, Amersham) that were incubated at 30°C for 1 h. After the reaction, the mixtures were analyzed on SDS–PAGE, and proteins were visualized by Coomassie blue staining. The same gel was dried and exposed to a phosphorimager screen to detect 3H labeling.

**Quantitative RT–PCR**

Cells were synchronized in S phase with hydroxyurea for 16 h. After release of synchronization, cells were grown in media without hydroxyurea for 5 h to collect cells in S phase. Total RNA was isolated with TRIzol reagent (Invitrogen). After the removal of contaminating genomic DNA by DNase I digestion, RNA was reversely transcribed using oligo(dT) primers and the First Strand System for RT–PCR kit (Invitrogen). Real-time PCR was performed using the Quantitect SYBR green PCR kit (QiAgen) on a LightCycler 1.2 instrument (Roche Diagnostics). A segment between nucleotides 962 and 1060 of ESCO2 was amplified using the forward primer 5′-AACTGCCACTGCTATCCAA-3′ and the reverse primer 5′-TCTGAAAGTTCAGATCCC-3′. For 3′-actin, a segment between nucleotides 699 and 780 was amplified with the forward primer 5′-TGCGTGACATTAAGGAGAAG-3′ and the reverse primer 5′-GCTGAGTACTGTTTCTCCA-3′. Real-time PCR efficiencies were determined by amplification of a standardized dilution series, and slopes were determined using the LightCycler software 3.5. The corresponding efficiencies
were calculated according to the equation \( E = 10^{[\text{1/slope}]} \). Efficiency for ESCO2 was 2.89 and for \( \beta\text{-actin} \) 3.55. The efficiencies were used for relative quantification using the equation ratio = \( (E_{\text{ESCO2}})^{\Delta C P(\text{control-sample})}/(E_{\text{\beta\text{-actin}}})^{\Delta C P(\text{control-sample})} \) (46).

**Western blot**

Cells were synchronized in S phase as described before. Then 3 \( \times 10^6 \) cells were washed in phosphate-buffered saline and lysed in 50 mM Tris–HCl (pH 7.5), 0.5\% Nonidet P-40, 10\% (v/v) glycerol, 100 mM NaCl, 5 mM \( \beta\)-glycerophosphate, 50 mM NaF, 0.3 mM \( \text{Na}_3\text{VO}_4 \), and 1 \( \times \) Complete protease inhibitor cocktail (Roche) for 30 min at 4°C. After centrifugation at 50,000g for 30 min at 4°C, the supernatant was removed. Then, protein concentration was estimated by Lowry assay and 45 \( \mu \)g of protein were separated on gradient 4–15\% SDS–polyacrylamide gels and transferred to a PVDF membrane. \( \beta\)-actin protein was used as loading control. Western blot was performed using a rabbit polyclonal antibody raised against a fragment of ESCO2 between residues 216 and 317 (33). Proteins were visualized using secondary peroxidase-labeled antibodies and ECL plus western blotting detection reagent (Amersham).

**Cell proliferation and viability**

For analysis of proliferation and cell viability, 0.5 \( \times 10^6 \) cells were plated in each well of 24-well plates and every 24 h for 4 days, one plate was removed and the cells were counted in log mode. For each measurement, 10\(^4\) cells were tripled using trypan blue or stained with FDA (fluorescein diacetate) for viability. FDA (Wako) was diluted in acetone (1 mg/ml stock solution). Stock solution was added to the cell suspensions to a final concentration of 5 \( \mu \)g/ml. Staining time was 5–10 min at 37°C. Cells were collected by pipetting without washes and analyzed using a FACScalibur flow cytometer (Becton-Dickinson). FDA fluorescence signal was collected from a PVDF membrane. \( \beta\)-actin protein was used as loading control. Western blot was performed using a rabbit polyclonal antibody raised against a fragment of ESCO2 between residues 216 and 317 (33). Proteins were visualized using secondary peroxidase-labeled antibodies and ECL plus western blotting detection reagent (Amersham).

**MMC sensitivity**

MMC (Wako) was diluted in ethanol and stored at −20°C. For each cell line, 2 \( \times 10^6 \) cells were treated with 0.8 \( \mu \)M for 3 h. Then, cells were washed, seeded in fresh medium and collected every 24 h for viability analysis as described before. The viability index was established as the viability percentage of cells treated divided by the percentage of cells untreated.

**Cell cycle span**

The average generation time (AGT) method that uses differential sister-chromatid stain was used to determine the cell cycle duration (47,48). Cells growing exponentially were washed and seeded in fresh medium supplemented with 5-bromodeoxyuridine (BrdU; Sigma) at 10 \( \mu \)M. Cells were harvested every 4 h from 16 to 60 h. Thirty minutes before harvest, colcemid (10 \( \mu \)g/ml; Gibco) was added and after collection cells were treated with hypotonic 0.075 M KCl and fixed in methanol:glacial acetic acid (3:1, v/v). Air-dried chromosome preparations were stained by the fluorescence-plus-Giemsa technique (49,50). To estimate the AGT, a replicative index (RI) based on 100 cells in each time point was calculated from the proportions of cells that had completed 1 (M1), 2 (M2) or 3 (M3) cycles in BrdU, \( RI = (%M1 \times 1) + (%M2 \times 2) + (%M1 \times 3)/100 \). AGT was calculated as the number of hours in BrdU divided by the RI.

**Mitotic index and fraction of cells at G2/M**

LCLs growing exponentially were washed, seeded in fresh medium and after 36–48 h in culture were collected for MI and cell cycle distribution analysis. For mitotic index, cells without colcemid treatment were collected by centrifugation, treated in 0.075 M KCl, fixed in methanol:glacial acetic acid (3:1, v/v). Cellular suspensions were spread on slides and stained in 5% Giemsa. The mitotic index, defined as the percent of cells at mitosis, was evaluated in 1000 cells/sample in repeated experiments. Percentage of cells in G2/M was determined by cell cycle distribution analysis using propidium iodide staining followed by flow cytometry analysis as described before (51).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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**Conflict of Interest statement.** None declared.

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