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

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SUMMARY STATEMENT: This study reveals an underlying mechanism of RBS in which ESCO2 mutation results in cohesin-dependent dysregulation of transcriptional programs that include the clinically relevant signaling molecule CX43.
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 cohesion 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 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. Importantly, Smc3-dependent bone and tissue regeneration defects are rescued by transgenic Cx43 overexpression, providing evidence that Smc3 directly contributes to RBS-type phenotypes (i.e. skeletal defects) in the regenerating fin model. Moreover, chromatin immunoprecipitation (ChIP) assays reveal that Smc3 binds to a discrete region of the cx43 promoter, suggesting that Esco2 exerts transcriptional regulation of cx43 through modification of
Smc3 bound to the cx43 promoter. These findings have the potential to unify RBS and CdLS as transcription-based mechanisms.
INTRODUCTION

Roberts syndrome (RBS) is a multi-spectrum developmental disorder characterized by severe skeletal deformities resulting in craniofacial abnormalities, long-bone growth defects and mental retardation (Van den Berg and Francke, 1993; Vega et al., 2005). Infants born with severe forms of RBS are often still-born and even modest penetrance of RBS phenotypes lead to significantly decreased life expectancy (Schule et al., 2005). Cornelia de Lange Syndrome (CdLS) patients exhibit phenotypes similar to RBS patients that include severe long-bone growth defects, missing digits, craniofacial abnormalities, organ 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; Schule et al., 2005; Gordillo et al., 2008; Krantz et al., 2004; Musio et al., 2006; Tonkin et al., 2004; Deardorff et al., 2007; Deardorff et al., 2012a, Deardorff et al., 2012b). 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; Arumagam 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/EFO2 (and ESCO1/EFO1 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; Schule et al., 2005; Musio et al., 2006; Deardorff et al., 2007; Deardorff et al., 2012a, Deardorff et al., 2012b; Gordillo et al., 2008; Yuan et al., 2015). NIPBL/Scc2 and MAU2/Scc4 heterodimer complex are required for cohesin ring opening/closing reactions that load cohesins onto DNA (Ciosk et al., 2004; Arumagam 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 such as transcriptional regulation via looping and chromosome condensation such that cohesins can associate with DNA throughout the genome in a site-specific manner (Kang et al., 2015; Poterlowicz et al., 2017; Phillips-Cremins et al., 2013; Rao et al., 2014; e Wit et al., 2015; Guo et al., 2015; Tang et al., 2015; Hansen et al., 2017). Intriguingly, cohesins associate with DNA throughout the genome and in a site-specific manner (Dorsett, 2016; Kawauchi et al., 2016; Watrin et al., 2016; Kang et al., 2015; Poterlowicz et al., 2017; Phillips-Cremins et al., 2013; Rao et al., 2014; e Wit et al., 2015; Guo et al., 2015; Tang et al., 2015; Hansen et al., 2017).

Formation of both cis and trans- DNA tethers throughout the cell cycle has hampered efforts to understand the molecular etiology of cohesinopathies. For instance, work from various model
systems strongly suggest that CdLS arises through transcriptional dysregulation that involve mostly cis-DNA tethers formed during the G1 portion of the cell cycle. In contrast, a predominant view is that RBS arises through trans-tethering defects that result in mitotic failure and loss of progenitor stem cells through apoptosis (Monnich 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; Xu et al., 2014).

The zebrafish regenerating caudal fin is a valuable model system for studies related to skeletal morphogenesis (Ton and Iovine, 2013a; Pfefferli and Jazwinska, 2015). The fin is comprised of 16-18 bony fin rays, each comprised of 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 (Monnich 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 (sof^{bl23}) phenotypes, which include reduced fin length, reduced bone segment length, and reduced cell proliferation (Iovine 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 cohesin 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 post amputated (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 fins and was negligible by 8 dpa (Fig...
1A). Thus, the *smc3* expression mirrors that of *esco2* - both of which peak 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 cryosectioned fins. The results reveal that the expression of *smc3* correlates with *esco2* localization (Fig 1B and 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 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 translation of Smc3 whereas MO2 alters the *smc3* pre-mRNA proper splicing. All results were compared to a standard negative control MO (Std-MO) as previously described (Banerji et al., 2016; Bhadra and Iovine, 2015).
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 RT-PCR. RT-PCR results reveal 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 post electroporation (dpe) or 4 dpa (i.e. shown in 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 3\textsuperscript{rd} fin ray. Quantification of bone segment length was based on measurements obtained from the first segment distal to the amputation plane of the 3\textsuperscript{rd} fin ray.
The Std-MO injected fins showed a high percent 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 and 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 and 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 (Figure 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-133sp<sup>edg</sup>*) that 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 up-regulated 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.

While rescue using Tg(hsp70:miR-133spp/dep) 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 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 0.5mM for both esco2 and smc3 MOs that alone did not cause skeletal phenotypes when compared to the standard control MO. We found that MO concentrations of 0.5mM for both esco2 and smc3 MOs that alone did not cause skeletal phenotypes when compared to the standard control MO. We found that MO concentrations of 0.5mM for both esco2 and smc3 were insufficient to cause skeletal defects (Fig 6). Next, we injected these sub-threshold doses of either the esco2 MO or the smc3
MO into regenerating fins of sof heterozygotes (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 blot in AB9 cells knocked down for either esco2 MO or smc3 MO. The results show that Esco2 or Smc3 proteins were each reduced using their respective knockdown morpholinos (Fig S3). Esco2 is reduced by about 65%, and Smc3 is reduced by about 60%. Critically, Cx43 protein levels also were reduced following either
knockdown (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 \textit{cx43} promoter. The \textit{cx43} promoter is approximately 6.7 kb in length, adjacent to an additional connexin gene (\textit{cx32.2}) that resides upstream of the \textit{cx43} coding sequence (Chatterjee et al., 2005; Fig 7A). We assayed Smc3 binding to the \textit{cx43} promoter by performing Chromatin Immunoprecipitation (ChIP) on AB9 cells. We first optimized the ChIP procedure by qualitative PCR analysis and using Smc3 as the target antibody and IgG as the negative control. We designed 31 primer pairs that, in overlapping fashion, span the entire 6.7 kb promoter (Table S2). Positive Smc3 binding was observed for primers 2-6 (800bp), primer 11 (250bp) and primers 18-28 (1.5kb) (Fig 7A). In contrast, the negative control (IgG) exhibited little to no binding throughout the promoter length.

To investigate in detail the specific regions of the \textit{cx43} promoter to which Smc3 binds, we next performed qPCR. We designed 5 primer pairs that spanned the Smc3 positive binding regions obtained from our qualitative PCR analysis (p2- p6) and 2 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 \textit{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 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 previous and current
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. Cx43 represents a valuable and informative target given that mutations in human *CX43* results in ODDD, and that CdLS models similarly report aberrant expression of *CX43* (Monnich et al., 2011; Kawauchi et al., 2009). Current mechanistic models of cohesin-based regulation of gene expression indicate that cohesin stabilizes looped DNA through which distant enhancer and a proximal promoter may be brought into registration (Kang et al., 2015; Poterlowicz et al., 2017; Phillips-Cremins et al., 2013; Rao et al., 2014; e Wit et al., 2015; Guo et al., 2015; Tang et al., 2015; Hansen et al., 2017). Our results, showing that cohesin (i.e. Smc3) binds to the *cx43* promoter, combined with the established role for Esco2 in Smc3-acetylation, are consistent with a similar model in which Esco2 and Smc3 may induce expression of skeletal genes (i.e. *cx43*) through changes in chromatin architecture (Fig 8). While speculative, this model is consistent with evidence that Esco2 functions during interphase, acetylates Smc3 and that cohesins stabilize DNA loops (Kim et al., 2008; Rahman et al., 2015; Xu et al., 2013; Monnich et al., 2011; Leem et al., 2011; Song et al., 2012; Kang et al., 2015; Poterlowicz et al., 2017; Phillips-Cremins et al., 2013; Rao et al., 2014; e Wit et al., 2015; Guo et al., 2015; Tang et al., 2015; Hansen et al., 2017). Future studies are required to provide further support for such a model, including identification of the distant enhancer element and demonstration of DNA looping through cohesion.

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; Deardorff et al., 2012a, Deardorff et al., 2012b; 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 (Monnich et al., 2011; Horsfield et al., 2012; Mehta 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. While the relative absence of mitotic failure in CdLS cells lead 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 cohesin 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 CdLS 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). The fish room has a 14:10 light: dark cycle with tightly regulated room temperature ranging from 27 to 29°C (Westerfield, 1993). Monitoring of the water quality is done 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 artemia cysts) and twice with...
flake food (Aquatox AX5) supplemented with 7.5% micropellets (Hikari), 7.5% Golden Pearl (300–500 micron, Brine Shrimp direct), and 5% Cyclo-Peeze (Argent).

**Zebrafish Strains and fin amputations**

Wild-type (C32), short fin (sof^{123}) and Tg (hsp70: miR-133sp^{add}) (Iovine and Johnson, 2000; Yin et al., 2012) *Danio rerio* animals were used. Males and females from 6 months-1 year of age were included. All procedures involving caudal fin amputations, fin regeneration, and harvesting were done as previously described (Banerji et al., 2016). Briefly, 0.1% tricaine solution was used for fish anaesthetization 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.

**Morpholino-mediated gene knockdown in regenerating fins**

The morpholinos (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′-TGTACATGGCGGTTTATGC -3′, (MO2) smc3-splice blocking MO: 5′-GCGTGAGTCGCATCTTACCTGTTTA-3′, esco2 MO and Standard Control MO (Std-MO) from Banerji et al., 2016. MOs were reconstituted to a final concentration of 1 mM 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 3 different concentrations- 0.75mM, 0.5mM and 0.25mM 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 0.5mM 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, histone-3-phosphate (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 minimum of 8-10 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 exon1 along with a portion of the intron1. The sequences of the control primers are as follows: C1 (forward primer) 5’- GACTGTTATGTCTTTTGCGTG-3’ and C2 (reverse primer) 5’
GCGGTTTATGCACAAAACACT-3’. The sequences of the targeting primers are as follows: P1 (forward primer) 5’-GGAGGAGGGTGTTTAATTGCAGC-3’ and P2 (reverse Primer) 5’-GCTTCGAAAGCCCTTGAATAATGAC-3’.

**Quantitative RT-PCR (qPCR) Analysis**

The qPCR analysis was performed on total mRNA extracted from 1 dpc/4 dpa harvested fins as described in the above section. The qPCR primers for *actin*, *cx43*, *hapln1a*, *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 Ct values represent expression levels normalized to *actin* values. Fold difference and standard deviation for the genes were determined using the method previously described (Sims 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’-CAAACCTGTGGATCCCTTCAGC and reverse primer 5’-TAATACGACTCAGGCTTTCTTCAATTTCTTCT-3’). The RNA polymerase T7 (RT7) binding site is highlighted in bold for the reverse primer. Digoxigenin-labelled 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 ‘sponge’ transgene (three miR-133 binding sites) results in the increase of _cx43_ levels (Yin et al., 2012).

Morpholino-mediated protein knockdown via electroporation in AB9 cells

AB.9 (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 1X PBS and trypsinized in 0.05% Trypsin-EDTA 1X (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 minutes. The pellet was re-suspended in 1-5ml of HEPES buffer (115mM NaCl, 1.2mM CaCl₂, 1.2mM MgCl₂, 2.4mM K₂PO₄ and 20mM HEPES with pH adjusted to 7.4) and put on ice. MOs were added to 400µl of re-suspended cells in the cuvettes on ice and incubated for 5 minutes. The cells were electroporated at 170V for 6-7 ms using an electroporater (BioRad Gene Pulser X Cell). Electroporated cells were added to 1ml of fresh media in 60mm culture dishes and incubated at 28°C for 24 hours.
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 et al., 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, sc-8198), anti-GFP (1:1000, Clontech) and anti-α-Tubulin (1:1000, Sigma, T9026) respectively. The primary antibody step was followed by incubation in fluorophore-conjugated secondary antibodies for fin lysates. These include anti-rabbit Alexa 488 or 546 (1:10,000, Invitrogen), anti-mouse Alexa 488 or 546 (1:10,000, Invitrogen) and anti-goat Alexa 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) and X-ray films were used for signal detection. For measurement of band intensities and the percent change calculation, the Image J software 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 done using 1% BSA for 1 hr 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 hour at room temperature (protected from light). The secondary antibodies used were as follows: anti-rabbit Alexa 488 or 568 (1:200, Invitrogen), anti-mouse Alexa 488 or 568 (1:200, Invitrogen). DAPI (1:1000, MP Biomedicals, LLC.) labels the nucleus. Cells were mounted with Vectashield (Vector Laboratories) and examined with Nikon Eclipse TE2000-U at 40X or 60X.

**Chromatin Immunoprecipitation (ChIP)**

ChIP protocol was performed on AB9 cells using the High-Sensitivity ChIP kit (Abcam, ab185913) according to manufacturer's instruction. The procedure for monolayer or adherent cells was followed with few modifications. Briefly, cells were grown to 80%-90% confluence on 100 mm dishes (around 4-6 dishes per round of ChIP), trypsinized and centrifuged at 1000 rpm for 20 minutes. The pellet was washed with 10 mL of 1X PBS and again centrifuged at the same speed and time. For cross-linking, 9 ml DMEM medium-containing formaldehyde (final concentration of 1%) was added to the cells and incubated at room temperature for 10 minutes on a rocker. After 10 minutes 1.25 M Glycine solution was added and centrifuged at 1000 rpm for 20 minutes followed by a washing step with 10 mL of ice cold 1X PBS. After another round of centrifugation, Lysis Buffer with protease inhibitor was used to re-suspend the cell pellet (200μL/1x10^6 cells) and incubated on ice for 30 minutes with periodic vortexing. The solution
was centrifuged at 3000 rpm for 20 minutes and the chromatin pellet re-suspended with the ChIP Buffer supplied in the kit (100 μl/1x10⁶ cells). Chromatin was sheared using a tip sonicator (Branson sonifier cell disrupter 200) with a 2.4 mm tip diameter microprobe, (Qsonica P-3) set to 25% power output. Sonication was carried out in 3-4 pulses of 10-15 seconds each, followed by 30-40 seconds rest on ice between each pulse. The sonicated chromatin was centrifuged at 12,000 rpm at 4°C for 10 minutes 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 manufactures instructions. Antibodies used were anti-IgG (kit) and anti-Smc3 (Santa Cruz, 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 mins at room temperature on an orbital shaker. ChIP reaction was set up according to the low abundance target criteria (details provided in the protocol booklet) overnight at 4°C on an orbital shaker. 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 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 done using Rotor-Gene 6000 series software (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 delta Cₜ (ΔCₜ). 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 the MINITAB 17 software.)

**Statistical analysis**

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

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Competing interests

The authors declare no competing interests.

Funding

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References


Figures

(Figures can be seen here)

Figure 1

Expression of smc3 in whole mount and cryosectioned regenerating fins. (A) Expression of smc3 by whole mount in situ hybridization at various time points (1, 3, 5 and 8 days post amputation; dpa) (n=6 per timepoint). A solid line indicates the amputation plane, except in 8 dpa, where it is out of the field of view. Brackets identify regions of smc3 expression. Scale bar is 50 µm. (B) In situ hybridization on a longitudinal cryosection of a 3 dpa fin showing the tissue-specific localization of smc3 mRNA. The expression is observed in most compartments of
the regenerating fin, and appears to be localized strongly in the blastemal compartment (b) with moderate expression in epidermis (e) and proximal mesenchyme (m), and including the skeletal precursor cells (*). The no probe control (right panel) shows no expression of smc3. Melanocytes are observed in the lateral mesenchyme. The amputation plane is out of the field of view. Three independent trials were performed with different fin sections from 3 different fins. (C) Schematic representation of a longitudinal section of a 3 dpa regenerating fin showing the overlapping expression patterns of esco2 and smc3 mRNA. Lighter purple areas indicate regions of smc3 expression and dark purple area represents both, smc3 and esco2 expression.
Figure 2

Validating the efficiency of *smc3* morpholinos. (A) Schematic representation of the zebrafish *smc3* pre-mRNA with exons (e) represented with grey boxes and the regions between the exons are 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 C1-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 3 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 MO2 injected sample) compared to lane 2 (marked with +) which amplified the spliced product (as expected for 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 (Standard control morpholino) 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 day post electroporation (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), histone-3-phosphate (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 3 independent trials were performed.
Figure 3

Morpholino mediated smc3 knockdown results in regenerate length, segment length and cell proliferation reduction. (A) Representative images of uninjected (UN), smc3 MO injected (MO1) and Std-MO injected fins. Total regenerate length was calculated by measuring the distance between the amputation plane (indicated by a solid black line) to the distal end of the 3rd fin ray (black arrows indicates the length measured). (B) Graph shows the significant reduction (indicated by *) of regenerate length in smc3 knockdown fins (for both MO1 and MO2) compared to the Std-MO injected fins using the percent similarity method. (C) Representative images of calcein stained fins of uninjected (UN), smc3 MO injected (MO1) and Std-MO injected fins. Segment length was calculated by measuring the distance between first two joints in the 3rd fin ray (black arrows indicates the length measured). Higher magnification images of segments are shown with joints indicated by white arrowheads. (D) Graph shows that significant reduction (indicated by *) of segment length in smc3 knockdown (for both MO1 and MO2)
compared to Std-MO injected fins using the percent similarity method. (E) Representative images of H3P-positive cells in uninjected (UN), smc3 MO injected (MO1) and Std-MO injected fins. Measurements were taken from the distal most 250μm of the 3rd ray. White brackets mark the defined area and n represents the number of H3P-positive cells in that area. Arrows identify H3P-positive cells. (F) Graph shows the significant reduction (indicated by *) in the number of H3P-positive cells in smc3 knockdown (for both MO1 and MO2) compared to Std-MO injected fins using the percent similarity method. For each experiment n=8 fins per trial and 3 independent trials were performed. For statistical significance, two tailed unpaired Student's t-test was used where P<0.05. Mean± s.e.m. is represented by error bars. Scale bar is 50 μm for panels A and C. Scale bar is 100 μm for panel C.
**Figure 4**

**smc3 regulates the expression of cx43 in regenerating fins.** (A) Representative image of a fin with smc3-knockdown side (smc3-MO) showing decreased cx43 staining compared to the uninjected side (UN). Higher magnification of the smc3 knockdown side of the same fin shows reduced levels of cx43 expression compared to the uninjected side (UN), which shows normal cx43 levels. (B) Representative image of Std-MO injected fin revealing similar cx43 levels in both injected and uninjected side (UN). Higher magnification of the same fin shows normal and similar levels of cx43 expression in both injected and uninjected sides (UN). For this experiment n=6 fins per trial and 3 independent trials were performed. A solid line indicates the amputation plane and scale bar is 100 µm and 50 µm. (C) Quantitative RT-PCR (qPCR) confirms downregulation of cx43 and cx43-dependent target genes (sema3d and hapln1a) following Smc3 knockdown. Fold difference values from the qPCR are shown in the graph. A fold difference of 1 is considered as no change with respect to Std-MO injected fins (indicated by the horizontal line). Three independent Smc3 knockdown samples were prepared. Each sample was tested in
duplicates (trials 1-3) for cx43, hapln1a, sema3d, shh, spry4 and mps1 (compared to the internal reference gene, actin). Each of the three trials are denoted by open circles and the averages are denoted by solid circles.
Figure 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:miR-133sp<sup>pol</sup> 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 hours, the heat shock process began. At this point there were total 3 groups of fish: (1) Tg+HS+ is the transgenic-positive fish that were heat shocked at 37°C for 1 hour, (2) Tg+HS- is the transgenic-positive fish but were not heat shocked and (3) Tg-HS+ were 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 (indicated by *) 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 3 independent trials were performed. For statistical significance, two tailed unpaired Student's t-test was used where $P<0.05$. Mean± s.e.m. is represented by error bars. Scale bar is 50 µm for all panels. (C) Smc3 protein expression is nearly similar (90%) in the Tg-HS+ (lane 1) and Tg+HS+ (lane 2) fin lysates (normalized to Tubulin). In contrast, Cx43 protein is increased in Tg+HS+ (lane 2) fin lysate compared to Tg-HS+ (lane 1) fin lysate, as expected. Similarly, GFP protein expression is also increased in Tg+HS+ fin lysate (lane 2) compared to Tg-HS+ fin lysate. Tubulin (50 kDa) was used as the loading control for all blots. Image J software was used for analysis of relative band intensity.
Synergy experiments demonstrate that both esco2 and smc3 act in a common pathway with cx43. (A) The graphs representing percent similarities show that the sub-threshold 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 that the sub-threshold dose of both MOs (esco2 and smc3) significantly reduce regenerate length when injected in sof heterozygotes (sof/+), compared with Std-MO (0.5 mM). (B) The graphs representing percent similarities show that the sub-threshold 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 (esc02 and smc3) significantly reduce segment length when injected in sof heterozygotes (sof/+) compared with Std-MO (0.5mM). For each experiment n=8 fins per trial and 3 independent trials were performed. For statistical significance (indicated by *), two tailed unpaired Student's t-test was used where P<0.05. Mean± s.e.m. is represented by error bars.
**Figure 7**

**Smc3 binds at a specific location of the cx43 promoter** (A) Schematic representation of the zebrafish cx43 promoter. It is approximately 6.7 kb in length, adjacent to an additional connexin gene (cx32.2). The horizontal bars indicate the binding regions of Smc3 inferred from qualitative PCR results. The positions of the seven qRT-PCR primer pairs (p1-p6) are indicated on the promoter region. The two primer pairs (p1 and p7) are the negative controls, since they lie at a region not predicted from previous PCR results. (B) The graph represents the fold enrichment of Smc3 binding (normalized to IgG) at different regions of the cx43 promoter. Significant enrichment was observed at p2 location of the promoter suggesting positive binding of Smc3 at the p2 region. Statistical significance was determined by one-way ANOVA test (P<0.001) with Tukey’s multiple comparison post hoc test (indicated with *). The mean± s.e.m. is represented by error bars for 3 independent trials.
**Figure 8**

**Esco2-dependent cis-DNA looping model underlying the etiology of RBS.** (A) Schematic representation of the cohesin ring complex. It is composed of two structural maintenance of chromosome (SMC) subunits (SMC1A and SMC3) and three non-SMC subunits (RAD21, SA1, 2 and PDS5). The cohesin auxiliary factor, NIPBL- MAU2 heterodimer complex helps in cohesin ring opening/closing reactions that loads cohesins onto DNA. Another auxiliary factor, **ESCO2** is a member of the **ESCO** family of N-acetyltransferases that acetylates the SMC3 cohesin subunit. (B) A model depicting the Esco2-dependent *cis*-DNA tethering mechanism underlying RBS in which the acetyltransferase Esco2 activates its target, Smc3 (denoted by Ac) that binds the *cx43* promoter, thus activating *cx43* transcription. This process is believed to occur through a *cis*-DNA looping mechanism that connects the enhancer (E) and promoter (P) of the *cx43* gene.
Table 1: qPCR Confirms Changes in Gene Expression Downstream of Smc3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average $C_T$ (smc3 KD)</th>
<th>Average $C_T$ (actin)</th>
<th>$\Delta C_T$ smc3 KD - actin$^a$</th>
<th>$\Delta C_T$ Std-MO - actin$^a$</th>
<th>$\Delta \Delta C_T$</th>
<th>$\Delta \Delta C_T$ (Std-MO) $^b$</th>
<th>Fold Difference relative to Std-MO $^c$</th>
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<td>cx43</td>
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<td>17.18 ± 0.16</td>
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<td>sema3d</td>
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<td>16.71 ± 0.15</td>
<td>7.62 ± 0.20</td>
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<td>0.94 ± 0.21</td>
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<td>hapln1a</td>
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<td>18.12 ± 0.08</td>
<td>6.64 ± 0.07</td>
<td>5.61 ± 0.32</td>
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<td>0.50 (0.35 - 0.68)</td>
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<tr>
<td>spry4</td>
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<td>-0.28 ± 0.33</td>
<td>1.21 (0.95 - 1.53)</td>
<td></td>
</tr>
</tbody>
</table>

a. The $\Delta C_T$ value is determined by subtracting the average actin $C_T$ value from the average gene $C_T$ 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 $\Delta \Delta C_T$ involves subtraction by the $\Delta C_T$ calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta \Delta C_T$ is the same as the standard deviation of the $\Delta C_T$ value.

c. The range given for gene relative to Std-MO is determined by evaluating the expression: $2^{\Delta \Delta C_T}$ with $\Delta C_T + s$ and $\Delta C_T - s$, where $s$ = the standard deviation of the $\Delta C_T$ value.
Figure S1

Smc3 knockdown using MO2 results in regenerate length, segment length and cell proliferation reduction. (Top) Representative images of uninjected (UN), smc3 MO injected (MO2) injected fins. Total regenerate length was calculated by measuring the distance between the amputation plane (indicated by a solid black line) to the distal end of the 3rd fin ray (black arrows indicates the length measured). (Middle) Representative images of calcein stained fins of uninjected (UN) and smc3 MO injected (MO2) injected fins. Segment length was calculated by measuring the distance between first two joints in the 3rd fin ray (white arrows indicates the length measured). Higher magnification images of the representing segments are shown with joints indicated by white arrowheads. (Bottom) Representative images of H3P-positive cells in uninjected (UN) and smc3 MO injected (MO2) injected fins. Measurements were taken from the distal most 250µm of the 3rd ray. White bracket marks the defined area and n represents the number of H3P-positive cells in that area. Higher magnification images of the representing H3P-positive cells are shown indicated by white arrows. For each experiment n=8 fins per trial and 3 independent trials were performed. Scale bar is 50 µm for top and middle panels. Scale bar is 100 µm for the bottom panel.
Smc3 knockdown does not increase the level of apoptosis. (A) Representative images of TUNEL-positive cells in MO1-injected and Std-MO injected fins. The MO1/Std-MO were injected in 3 dpa fins and harvested at 1 dpe/4 dpa. TUNEL-positive cells were counted by eye from the distal-most 250μm of the 3rd ray. Arrows identify TUNEL-positive cells and the white horizontal line indicates the plane of amputation. (B) The graph reveals no significant difference between the MO1-injected fins compared to Std-MO injected fins using the percent similarity method. For each experiment n=8 fins. For statistical significance, two tailed unpaired Student’s t-test was used where $P<0.05$. Mean± s.e.m. is represented by error bars. Scale bar is 50 μm.
**Figure S3**

**AB9 cells as a system to evaluate cohesin-binding at the cx43 promoter.** (A) Expression of Esco2 and Smc3 are detected in AB9 cells by immunofluorescence. The anti-Esco2 antibody and anti-Smc3 antibody stains the nuclei of the cells (DAPI, blue), indicated with arrows. For each protein, 3 independent trials were performed. Scale bar is 10 µm (B) Western blot analysis detects the Esco2 in Std-MO electroporated control cell lysates (Lane 1: Control) at a predicted size of 68 kDa. A reduction in Esco2 protein levels in MO1-electroporated esco2 knockdown cell lysate (lane 2: Esco2 KD) was observed when compared to the control sample (lane 1: Control). The results with anti-Cx43 antibody reveals reduced Cx43 protein levels (detected at 43 kDa as predicted) in Esco2 KD compared to control. Western blot analysis detects Smc3 in Std-MO electroporated control cell lysates (lane 1: Control) at a predicted size of 142 kDa. A reduction in Smc3 protein levels in MO1-electroporated smc3 knockdown cell lysate (lane 2: Smc3 KD) was observed when compared to the control sample (lane 1: Control). The results with anti-Cx43 antibody reveals reduced Cx43 protein levels (detected at 43 kDa as predicted) in Smc3 KD compared to control. Tubulin detected at 50 kDa was used as the loading control for both blots. Image J software was used for analysis of relative band intensity from data of 3 independent trials.
### Table S1. qPCR Primer sequences

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### Table S2. PCR Primer sequences

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Forward primer-fp and reverse primer-rp. Positive indicates the presence of a amplified PCR product for that primer pair and negative indicates no PCR product for that primer pair.

**Table S3. PCR Primer sequences for ChIP- qRT PCR**

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      | rp- 5'-GATCTCATGGCTGTGACGTAG-3’ |
| p2   | fp- 5'-GGGTGTGAAGATTTATGGTTGCC-3’  
      | rp- 5'-AGGTGTGGAACTAGAGGAGTAG-3’ |
| p3   | fp- 5'-GCATGTTCTCCTTGGCTTGTTG-3’  
      | rp- 5'-CATCCACACACACACTCATACA-3’ |
| p4   | fp- 5'-CAGACTTGCCATTGTAGCCTAT-3’  
      | rp- 5'-GGCGAGCCCTGTGAAATAAA-3’ |
| p5   | fp- 5'-AGGATCAAGTGAAGCGAGTTTAG-3’  
      | rp- 5'-TCAGAGGGAAGAAGTGGAAGAAG-3’ |
| p6   | fp- 5'-GATGGCTTTTCTCCTCCGCTTAT-3’  
      | rp- 5'-CTATCTGGAACCTAGCATGAC-3’ |
| p7   | fp- 5'-TATCCCAGACCAGGTGTAGTT-3’  
      | rp- 5'-GCACCTGCTGAGGATGTAT-3’ |