Cohesin mediates Esco2-dependent transcriptional regulation in a zebrafish regenerating fin model of Roberts Syndrome

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ABSTRACT
Robert syndrome (RBS) and Cornelia de Lange syndrome (CdLS) are human developmental disorders characterized by craniofacial deformities, limb malformation and mental retardation. These birth defects are collectively termed cohesinopathies as both arise from mutations in cohesin genes. CdLS arises due to autosomal dominant mutations or haploinsufficiencies in cohesin subunits (SMC1A, SMC3 and RAD21) or cohesin auxiliary factors (NIPBL and HDAC8) that result in transcriptional dysregulation of developmental programs. RBS arises due to autosomal recessive mutations in cohesin auxiliary factor ESCO2, the gene that encodes an N-acetyltransferase which targets the SMC3 subunit of the cohesin complex. The mechanism that underlies RBS, however, remains unknown. A popular model states that RBS arises due to mitotic failure and loss of progenitor stem cells through apoptosis. Previous findings in the zebrafish regenerating fin, however, suggest that Esco2-knockdown results in transcription dysregulation, independent of apoptosis, similar to that observed in CdLS patients. Previously, we used the clinically relevant CX43 promoter to demonstrate a transcriptional role for Esco2. CX43 is a gap junction gene conserved among all vertebrates that is required for direct cell-cell communication between adjacent cells such that cx43 mutations result in oculodentodigital dysplasia. Here, we show that morpholino-mediated knockdown of smc3 reduces cx43 expression and perturbs zebrafish bone and tissue regeneration similar to those previously reported for esco2 knockdown. Also similar to Esco2-dependent phenotypes, SMC3-dependent bone and tissue regeneration defects are rescued by transgenic Cx43 overexpression, suggesting that SMC3 and Esco2 cooperatively act to regulate cx43 transcription. In support of this model, chromatin immunoprecipitation assays reveal that Smc3 and Esco2 cooperatively act to modulate Smc3 expression and perturb SMC3-dependent bone and tissue regeneration defects and severe mental retardation (Tonkin et al., 2004; Krantz et al., 2004; Gillis et al., 2004; Musio et al., 2006). Collectively, RBS and CdLS are termed cohesinopathies as they arise due to mutations in genes predominantly identified for their role in sister chromatid tethering reactions (termed cohesion) (Vega et al., 2005; Schüle et al., 2005; Girdullo et al., 2008; Krantz et al., 2004; Musio et al., 2006; Tonkin et al., 2004; Deardorff et al., 2007, 2012a,b). Cohesins are composed of two structural maintenance of chromosome (SMC) subunits, SMC1A and SMC3, and several non-SMC subunits that include RAD21 (Mcd1/Scc1), SA1, 2 (stromal antigen/Scc3/Irr1) and PDS5. At least a subset of cohesin subunits form rings that appear to topologically entrap individual DNA segments (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999; Hartman et al., 2000; Panizza et al., 2000; Haering et al., 2002; Gruber et al., 2003; Arumugam et al., 2003; Tong and Skibbens, 2014; Eng et al., 2015; Stigler et al., 2016).

RBS is an autosomal recessive disease that arises due to loss of function mutations in the ESCO2 gene that encodes an N-acetyltransferase (Ivanov et al., 2002; Bellows et al., 2003; Hou and Zou, 2005; Vega et al., 2005). ESCO2/EF02 (and ESCO1/EF01 paralog) are the human orthologues of the ECO1/CTF7 first identified in budding yeast (Skibbens et al., 1999; Toth et al., 1999; Bellows et al., 2003; Hou and Zou, 2005). All ESCO/EFO family N-acetyltransferases modify the SMC3 cohesin subunit (Zhang et al., 2008; Unal et al., 2008; Rolef Ben-Shahar et al., 2008). ESCO2 plays an essential role in sister chromatid cohesion during S phase and ensures proper chromosome segregation during mitosis. In contrast, CdLS arises due to autosomal dominant mutations in cohesin subunits (SMC1A, SMC3 and RAD21) and cohesin auxiliary factors (NIPBL and HDAC8) (Krantz et al., 2004; Tonkin et al., 2004; Schüle et al., 2005; Musio et al., 2006; Deardorff et al., 2007, 2012a,b; Girdullo et al., 2008; Yuan et al., 2015). NIPBL/Sc2 and MAU2/Sc4 heterodimer complex are required for cohesin ring opening/closing reactions that load cohesins onto DNA (Ciосk et al., 2000; Arumugam et al., 2003; Watrin et al., 2006; Bernard et al., 2006).

Extensive research provides fascinating evidence that cohesin functions beyond sister chromatid cohesion (trans-tethering that brings together two DNA molecules). Cohesins (often in combination with CTCF) also participate in various cis-tethering events including transcriptional regulation via looping and chromosome condensation through intramolecular looping such that cohesins can associate with DNA throughout the genome and in a site-specific manner (Kang et al., 2015; Poterlowicz et al., 2017; Phillips-Cremins et al., 2013; Rao et al., 2014; de Wit et al., 2015; Phillips-Cremins et al., 2013; Rao et al., 2014; de Wit et al., 2015;
Esco2 is critical for regrowth (Banerji et al., 2016). Importantly, that study revealed that 2012). Previously, we reported on a novel regenerating fin model of cohesion gene knockdowns (Mönnich et al., 2011; Morita et al., 2012; Percival et al., 2015). More recent evidence, however, is consistent with an emerging model that transcriptional dysregulation may underlie RBS as well as CdLS such that mitotic failure is present but not a causative agent of RBS (Banerji et al., 2016; Xu et al., 2013, 2014).

The zebrafish regenerating caudal fin is a valuable model system for studies related to skeletal morphogenesis (Ton and Lovine, 2013a; Pfefferl and Jaźwińska, 2015). The fin consists of 16-18 bony fin rays, each comprising bony segments flanked by fibrous joints. The tissue itself is relatively simple, with an epidermis surrounding two hemi-rays of bone matrix that in turn surround a mesenchyme that includes blood vessels, undifferentiated fibroblasts and nerves. Upon amputation, the fin regenerates rapidly via the establishment of a proliferative compartment called the blastema.

Because gene knockdown does not require systemic treatment, evaluating gene function in the regenerating fin eliminates any potentially confounding effects of embryonic lethality upon cohesion gene knockdowns (Mönnich et al., 2011; Morita et al., 2012). Previously, we reported on a novel regenerating fin model of RBS and documented the role of esco2 in skeletal and tissue regrowth (Banerji et al., 2016). Importantly, that study revealed that Esco2 is critical for connexin43 (cx43) expression. Cx43 comprises gap junctions which confer direct communication between cells through channels that allows small signaling molecules (<1000 Da) to pass (Goodenough et al., 1996). CX43 function is conserved among vertebrates, is the most abundant connexin in bone cells, and is important for skeletal development such that CX43 mutations lead to the skeletal disorder oculodentodigital dysplasia (ODDD) in humans and mice (Paznekas et al., 2003; Flenniken et al., 2005; Jones et al., 1993). In zebrafish, hypomorphic mutations in cx43 cause the short fin (srf) phenotype, which include reduced fin length, reduced bone segment length, and reduced cell proliferation (Lovine et al., 2005). Here, we provide evidence that smc3 knockdown recapitulates both esco2 and cx43 knockdown phenotypes (i.e. reduced fin and bone segment length). Critically, smc3 is required for cx43 expression. Moreover, we mapped Smc3 binding within the cx43 promoter, consistent with the model that Smc3 directly impacts cx43 expression. These studies provide proof-of-concept for a model suggesting that Esco2 activated Smc3 binds to clinically relevant skeletal regulatory genes.

RESULTS
Expression of smc3 in the regenerating fin
Esco2 is a critical regulator of fin skeletal and tissue regeneration that is required for expression of the developmental signaling factor cx43 (Banerji et al., 2016). While Esco2 is essential for modifying the cohesion subunit Smc3 to produce sister chromatid tethering and high fidelity chromosome segregation, a role for Smc3 in mediating Esco2-dependent RBS-like skeletal and tissue defects remains unknown. To address this gap in knowledge, we evaluated smc3 expression and function during fin regeneration. First, we completed in situ hybridization to monitor the temporal expression of smc3 mRNA in 1, 3, 5 and 8 days postamputated (dpa) fins. The results reveal that smc3 mRNA is strongly expressed at 3 dpa, similar to esco2 expression (Fig. 1A). smc3 expression decreased by 5 dpa and was negligible by 8 dpa (Fig. 1A). Thus, the smc3 expression mirrors that of esco2, peaking in expression at 3 dpa when regeneration is at its peak (Banerji et al., 2016; Lee et al., 2005; Hoptak-Solga et al., 2008).

Expression of esco2 mRNA is localized to the highly proliferative blastemal compartment of the fin (Banerji et al., 2016). To test whether smc3 expression is localized similarly to the blastema, we performed in situ hybridization on 3 dpa cyrosectioned fins. The results reveal that the expression of smc3 correlates with esco2 localization (Fig. 1B,C), but that smc3 also extends to the epidermis, mesenchyme and skeletal precursor cells (Fig. 1B, left panel). No staining was detected in 3 dpa...
cryosectioned fins in the absence of the smc3 probe (Fig. 1B, right panel). In combination, our studies reveal that smc3 expression temporally and, in part, spatially coincides with that of esco2 expression, consistent with a requirement during the early stage of regeneration specifically in the proliferative blastemal compartment of the regenerating fin.

**Knockdown of smc3 results in reduced regenerate length, segment length and cell proliferation**

We previously reported that Esco2 is essential for regenerate length, segment length and cell proliferation in regenerating fins (Banerji et al., 2016). Similar to esco2, smc3 is essential. This precludes the use of zygotic mutants to define gene function during adult regeneration. Therefore, we designed two independent non-overlapping morpholinos (MOs) that target Smc3: one targeting the smc3 ATG (MO1) and the second targeting the first splice site junction (exon1-intron1; e1i1) of smc3 (MO2) (Fig. 2A). Thus, MO1 blocks the translation of Smc3 whereas MO2 alters the proper splicing of smc3 pre-mRNA. All results were compared to a standard negative control MO (Std-MO) as previously described (Banerji et al., 2016; Bhadra and Iovine, 2015).

**Fig. 2. Validating the efficiency of smc3 MOs.** (A) Schematic representation of the zebrafish smc3 pre-mRNA with exons (e) represented by grey boxes and the regions between the exons the introns (i). The position of MO1 (ATG blocker) at the start codon of the smc3 gene is indicated by a blue bar (indicated on e1 with a vertical line). MO2 is positioned at the first exon and intron junction of the splice donor site (e1i1). The positions of the control primer pairs (C1-C2) are indicated with blue arrows, whereas the position of the target primer pairs (P1-P2) is indicated with red arrows. (B) Western blot analysis detects Smc3 at a predicted size of 142 kDa. Smc3 protein levels are reduced in both MO1 (62%) and MO2 (83%) fin lysates (lanes 2 and 3, respectively) compared to the Std-MO injected fin lysate (lane 1). Tubulin was used as a loading control at a predicted size of 50 kDa. Similar findings were observed in each of three trials (n=10 fins per trial). (C) Results of RT-PCR analysis using CI-C2 and P1-P2 primer pairs for verifying the efficiency of MO2. The templates for both these primer pairs are numbered from 1 to 4 as follows: (1) genomic DNA extracted from regenerating fins, (2) cDNA from fins injected with Std-MO, (3) cDNA from fins injected with MO2 and (4) no template control (NTC). We used three fins to generate genomic DNA and 10 fins to generate cDNA. The C1-C2 primer pair amplified an expected 210 bp product. In contrast, the P1-P2 pair amplified a 729 bp product in lane 3 (marked with *) due to the inclusion of intron1 (as predicted for the MO2-injected sample) compared to lane 2 (marked with +), which amplified the spliced product (as expected for the Std-MO injected sample). (D) Schematic outline of knockdown experiments. Fins are amputated (50% level) and permitted to regenerate for 3 days. At 3 dpa, either smc3 MOs (MO1 and MO2) or Std-MO was microinjected to one half of the regenerating fin keeping other half uninjected. This was immediately followed by electroporation on both injected and uninjected sides of the fin. The next day, i.e. 1 dpe or 4 dpa, the injected part of the fins were evaluated for MO uptake using a fluorescence microscope. Only those fish that showed a strong signal of the fluorescein-tagged MO were used for further experiments. For experiments such as in situ hybridization (ISH), H3P and RNA extraction for RT-PCR, the fins were harvested at 1 dpe or 4 dpa. Note that for RNA extraction, all fin rays across the fin were injected with MO and electroporated before harvesting. For regenerate length and segment length measurement and analysis, fins were allowed to regenerate for longer period and were calcein stained at 4 dpe or 7 dpa. For each experiment n=8 per trial and at least three independent trials were performed.
We first validated the efficiency of the two smc3 MOs (MO1 and MO2) by monitoring Smc3 protein levels in fins treated with MO1, MO2 or Std-MO. The results reveal that the Smc3 protein levels were significantly reduced in the Smc3 knockdown (MO1 and MO2) lysates compared to the Std-MO control lysates (Fig. 2B). To confirm the effectiveness of MO2 to block proper splicing, we performed reverse transcription polymerase chain reaction (RT-PCR). RT-PCR results revealed that intron1 was retrieved only when fins were injected with MO2 and not when injected with Std-MO (Fig. 2C). Sequencing confirmed that the products represent the smc3 gene (not shown). These analyses provide strong evidence for target specificity for both MO1 and MO2 (Eisen and Smith, 2008).

Using both MOs we carried out microinjection and electroporation as previously described (Govindan et al., 2016; Banerji et al., 2016) (Fig. 2D). All MOs are tagged with fluorescein, allowing us to validate cellular uptake microscopically 1 day postelectroporation (dpe) or 4 dpa (Ton and Iovine, 2013b). All MO-positive fins were selected for further experiments, while MO-negative fins were excluded (i.e. these fins likely represent failed electroporation). For measurement of regenerate length and segment length, smc3 knockdown/Std-MO fins were calcein stained at 4 dpe/7 dpa and measured. To reduce the effect of fin-to-fin variation, we utilized the percent similarity method in which values close to 100% indicates no difference between injected and non-injected sides of the same fin. Values less than 100% indicate reduced growth of the injected fin side compared to the non-injected side of the same fin, whereas values greater than 100% indicate increased growth of the injected fin side compared to the non-injected side (Govindan et al., 2016; Bhadra and Iovine, 2015; Banerji et al., 2016). Quantification of regenerate length was based on the distance from the plane of amputation to the distal end of the 3rd fin ray. Quantification of bone segment length was based on measurements obtained from the first segment distal to the amputation plane of the 3rd fin ray. The Std-MO injected fins showed a high percentage similarity to the uninjected side, indicating that the control MO had no effect on regenerate and bone segment length as expected. In contrast, both MO1 and MO2 showed low percentage of similarities, indicating significantly reduced growth for both regenerate length and segment length in injected fins compared to internal controls of the non-injected sides of the same fins (Fig. 3A-D; Fig. S1).

Esco2 knockdown also results in reduced cell proliferation but not elevated levels of apoptosis (Banerji et al., 2016). Thus, we next addressed whether the effect of smc3 knockdown on both regenerate length and segment length was based on altered levels of either cell proliferation or apoptosis. To test the first of these possibilities, we quantified the number of mitotic cells by staining for Histone-3 phosphate (H3P) on 1 dpe smc3 knockdown (MO1 and MO2) and Std-MO injected fins. The results reveal significant reduction in H3P-positive cells in smc3 knockdown fins compared to the control fins (Fig. 3E,F; Fig. S1). We then tested the possibility that apoptosis or programmed cell death (PCD) is increased in Smc3 depleted fins. TUNEL assays were performed on fins injected with either smc3 MO1 or Std-MO in one half of the fin, keeping the other half uninjected. Fins were harvested at 1 dpe/4 dpa for TUNEL staining. The results failed to reveal any statistically significant difference in the number of apoptotic cells between the MO1 injected and Std-MO injected fins (Fig. S2). Thus, Smc3-dependent regeneration defects in reducing cell proliferation but not elevating PCD are similar to those previously reported for Esco2 (Banerji et al., 2016). Having validated smc3-knockdown phenotypes (reduced regenerate length, segment length and cell proliferation) using two non-overlapping MOs, all subsequent experiments were performed using a single targeting smc3-MO (MO1).

smc3 and esco2 function together during skeletal regeneration

esco2 is critical for cx43 expression, although the basis for this regulation remains unknown (Banerji et al., 2016). Thus, it became important to determine if smc3-knockdown also influences cx43 expression. We performed whole-mount in situ hybridization with cx43 probe on smc3 knockdown fins. Half of the fin was injected with MO1 or Std-MO and the other half was uninjected as an internal control. The smc3 knockdown side exhibited significantly reduced expression of cx43 compared to the uninjected side (Fig. 4A). In contrast, the Std-MO injected side showed no difference in cx43 expression compared to the uninjected side (Fig. 4B). Because reduced cell proliferation is not sufficient to reduce cx43 expression (Govindan and Iovine, 2014; Bhadra and Iovine, 2015), the observed reduction of cx43 expression in smc3 knockdown fins is likely not the result of reduced cell proliferation.

To complement these studies, we next completed quantitative RT-PCR (qPCR) to confirm that cx43 expression is reduced following smc3 knockdown (Fig. 4C and Table 1; primers in Table S1). Importantly, we found that cx43, in addition to its downstream target genes sema3d and hapln1a (Ton and Iovine, 2012; Govindan and Iovine, 2014), are reduced following smc3 knockdown. Moreover, we found that expression of mono polar spindle (mps1), sonic hedgehog (shh) and sprouty4 (spry4) (Poss et al., 2002; Laforest et al., 1998; Lee et al., 2005) are not reduced in smc3 knockdown fins. Together, these findings are remarkably similar to our prior findings regarding changes in cx43 and downstream gene expressions in fins knocked down for esco2 (Banerji et al., 2016).

To provide further evidence that smc3 acts upstream of cx43, we tested for rescue of smc3-MO phenotypes by overexpressing Cx43 (Banerji et al., 2016). For this purpose, we used the transgenic line Tg(hsp70:miR-133spdp48), which overexpresses Cx43 in both regenerating heart and fins. In this line, heat shock induces expression of the miR-133 target sequence fused to EGFP and therefore sequesters the miR-133. This causes increased expression of miR-133 target genes such as cx43 (Yin et al., 2012; Banerji et al., 2016). We tested three groups of fish, as follows: (1) transgene positive and heat shocked (Tg+HS+), (2) transgene negative and heat shocked (Tg−HS+) and (3) transgene positive but not heat shocked (Tg+HS−) (Fig. 5A). Importantly, three independent heat shock trials revealed that both regenerate length and bone segment length defects otherwise exhibited in smc3 knockdown were significantly rescued in the Tg+HS+ group (Fig. 5B). This rescue was specific to transgene activation and was not induced by heat shock alone or in combination with any other group. We previously confirmed up-regulation of both cx43 mRNA and Cx43 protein levels in Tg+HS+ fins and also demonstrated that the esco2 mRNA and Esco2 protein levels are comparable between the Tg+HS+ and Tg−HS+ fins (Banerji et al., 2016). Similarly, to rule out the possibility that the transgene induces Smc3 expression, we further confirmed that Smc3 protein is not upregulated in Tg+HS+ fins compared to the Tg−HS+ fins. (Fig. 5C). These findings support an exciting model that Esco2 and Smc3 function together upstream to regulate cx43 gene expression.

Although rescue using Tg(hsp70:miR-133spdp48) supports our model that cx43 is functionally activated downstream of Esco2 and Smc3, because miR-133 has multiple targets (Yin et al., 2008), we cannot rule out the possibility that a different target gene is responsible for this rescue effect.
for the rescue. Therefore, to complement these studies we tested for synergistic interactions between esco2 and cx43, and between smc3 and cx43. First, we identified doses of the esco2 and smc3 MOs that alone did not cause skeletal phenotypes when compared to the standard control MO. We found that MO concentrations of 0.5 mM for both esco2 and smc3 were insufficient to cause skeletal defects (Fig. 6). Next, we injected these subthreshold doses of either the esco2 MO or the smc3 MO into regenerating fins of sofheterozygotes (sof/+, which carry a hypomorphic mutation in cx43 (Iovine et al., 2005). The growth and regeneration of sof/+ fins are only marginally shorter than wild-type fins and therefore represent a subthreshold activity of cx43 function. Remarkably, injection of subthreshold levels of esco2 MO significantly reduced regenerate and bone segment growth in sof/+ fins, compared to wild-type fins (Fig. 6). Moreover, injection of subthreshold levels of smc3 similarly reduced regenerate and bone segment growth in sof/+ fins, compared to wild-type fins (Fig. 6). These findings provide compelling evidence of synergy and demonstrate that esco2 and smc3 act in a common genetic pathway with cx43.

Smc3 directly binds to a specific region of the cx43 promoter
What is the basis through which both Esco2 and Smc3 regulate cx43 expression? To address this issue, we switched to a less complex AB9 fibroblast cell line previously reported to complement in vivo regenerating fin studies and express Cx43 (Bhadra et al., 2015). AB9 cells are primary fibroblasts derived from regenerating caudal fins of the adult zebrafish. We first tested whether AB9 cells also express Esco2 and Smc3. AB9 cells grown on a coverslip were fixed and processed for immunofluorescence. The results show that anti-Esco2 antibody and anti-Smc3 antibody both overlap with the DAPI-stained nuclei, revealing that both Esco2 and Smc3 are located in cell nuclei (Fig. S3). Having validated the AB9 cell system, we next tested whether either esco2 or smc3 similarly regulate Cx43 protein levels as occurs in regenerating fins. Cx43 protein levels were monitored by western blotting in AB9 cells knocked down for either esco2 MO or smc3 MO. The results show that Esco2 is reduced by ∼65%, and Smc3 is reduced by ∼60%. Critically, Cx43 protein levels also were reduced following knockdown with...
either MO (Fig. S2). Cx43 is reduced by 92% following Esco2 knockdown, and is reduced by about 68% following Smc3 knockdown. Therefore, this tissue culture AB9 system recapitulates the reduced Cx43 protein levels upon Esco2 and Smc3 knockdowns in regenerating fins (Banerji et al., 2016).

It is well established that cohesins bind directly and stabilize DNA-tethering structures required for efficient gene expression (Dorsett, 2016; Merkenschlager and Nora, 2016; Jeppsson et al., 2014). Thus, we hypothesized that Smc3, as a part of the cohesin complex, directly binds to a segment of the Cx43 promoter. The Cx43 promoter is ∼6.7 kb in length, adjacent to an additional connexin gene (cx32.2) that resides upstream of the Cx43 coding sequence (Chatterjee et al., 2005; Fig. 7A). We assayed Smc3 binding to the Cx43 promoter by performing chromatin immunoprecipitation (ChIP) on AB9 cells. We first optimized the ChIP procedure by performing ChIP on AB9 cells. We first optimized the ChIP procedure by performing ChIP on AB9 cells. We first optimized the ChIP procedure by performing ChIP on AB9 cells. We first optimized the ChIP procedure by performing ChIP on AB9 cells. We first optimized the ChIP procedure by performing ChIP on AB9 cells.

To investigate in detail the specific regions of the Cx43 promoter to which Smc3 binds, we next performed qPCR. We designed five primer pairs that spanned the Smc3 positive binding regions obtained from our qualitative PCR analysis (p2-p6) and two primer pairs as negative controls that fall within the no binding zone (p1 and p7) (Table S3). The results reveal significant binding of Smc3 specifically within one region (p2) of the Cx43 promoter (Fig. 7B). Binding was also observed at p3-p6, but at levels that did not rise to statistically significant levels. The negative controls (p1 and p7) showed negligible binding. These ChIP results provide strong evidence that Smc3 binds directly to the Cx43 promoter.

**DISCUSSION**

Esco2 mutations are the only known etiologic agent for RBS. Previously, we established esco2 knockdown in regenerating fin as a powerful system from which to elucidate the molecular basis of RBS. One major revelation of the current study is that Smc3 functions in a similar manner as Esco2 during fin regeneration. First, smc3 mRNA expression downstream of Smc3.

**Table 1.** qPCR confirms changes in gene expression downstream of Smc3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average ΔCT (smc3-MO)</th>
<th>Average ΔCT (actin)</th>
<th>ΔΔCT smc3 KD - actin</th>
<th>ΔΔCT Std-MO - actin</th>
<th>Fold difference relative to Std-MO</th>
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<tr>
<td>cx43</td>
<td>22.74±0.11</td>
<td>17.18±0.16</td>
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<td>4.37±0.15</td>
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<td>16.71±0.15</td>
<td>7.62±0.20</td>
<td>6.95±0.13</td>
<td>1.04 (0.94-1.14)</td>
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<tr>
<td>hapln1a</td>
<td>24.76±0.08</td>
<td>18.12±0.08</td>
<td>6.64±0.07</td>
<td>5.61±0.32</td>
<td>1.02 (0.94-1.11)</td>
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<tr>
<td>spry4</td>
<td>22.27±0.08</td>
<td>17.72±0.08</td>
<td>5.14±0.17</td>
<td>4.85±0.34</td>
<td>0.93 (0.85-1.02)</td>
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<tr>
<td>shh</td>
<td>22.70±0.10</td>
<td>19.51±0.05</td>
<td>5.36±0.12</td>
<td>5.24±0.30</td>
<td>0.92 (0.85-1.00)</td>
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<tr>
<td>mps1</td>
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<td>17.62±0.11</td>
<td>4.80±0.12</td>
<td>5.10±0.32</td>
<td>0.93 (0.85-1.01)</td>
</tr>
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a. The ΔCT value is determined by subtracting the average actin CT value from the average gene CT value. The standard deviation of the difference is calculated from the standard deviations of the gene and actin values using the comparative method.

b. The calculation of ΔΔCT involves subtraction by the ΔCT calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of ΔΔCT is the same as the standard deviation of the ΔCT value.

c. The range given for a gene relative to Std-MO is determined by evaluating the expression 2^−ΔΔCT with ΔΔCT+s and ΔΔCT−s, where s=the standard deviation of the ΔΔCT value.
expression coincides with esco2 expression in the proliferative blastemal compartment of the regenerating fin. Second, morpholino-mediated smc3 knockdown revealed that Smc3-dependent phenotypes (i.e. reduced regenerate length, bone segment length and cell proliferation in the absence of increased PCD) recapitulate the esco2/cx43-dependent phenotypes. Third, we see a reduction in the cx43 expression levels, and in cx43 target genes, in smc3 knockdown fins. Fourth, transgene dependent overexpression of target genes that include cx43 rescues Smc3-dependent phenotypes to a similar degree as Esco2-dependent phenotypes. Finally, we find evidence of synergistic interactions between esco2, smc3, and cx43. Thus, the combination of our current and previous findings (Banerji et al., 2016) provide compelling evidence that Esco2, Smc3, and Cx43 function in a common pathway, and suggest that RBS may be a transcriptional malady similar to that of CdLS.

A popular model is that Esco2 deficiency results in mitotic failure and progenitor cell death through apoptosis. A second revelation of the current study is that RBS developmental phenotypes may instead arise directly from reduced or altered cohesin (Smc3) binding to the promoter of clinically relevant skeletal development genes. As proof-of-concept, our ChIP experiments demonstrate that Smc3 physically binds to the cx43 promoter and is required, along with Esco2, for efficient cx43 expression.

Fig. 5. Overexpression of cx43 rescues smc3-dependent skeletal phenotypes. (A) Experimental timeline providing details of the fin amputation, MO injection/electroporation, heat shock and data analysis process. Fin amputation (50% level) was performed on transgenic hsp70:murl-1333spdd48 fish (Tg+) and their siblings (Tg−). At 3 dpa, smc3 MO was injected in one half of the fin keeping the other half uninjected. This step was immediately followed by electroporating both sides of the fin. After a period of 4 h, the heat shock process began. At this point there were three groups of fish: (1) Tg+HS+, the transgenic-positive fish that were heat shocked at 37°C for 1 h; (2) Tg+HS−, the transgenic-positive fish but were not heat shocked; (3) Tg−HS+, the siblings (transgenic-negative) that were similarly heat shocked as Tg+HS+. At 4 dpa or 1 dpe, the Tg+HS+ fins were screened for positive GFP expression, which indicated transgene induction. The control groups (Tg−HS− and Tg−HS−) were GFP negative, indicating absence of transgene induction. For regenerate length and segment length measurement and data analysis, fins were calcein stained at 7 dpa or 4 dpe. (B) The graph reveals significant rescue of smc3-dependent regenerate and segment length defects in Tg+HS− smc3 knockdown fins compared to the control groups (Tg−HS− and Tg+HS−). For each experiment n=8 fins per trial and three independent trials were performed. *P<0.05, two tailed unpaired Student’s t-test. Data are mean±s.e.m.

Fig. 6. Synergy experiments demonstrate that both esco2 and smc3 act in a common pathway with cx43. (A) The graphs representing percent similarities show that the subthreshold doses of esco2 MO (0.5 mM) and smc3 MO (0.5 mM) do not cause significant reduction in regenerate length of wild-type fins (+/+ when compared to Std-MO (0.5 mM) injected into wild-type fins (+/+). The graphs representing percent similarities show the subthreshold dose of both MOs (esco2 and smc3) significantly reduces regenerate length when injected in sof heterozygotes (sof+), compared with Std-MO (0.5 mM). (B) The graphs representing percent similarities show that the subthreshold doses of esco2 MO (0.5 mM) and smc3 MO (0.5 mM) do not cause significant reduction in segment length of wild-type fins (+/+ when compared to Std-MO (0.5 mM) injected into wild-type fins (+/+). The graphs representing percent similarities show that the sub-threshold dose of both MOs (esco2 and smc3) significantly reduce segment length when injected in sof heterozygotes (sof+), compared with Std-MO (0.5 mM). For each experiment n=8 fins per trial and 3 independent trials were performed. *P<0.05, two tailed unpaired Student’s t-test. Data are mean±s.e.m.
Both CdLS and RBS are grouped under a similar disease category of cohesinopathies, yet the etiologies of these sister maladies are considered different. Transcriptional dysregulation is considered to be the primary mechanism underlying CdLS (Krantz et al., 2004; Tonkin et al., 2004; Gillis et al., 2004; Musio et al., 2006; Deardorff et al., 2007, 2012a,b; Zhang et al., 2009). For example, defects in cis-DNA tethering events result in severe to mild phenotypes observed in CdLS. Cohesin subunits (SMC1A and SMC3) and NIPBL interact with Mediator complexes along with RNA polymerase II that bring long-distance enhancers to close proximity of the promoter of transcriptionally active genes via a cis-mediated DNA looping event (Kagey et al., 2010). The molecular mechanism underlying RBS is thought to occur through trans-tethering mitotic defects. It is true that mitotic failure and modest levels of apoptotic are often accompanied in mouse and zebrafish embryo studies of RBS and our current findings do not rule out the possibility that these can contribute to developmental defects (Mönch et al., 2011; Horsfield et al., 2012; Mönnich et al., 2011; Leem et al., 2011; Song et al., 2012; Kang et al., 2015; Poterlowicz et al., 2017; Phillips-Cremins et al., 2013; Whelan et al., 2012). However, our findings that RBS-type phenotypes (skeletal defects) can occur in the absence of apoptosis greatly diminishes these models. Instead, our data suggests a unified mechanism for both RBS and CdLS through transcriptional dysregulation (Banerji et al., 2017).

Despite the similar and overlapping phenotypes of RBS and CdL, only cells from RBS typically exhibit mitotic failure and elevated levels of apoptosis. Although the relative absence of mitotic failure in CdLS cells led researchers to propose a transcriptional dysregulation mechanism, this conclusion failed to translate to models of RBS. Based on our findings, we suggest that changes in gene dosage is a critical aspect of both CdLS and RBS phenotypes. For instance, an elegant study performed in yeast revealed differential dosage effects on a subset of cohesion-related functions (Heidinger-Pauli et al., 2010). In humans, CdLS arises due to heterozygous dominant mutations in cohesion pathway genes. Thus, one functional copy of the gene may be sufficient to support cohesion but may not be
sufficient to prevent changes in gene transcription. In contrast, RBS arises due to homozygous recessive mutations. Therefore, both copies of the ESCO2 gene are defective, which blocks all cohesion pathway function such that mitotic defects appear more prevalent and thus obscures contributions provided by transcription dysregulation. Our studies demonstrating that Esco2 and Smc3 function together to regulate cx43 expression provide compelling evidence for a more unified model linking the underlying mechanisms of Cdc5s and RBS cohesinopathies.

MATERIALS AND METHODS

Statement on the ethical treatment of animals

This study was performed strictly according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Lehigh’s Institutional Animal Care and Use Committee (IACUC) approved the protocols performed in the manuscript (Protocol identification # 190, approved 05/19/16). Lehigh University’s Animal Welfare Assurance Number is A-3877-01. All experiments were performed to minimize pain and discomfort.

Housing and husbandry

Zebrafish (Danio rerio) were housed in a re-circulating system built by Aquatic Habitats (now known as Pentair Aquatic Habitats, Apopka, FL, USA). The fish room has a 14:10 light-dark cycle with tightly regulated room temperature ranging from 27°C to 29°C (Westerfield, 1993). Monitoring of the water quality is performed automatically to maintain conductivity of 400–600 µS and pH in the range of 6.95–7.30. A biofilter is used to maintain nitrogen levels and a 10% water change occurs daily. Sequential filtration of recirculating water was carried out using pad filters, bag filters and a carbon canister before circulating over ultraviolet lights for sterilization. Fish feeding schedule was as follows: fed three times daily, once with brine shrimp (hatched from INVE arteria cysts) and twice with flake food (Aquatox AX5, Aquaneering, San Diego, CA, USA) supplemented with 7.5% micropellets (Hikari, Hayward, CA, USA), 7.5% Golden Pearl (300–500 µm, Brine Shrimp Direct, Ogden, UT, USA) and 5% Cyclo-Peeze (Argent Labs, Redmond WA, USA).

Zebrafish strains and fin amputations

Wild-type (C32), short fin (sof)123 and Tg (hsp70: miR-133sppd48) (Iovine and Johnson, 2000; Yin et al., 2012) Danio rerio animals were used. Males and females from 6 months to 1 year of age were included. All procedures involving caudal fin amputations, fin regeneration, and harvesting were performed as previously described (Banerji et al., 2016). Briefly, 0.1% tricaine solution was used for fish anesthesia and their caudal fin rays amputated at 50% level using a sterile razor blade. Regenerating fins were harvested at the required time points and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. The fixed fins were dehydrated in methanol (100%) and stored at 20°C until further use.

MO-mediated gene knockdown in regenerating fins

The MOs used in the study were all fluorescein-tagged and purchased from Gene Tools, LLC. The sequences for MOs are as follows: (MO1) smc3-ATG blocking MO: 5′-TGATCGCTATGCTAGCGCTTATGC-3′, (MO2) smc3-splice blocking MO: 5′-GCGTGGAGGTGACATCTTACGTTTATTTTT-3′, esco2 MO and Standard Control MO (Std-MO) from Banerji et al. (2016). MOs were reconstituted to a final concentration of 1 nM in sterile water. Microinjection and electroporation procedures were carried out as described in the previous studies (Banerji et al., 2016).

For synergy experiments between esco2 or smc3 and cx43, first the esco2 and smc3 MOs were tested at three different concentrations– 0.75 mM, 0.5 mM and 0.25 mM versus the Std control MO. No significant effect was observed in regenerate length and segment length for the 0.5 mM and 0.25 mM concentrations for both esco2 MO and smc3 MO1. Thus, the subthreshold concentration of 0.5 mM was selected for injecting and electroporating in 3 dpa sof123 heterozygote (sof+) regenerating fins. Microinjection and electroporation procedures were carried out as described previously (Banerji et al., 2016).

Measurements (regenerate length, segment length, cell proliferation and cell death)

MO-injected fins were calcein stained at 4 dpe/7 dpa, and regenerate length and segment length was determined as described (Du et al., 2001; Banerji et al., 2016). For detection of mitotic cells, H3P assay was performed on fins harvested at 1 dpe/4 dpa as described (Banerji et al., 2016). For detection of apoptotic cells, the TUNEL assay was performed as described in Banerji et al., 2016.

RNA extraction and RT-PCR analysis on regenerating fins

RT-PCR analysis was performed on total mRNA extracted from 1 dpe/4 dpa harvested fins that were either injected with smc3 splice blocking MO (MO2) or Std-MO injected. Trizol reagent (Gibco) was used to extract mRNA from a minimum of eight to 10 fins. For making cDNA, 1 mg of total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) using oligo (dT) primers. Two pairs of primers were used for testing the splicing efficiency. The control primer pair (C1-C2) was designed to amplify a portion of the exon 1 of smc3 mRNA whereas the targeting primer pair (P1-P2) was designed to amplify the exon 1 along with a portion of the intron1. The sequences of the control primers are as follows: C1 (forward primer) 5′-GACTGTGATGTGCTTGGCGGTTTATGC-3′ and C2 (reverse primer) 5′ GCGTGGATGTAGCAAAACTCT-3′. The sequences of the targeting primers are as follows: P1 (forward primer) 5′-GGAGGAGGTTTTATTCAAG-3′ and P2 (reverse Primer) 5′-GCTGCGAGAGCATATGAATACGAC-3′.

qPCR analysis

qPCR analysis was performed on total mRNA extracted from 1 dpe/4 dpa harvested fins as described in the above section. The qPCR primers for actin, cx43, hsp70, luna, sema3d, shh, spry4, mps1 were used at a concentration of 2.5 µM (Banerji et al., 2016; Govindan and Iovine, 2014, Table S1). Data from three biological replicates (3 dpa esco2 MO, smc3 MO2 and Std-MO injected fins) were used, with qPCR for each gene performed in duplicate as described in Banerji et al., 2016. Actin was used as a housekeeping gene and the delta C_{t} values represent expression levels normalized to actin values. Fold difference and standard deviation for the genes were determined using the method previously described (Simps et al., 2009; Ton and Kathryn Iovine, 2012; Banerji et al., 2016).

RNA probe preparation for in situ hybridization on whole-mount and cryosectioned fin

The cx43 template was made as described (Iovine et al., 2005). The smc3 template was generated using gene-specific primers (Forward primer 5′-CA-CCTTGTCTCCTTCTTATATT-3′, Reverse primer 5′-AACTTACGCCGTTGCTTCGAC-3′). The RNA polymerase T7 (RT7) binding site is highlighted in bold for the reverse primer. Digoxigenin-labeled RNA probes were generated and whole mount/ cryosection in situ hybridization was completed as previously described (Banerji et al., 2016).

Transgenic overexpression of cx43

Tg(hsp70:miR-133sp pd48) denoted as transgene-positive (Tg+) and their siblings denoted as transgene-negative (Tg−) were used in the heat shock experiment as previously described (Banerji et al., 2016). Knocking down miR-133 (which targets cx43 for degradation) via the 'spoon' transgene (three miR-133 binding sites) results in the increase of cx43 levels (Yin et al., 2012).

MO-mediated protein knockdown via electroporation in AB9 cells

AB9 (ATCC® CRL-2298™) is a primary fibroblast cell line originating from the zebrafish caudal fins. Once the cells were at 80–90% confluency in 100 mm dishes (28°C with 5% CO2) knockdown procedure was completed (Bhadra et al., 2015). Briefly, the adherent cells were washed with 1× PBS and trypsinized in 0.05% Trypsin-EDTA 1× (Gibco) for 5 min at 28°C. DMEM media supplemented with 15% heat inactivated FBS, antibiotics-antimycotics (Gibco) were added to inactivate the trypsin. The cells were collected by centrifugation at 750 rpm for 5 min. The pellet was re-suspended in 1-5 ml of HEPES buffer (115 mM NaCl, 1.2 mM CaCl2, 1810

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1.2 mM MgCl₂, 2.4 mM K₂HPO₄ and 20 mM HEPES with pH adjusted to 7.4) and put on ice. MOs were added to 400 µl of re-suspended cells in the cuvettes on ice and incubated for 5 min. The cells were electroporated at 170 V for 6-7 ms using an electroporator (Gene Pulser X Cell, BioRad). Electroporated cells were added to 1 ml of fresh media in 60 mm culture dishes and incubated at 28°C for 24 h.

**Lysate preparation and immunoblotting**

Smc3 knockdown validation was confirmed by preparing MO1, MO2 and Std-MO injected fin lysates as described in Farwell et al. (2017). For evaluating the protein expression, western blotting technique using fluorescent secondary antibody was used as previously described (Farwell and Lowe-Krentz, 2016). AB9 cell lysate was prepared and western blots performed as previously described (Bhadra et al., 2015). The antibodies used for the western blots are as follows: Cx43, Esco2, Smc3, GFP and Tubulin were detected using anti-Cx43 (1:1000, Hoptak-Solga et al., 2008), anti-Esco2 (1:1000, Banerji et al., 2016), anti-Smc3 (1:1000, Santa Cruz Biotechnology, sc-8198), anti-GFP (1:1000, Clontech) and anti-α-Tubulin (1:1000, Sigma-Aldrich, T9026) respectively. The primary antibody step was followed by incubation in fluorophore-conjugated secondary antibodies for fin lysates. These include anti-rabbit Alexa Fluor 488 or 546 (1:10,000, Invitrogen), anti-mouse Alexa Fluor 488 or 546 (1:10,000, Invitrogen) and anti-goat Alexa Fluor 488 or 546 (1:10,000, Invitrogen). For western blots using heat-shocked fin lysates and cell lysates, the primary antibody step was followed by incubation in IgG-HRP (1:10,000, BioRad) secondary antibodies. The ECL chemiluminescent reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL, USA) and X-ray films were used for signal detection. For measurement of band intensities and the percent change calculation, ImageJ software (https://imagej.nih.gov/ij/) was used. Relative pixel densities of gel bands were measured using the gel analysis tool in ImageJ software as previously described (Bhadra and Iovine, 2015). Tubulin was used as a loading control and thus the relative expression calculations were based on the ratio of Smc3 or Cx43 to Tubulin.

**Immunofluorescence on AB9 cells**

Poly-L-lysine cover glasses were used for seeding the cells as previously described (Bhadra et al., 2015). Blocking was performed using 1% BSA for 1 h at room temperature. The cover slips were incubated with the primary antibody (see above) overnight at 4°C (in a covered chamber surrounded with damp Kim wipes). Cells were incubated with the secondary antibody for 1 h at room temperature (protected from light). The secondary antibodies used were as follows: anti-rabbit Alexa Fluor 488 or 568 (1:200, Invitrogen), anti-mouse Alexa Fluor 488 or 568 (1:200, Invitrogen). DAPI (1:1000, MP Biomedicals, LLC, Santa Ana, California, USA) labels the nucleus. Cells were mounted with Vectashield (Vector Laboratories) and examined with an Eclipse TE2000-U (Nikon, Melville, NY, USA) at 40× or 60×.

**ChIP**

The ChIP protocol was performed on AB9 cells using a High-Sensitivity ChIP kit (Abcam, ab185913) according to the manufacturer’s instruction. The procedure for monolayer or adherent cells was followed with few modifications. Briefly, cells were grown to 80% confluence on 100 mm dishes (around four to six dishes per round of ChIP), trypsinized and DNA release buffer (kit) was added to the cells and incubated at 28°C for 24 h. Electroporated cells were added to 1 ml of fresh media in 60 mm culture dishes and incubated at 28°C for 24h. ChIP was subjected to sonication in three to four pulses of 10-15 s each, followed by 30-40 s rest on ice between each pulse. The sonicated chromatin was centrifuged at 12,000 rpm at 4°C for 10 min and stored at −20°C. A small amount of chromatin solution was used for DNA extraction in order to verify the size of the sheared DNA before starting the immunoprecipitation procedure (100-700 bp with a peak size of 300 bp). Antibody binding to assay wells and ChIP reactions was performed according to the manufacturer’s instructions. Antibodies used were anti-IgG (kit) and anti-Smc3 (Santa Cruz Biotechnology, sc-8198) with a concentration of 0.8 µg/well for both antibodies. The sealed strip wells with the respective antibodies and Antibody Buffer (kit) were incubated for 90 min at room temperature on an orbital shaker. The ChIP reaction was set up using the low abundance target criteria (details provided in the protocol booklet) overnight at 4°C on an orbital shaker. The next day, the wells were washed with Wash buffer (kit) and DNA release buffer and cross-links were reversed (according to the manual). The released DNA was used in PCR or qPCR reactions.

**ChIP primer design and qPCR**

The zebrafish cx43 promoter sequence was obtained from the BAC clone (DKEY-261A18). Overlapping 31 primer pairs were designed spanning the entire 6.7 kb region of the cx43 promoter (Table S2). For qPCR analysis, the primers were designed using the Primer Quest tool software (https://www.idtdna.com/PrimerQuest/Home/Index) from IDT (Table S3). Three independent samples (biological replicates) were prepared for ChIP, and qPCR reactions were performed in duplicate. ChIP DNA for non-immune IgG served as the negative control. The templates were a 1:10 dilution following ChIP using either IgG or Smc3 antibodies. PCR reactions were set up using SYBR green kit (Qiagen). Analyses of the amplified samples were performed using Rotor-Gene 6000 series software (https://www.qiagen.com/us/resources/resourcedetail?id=9dbd8ae1-1d7-4519-a1ff-b6b0b526b570-lang=en) (Corbette Research) and the average cycle number (Ct) determined for each amplicon. For fold enrichment calculation the smc3 Ct values were normalized relative to IgG control values and were represented as t (ΔΔCt). The fold enrichment was determined using the ΔΔCt method (2−ΔΔCt) as described previously (Sims et al., 2009; Ton and Kathryn Iovine, 2012; Banerji et al., 2016). Statistical significance was determined by one-way ANOVA test (P<0.001) with Tukey’s multiple comparison post hoc test (using MINITAB 17 software).

**Statistical analysis**

All graphs and error bars were generated using Microsoft Excel (2013) software. For statistical significance calculation, two-tailed unpaired t-test was performed using Graphpad software (www.graphpad.com). Statistical significance was also determined by one-way ANOVA (P<0.001) with Tukey’s multiple comparison post hoc test (using MINITAB 17 software).

**Acknowledgements**

We thank Rebecca Bowman for taking care of the zebrafish colony, Shashwati Bhattacharya for technical help during the revisions, and members of the Iovine and Skibbens laboratories for critical discussions.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


**Funding**

This work was supported by a Lehigh University Faculty Innovation Grant and a Nemes Research Laboratory Fellowship.

**Supplementary information**

Supplementary information available online at http://bio.biologists.org/lookup/doi/10.1242/bio.026013.supplemental.

This article has an associated First Person interview with the first author of the paper available online at http://bio.biologists.org/lookup/doi/10.1242/bio.026013.supplemental.
Skibbens, R. V., Corson, L. B., Koshland, D. and Hieter, P.


