IRX3/5 regulate mitotic chromatid segregation and limb bud shape

HIROTA TAO1, JEAN-PHILIPPE LAMBERT2,*, THEODORA M. YUNG1, MIN ZHU3, NOAH A. HAHN1,4, DANYI LI1,4, KIMBERLY LAU1, KENDRA STURGEON1, VJITHA PUVIDRAN1, XIAOYUN ZHANG1, WUMING GONG5, XIAO XIAO CHEN4, GREGORY ANDERSON6, DANIEL J. GARRY5, R. MARK HENKELMAN6, YU SUN3, ANGELO IULIANELLA7, YASUHIKO KAWAKAMI8, ANNE-CLAUDE GINGRAS2,4, CHI-CHUNG HUI1,4,‡ AND SEVAN HOPYAN1,4,9,‡

ABSTRACT
Pattern formation is influenced by transcriptional regulation as well as by morphogenetic mechanisms that shape organ primordia, although factors that link these processes remain under-appreciated. Here we show that, apart from their established transcriptional roles in pattern formation, IRX3/5 help to shape the limb bud primordium by promoting the separation and intercalation of dividing mesodermal cells. Surprisingly, IRX3/5 are required for appropriate cell cycle progression and chromatid segregation during mitosis, possibly in a nontranscriptional manner. IRX3/5 associate with, promote the abundance of, and share overlapping functions with co-regulators of cell division such as the cohesin subunits SMC1, SMC3, NIPBL and CUX1. The findings imply that IRX3/5 coordinate early limb bud morphogenesis with skeletal pattern formation.

KEY WORDS: Iroquois, Cohesin, Limb development, Mesodermal cell intercalations, Mitotic chromatid segregation, Morphogenesis

INTRODUCTION
Iroquois (Irx) genes were first identified as regulators of pre-pattern that control specification of large territories during Drosophila development (Cavodeassi et al., 2001). They encode proteins of the TALE subclass of atypical homeodomains (Burglin, 1997; Cavodeassi et al., 2001), other members of which include the MEIS and PBX proteins that are characterised by three additional amino acid residues (proline-tyrosine-proline) between helix 1 and helix 2 (Burglin, 1997). In mice, six Irx genes are found in two clusters: IrxA cluster contains Irx1, Irx2 and Irx4, and Irgb cluster consists of Irx3, Irx5 and Irx6 (Gómez-Skarmeta and Modolell, 2002).

IRX3/5 proteins are recognised for their ability to activate and repress transcription in a wide variety of developmental and physiological contexts (Costantini et al., 2005; Lee et al., 2004; Matsumoto et al., 2004; Kim et al., 2012; Biltoni et al., 2005). Irx3 is a target of obesity-associated variants within the FTO locus (Smemo et al., 2014) and regulates cardiac conduction (Zhang et al., 2011). Irx5 influences terminal differentiation of retinal bipolar neurons (Cheng et al., 2005) and of cardiomyocytes (Costantini et al., 2005). Irx3 and Irx5 are located about 550 kb apart on mouse chromosome 8 and are expressed in overlapping domains in the developing heart and limb, in which they have partially redundant functions (Gaborit et al., 2012; Li et al., 2014).

In the developing limb, anteroposterior polarity of the early bud is essential for the formation of appropriate skeletal pattern and is marked by domains of anterior Gli3 and posterior Hand2 expression (Te Welscher et al., 2002; Zhulyn et al., 2014; Tao et al., 2017). Irx3/5 are required for the expression of anterior mesodermal markers and for the formation of proximal (femur) and anterior (tibia, first and second rays) skeletal elements, specifically in the hindlimb. Irx3/5 counterbalance the posterior pattern regulator sonic hedgehog (Shh) that is downstream of Hand2, by directly upregulating transcription of the hedgehog antagonist Gli3. Hindlimb specificity of the mutant phenotype may be attributable to the greater Gli3-rich anterior domain in the forelimb that protects against diminished expression due to Irx3/5 deficiency. Interestingly, Irx3/5 are required during limb initiation before there is molecular evidence of emergent skeletal pattern (Li et al., 2014; Zhulyn et al., 2014). At that early stage, the Irx3/5 mutant limb bud is deformed and has apparently diminished anterior mesodermal volume, although it is unclear what cellular functions are affected.

Some of the key cell behaviours that are known to underlie early limb bud morphogenesis include directional mesenchymal cell movements and oriented cell divisions (Boehm et al., 2010; Gros et al., 2010; Wyngaarden et al., 2010; Mao et al., 2015). For limb bud ectoderm, the immediate intercalation of daughter cells among neighbouring cells is an important process that precipitates cell rearrangements to orient tissue growth (Lau et al., 2015). The separation of daughter cells, or cytokinesis, involves well defined changes in cellular morphology that include remodelling of the cortex, constriction of a cleavage furrow, and severing, or abscission, of the plasma membrane (Mohan et al., 2012; Liu and Robinson, 2018). During this process, sister chromatids are separated and any lagging chromosomes must be cleared (Morales and Losada, 2018; Liu and Robinson, 2018). Although specific pathways such as the NoCut checkpoint control that process (Mendoza et al., 2009), the loss of function of various proteins and complexes disrupts sister chromatid segregation and results in chromosome bridge formation at anaphase that delays or prevents cytokinesis. Cohesin and condensin complexes that are composed
of structural maintenance of chromosomes (SMC1A/3, SMC2/4, respectively) family proteins (DeMare et al., 2013; Jeppsson et al., 2014; Kschonsak and Haering, 2015) are responsible for the establishment of interphase chromatin structure and the formation of condensed chromosomes in mitosis (Kakui and Uhlmann, 2018). The cohesin complex regulates embryonic development in plants and animals by pairing sister chromatids during cell division and by influencing transcription (Minina et al., 2017; Smith et al., 2014; Marsman et al., 2014; Tedeschi et al., 2013; Mouri et al., 2012; Kawauchi et al., 2009). During S phase, replicated sister chromatids are connected with one another by the monomeric ring-like cohesin complex, and this cohesion is necessary for bioreorientation and orderly segregation of chromosomes on the mitotic spindle (Peters and Nishiya, 2012; Skibbens, 2008; Liu et al., 2019). Interestingly, deficiency of nipped-B-like (Nipbl), the product of which facilitates cohesin loading (Hirano, 2006), results in fin bud undergrowth and transcriptional misregulation in the mouse limb bud (Muto et al., 2014; Hirano, 2006), results in fin bud undergrowth and orderly segregation of chromosomes on the mitotic spindle (Peters and Nishiya, 2012; Skibbens, 2008; Liu et al., 2019). Interestingly, deficiency of nipped-B-like (Nipbl), the product of which facilitates cohesin loading (Hirano, 2006), results in fin bud undergrowth and transcriptional misregulation in the mouse limb bud (Muto et al., 2014; Hirano, 2006), results in fin bud undergrowth and orderly segregation of chromosomes on the mitotic spindle (Peters and Nishiya, 2012; Skibbens, 2008; Liu et al., 2019).

To assess early limb bud morphology in the mouse embryo, we employed optical projection tomography (OPT). Irx3+/− mutants are required for mitotic chromatid separation specifically in the anterior region of the limb bud. IRX3/5 are in close proximity to cohesin subunits and CUX1 and promote their abundance in that region. These data suggest that IRX3/5 share overlapping functions with regulators of cell division and contribute to early limb bud morphogenesis in addition to their recognised role in skeletal pattern formation.

RESULTS

Irx3/5 regulate limb bud morphogenesis and mesodermal daughter cell intercalation

To assess early limb bud morphology in the mouse embryo, we employed optical projection tomography (OPT). Irx3/5−/− mutant hindlimb buds were short and bulbous relative to those of wild-type (WT) littermates owing to disproportionate undergrowth of their anteriorposterior (AP) and proximodistal (PD) axes and overgrowth of their dorsosventral (DV) axes between somite stages (som.) 31 and 37 (Fig. 1A-D, Movies 1 and 2). Previously, we have shown that oriented cell motility and division are important for early limb bud morphology (Wyngaarden et al., 2010). To examine dynamic cell behaviours that might underlie this dysmorphology, we performed live time-lapse light sheet imaging of intact mouse embryos that harbour an H2B-GFP transgene (Hadjantonakis and Papaioannou, 2004) for 2.5 h periods at som. 32 stage (Movies 3 and 4). Limb bud mesodermal cell tracks in 3D (documented using Imaris, Movies 5 and 6) were converted to cell displacements using particle image velocimetry in Matlab (Movies 7 and 8) to generate 3D ‘dandelion’ plots for analysis. In contrast to the predominantly distalward displacement of mesodermal cells in the WT background, H2B-GFP; Irx3/5−/− mutant cells moved more radially (Fig. 1E,F, Movies 9 and 10), and these movements are qualitatively consistent with the relatively bulbous shape of the mutant bud. To assess the longer-term consequences of cell movement differences, we activated the transgenic multi-colour reporter Confetti (Snippert et al., 2010) using tamoxifen-responsive Esr1:Cre (Lee et al., 2014) to facilitate the identification of changes in cellular position over time. Approximately 24 h after tamoxifen delivery, these embryos were harvested and wide-field confocal analysis of the entire 29-30 som. hindlimb field was performed before and after 8 h in aerated roller culture supplemented with rat serum. Overall mesodermal cell displacements were diminished in Irx3/5−/− mutants (Fig. S1A-D), suggesting that mechanisms of cell movement were impaired (labelling of cells with Confetti was random as no distinct population was reproducibly labelled in different experiments, and variation of tamoxifen dose did not systematically affect the labelled population; Fig. S1E). These observations imply that dysmorphology in the Irx3/5−/− mutants is attributable to disoriented and diminished mesodermal cell displacements in developing limb buds.

To more precisely define dynamic mesodermal cell behaviours, we examined Esr1:Cre;Confetti transgenic embryos at higher resolution. During confocal live imaging of an anterior hindlimb field of ~150 mesodermal cells through 20 frames at 5 min intervals, WT daughter cells frequently separated from one another and intercalated among their neighbours immediately following cell division. In contrast, Irx3/5−/− mutant daughter cells more often remained adjacent to one another and failed to intercalate among neighbouring cells (Fig. 1G,H, Movies 11 and 12). Daughter cell intercalation contributes to reshaping limb bud ectoderm (Lau et al., 2015), and these observations suggest that Irx3/5 facilitate the same process in limb bud mesoderm, possibly by affecting cell division or daughter cell separation.

To ask whether cell division is affected by Irx3/5, we measured cell cycle time in vivo by assessing the proportion of cells that exited S phase between timed CidU and IddU injections of pregnant females as described previously (Boehm et al., 2010) (Fig. S2A). Consistent with the anterior domain of Irx3/5 expression (Li et al., 2014), cell cycle time was lengthened (11.3 versus 9.7 h) in anterior, but not posterior (10.2 versus 10.2 h), mesoderm of mutant 29 som. hindlimb buds (Fig. 2A). These cell cycle times are at the short end of the range documented previously for the later stage embryonic day (E) 11.0-11.25 forelimb bud (Boehm et al., 2010), suggesting mesodermal cell division slows during development. Flow cytometry of DAPI-stained cells that we dissociated from limb buds demonstrated that 21.3% of mutant cells were in G2/M phase compared with 13.4% for WT (Fig. 2B), implying that mitotic transit was delayed in mutant embryonic cells.

To seek further insight into the source of mitotic delay and lack of daughter cell separation that we had observed, we analysed mitotic cells in live H2B-GFP reporter embryo movies. To our surprise, 61.5% of mitotic cells in the anterior hindlimb mesoderm of 27-29 som. Irx3/5−/− mutant embryos (versus 9.5% in WT) exhibited chromatid bridges during the metaphase-anaphase transition (Fig. 2C, Movies 13-16). Chromatin bridges resolved in one of three primary outcomes. Some bridges resolved after a delay and allowed for clear chromatid separation (that we defined as a lagging chromatid; Movie 14). Some bridges persisted until chromatin decondensation was apparent at the beginning of interphase, resulting in failure of daughter cell separation, or until chromatin fragmentation was apparent (that we defined as a persistent bridge; Movie 15). Complete failure of metaphase chromatin separation was also noted in a small proportion of cases (Fig. 2D, Movie 16). We documented the duration of mitotic phases that were clearly observable using the H2B-GFP reporter for live imaging in vivo. Among Irx3/5−/− mutant mesodermal cells, the metaphase-telophase transition period was extended, and a greater proportion of those in telophase subsequently exhibited chromatin fragmentation.

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instead of transitioning to interphase (Fig. S2B, Movie 17). Therefore, Irx3/5 regulate mitotic progression by promoting chromatid segregation.

Time-lapse evidence of daughter cell fragmentation suggested that the fate of dividing cells with persistent chromatin bridges was apoptosis. We have previously shown that apoptotic cells were more numerous in the Irx3/5−/− mutant anterior hindlimb bud by 37 som. (Li et al., 2014), but we did not clearly identify a difference among caspase 3-positive cells at 29 som. (Fig. S2C) despite evident dysmorphology at that stage. It is uncertain whether the proportion of apoptotic cells is attributable entirely to failed mitoses, as we cannot directly compare the period of time represented by our time-lapse movies with our immunostains. Mutant mesodermal cells that did complete cytokinesis (a group that includes normal division and lagging chromosomes) exhibited disoriented planes of division (Fig. 2E). Not surprisingly, these findings underscore the importance of chromatid segregation for morphogenesis.

**IRX3/5 bind with regulators of chromatid segregation**

To test whether Irx3/5 regulate the transcription of genes that promote chromatid segregation, we performed RNA-seq of whole tissue posterior to somite 20 that includes the hindlimb fields of 26/27 som. Irx3/5−/− mutant embryos. Relative to WT specimens, Irx3/5−/− mutants exhibited significant changes in the expression of many genes including those that regulate translation, fatty acid β-oxidation, megakaryocyte differentiation and hematopoietic progenitor cell differentiation. However, the expression of genes that regulate chromatid segregation or cell cycle progression was not significantly altered in mutants (Fig. S3, Table S1), raising the possibility that IRX3/5 regulate cell division in a nontranscriptional manner. NCBI BioSample accession numbers to the full RNA-seq dataset are noted in Table S2.

To identify proteins that associate with IRX3 and IRX5 in an unbiased fashion, we performed proximity-dependent biotin identification (BioID) coupled to mass spectrometry, a method that captures proximate (within 10 nm) and interacting proteins in living cells (Roux et al., 2012; Kim et al., 2014). HEK 293 cells were chosen based on our experience with them for BioID. Although these cells are not a developmental model, we note they express transcription factors that are important for establishing limb pattern at an early stage which is the focus of our study, such as Gli2, Gli3 (Mo et al., 1997; Zhulyn et al., 2014), Meis1, Meis2 (Mercader et al., 1999; Capdevila et al., 1999) and Sall1-3 (Kawakami et al., 2009) according to proteinatlas.org and proteomicsdb.org. As expected, based on the
known function of IRX3/5, multiple regulators of transcription that bind to DNA or to other transcription factors, including TLE, BCOR, ZNF, FOX and TCF, were among the highest probability neighbours of IRX3 and IRX5 in HEK 293 cells (Fig. 3A). Among these transcription factors were well-recognised regulators of limb development, such as DLX6, PITX1 and 5’ HOX proteins (Fig. 3B).

Given the cell cycle progression defects among Irx3/5 deficient cells, we scrutinised BioID partners of IRX3/5 that regulate cell division. Two high significance IRX3/5 binding partners observed at a false discovery rate of less than 1% were the CUX1 proteins that regulate condensin (Seipold et al., 2009), were also identified at lower abundance in the BioID assay primarily of IRX3 and several of our BioID hits, including cohesin subunits and CUX1 (Fig. 3C). Although the latter is not commonly referred to as contacts (Fig. 4C). To test whether those endogenous proteins were physically bound in vivo, we used the proximity ligation assay (PLA), a method that detects protein-protein interactions with high specificity (Ke et al., 2013). IRX3 associated with SMC1 in 9.7% and with CUX1 in 12.5% of anterior mesodermal hindlimb nuclei (Fig. 4D,E). We interpret these findings to imply that IRX3 physically recognises both SMC1a and SMC1b proteins) and CUX1 (Fig. 4A), supporting the binding of these proteins in vitro. To extend these analyses in vivo, we dissected and lysed 30 WT hindlimb buds. Under endogenous conditions, anti-IRX3 antibody co-immunoprecipitated SMC1, SMC3 and CUX1 (Fig. 4A). To study the spatial distribution of these proteins, we performed whole-mount in situ hybridisation (ISH) with H2B-GFP (28-29 som., n=4 embryos per condition). Arrowheads indicate anaphase bridge in anterior limb bud mesoderm. (E) Cell division planes were disoriented in anterior Irx3/5−/− mutant limb bud mesoderm (30-35 cell divisions, n=3 embryos per condition, 29-30 som.). Scale bars: 10 µm. Error bars indicate s.e.m.

Irx3/5 promotes cohesin subunit and CUX1 protein abundance

We asked whether the presence of Irx3/5 impacted cohesin subunits and CUX1. Immunostain intensities of SMC1, SMC3, NIPBL and CUX1 were diminished specifically in anterior hindlimb mesoderm in Irx3/5−/− mutants in vivo relative to WT controls (Fig. 5A-C,
We note that delayed mitosis could result in some of the SMC1 depletion that we observed in mutants, as SMC1 is removed from chromosome arms during mitosis (Waizenegger et al., 2000; Sumara et al., 2000). Consistent with our RNA-seq data, RT-quantitative PCR of tissue dissected from the anterior hindlimb indicated that transcription of the Smc1a, Smc1b and Cux1 genes was unaltered in Irx3/5−/− mutants (Fig. 5D,E). Therefore, Irx3/5 contribute to the abundance of at least some cohesin subunits and CUX1 in a regional, and likely nontranscriptional, fashion.

**Irx3/5 exhibit overlapping function with Cux1/2**

The preceding datasets suggest that IRX3/5 have overlapping functions and might cooperate with cohesin and CUX1 to promote regionally appropriate cell division. To further address this possibility, we asked whether removal of Cux1/2 results in a limb bud phenotype that resembles that of Irx3/5−/− mutants. Although Cux1 is widely expressed, Cux2 is restricted to the interlimb lateral plate and anterior aspect of the early hindlimb field (Iulianella et al., 2003). We crossed Cux1+/− (Luong et al., 2002) and Cux2+/− (Iulianella et al., 2008) mutants and observed that single homozygous and double heterozygous Cux1+/2+ mutants did not exhibit a limb bud phenotype. Double homozygous Cux1+/2− stage-matched 29 and 32 som. hindlimb buds were small with shortened PD axes that was most pronounced in the anterior region and resembled those of Irx3/5−/− mutants (Fig. 6A). The DV axis is broader in Irx3/5 mutants, suggesting these regulators have overlapping but distinct morphogenetic functions. As with Irx3/5−/− mutants, the forelimb buds of Cux1+/2− mutants were not affected (not shown), the proportion of pHH3-positive cells was diminished (Fig. S6A), and caspase 3-positive cells were rare (Fig. S6B). Interestingly, chromatin bridges were evident among Cux1+/2− mutant mesodermal cells, although their fates could not be followed owing to the static nature of this analysis (Fig. 6B). Moreover, intensities of SMC1 and NIPBL immunostaining were...
diminished specifically in the anterior region of the hindlimb bud (Fig. 6C). At later stages, limb shape and skeletal pattern were normal in E13.5 and E14.5 Cux1^−/− mutants (Fig. S6C), implying the early morphogenetic deficiency had recovered. These data indicate that Irx3/5 and Cux1/2 have overlapping, though not necessarily interactive, morphogenetic functions in the early limb bud. Skeletal pattern, however, is regulated separately by Irx3/5.

**DISCUSSION**

The use of an unbiased protein binding assay and with live imaging were keys to identifying unexpected partners and processes regulated by IRX3/5. These approaches allowed us to identify cohesin subunits and CUX1 in proximity to IRX3 in vitro and in vivo, although we cannot state which proteins bind directly to IRX3. The lack of transcriptional change of cell cycle regulators in Irx3/5 mutants combined with their physical association with regulators of sister chromatid segregation raises the possibility that IRX3/5 have nontranscriptional functions in addition to their transcriptional roles during pattern specification. Cohesin itself plays a dual role in segregating sister chromatids and marking gene enhancers throughout the cell cycle to facilitate transcriptional memory after cell division (Yan et al., 2013). Our data do not distinguish whether the binding of IRX proteins with cohesin regulators and with CUX1 occurs in a complex that regulates chromatid segregation directly or upon chromatin at developmentally active loci. Therefore a coincidental role for IRX3/5 in the transcriptional regulation of other signalling pathways, or potential participation of IRX3/5 in an indirect nontranscriptional process such as CUX1/2-mediated DNA damage...
repair (Pal et al., 2015; Ramdzan et al., 2015, 2014; Iulianella et al., 2008) that regulates mitosis is possible. In principle, diminished cohesin may be associated with premature chromatid separation and/or chromatin bridges. Cohesin was originally identified as a regulator of sister chromatid cohesion, but it also organises interphase chromatin loops (Hadjur et al., 2009; Mishiro et al., 2009), spatial organisation of DNA (Guillou et al., 2010) and telomere replication (Remeseiro et al., 2012), making it somewhat challenging to predict the outcome of downregulation in different contexts. Genetic loss of cohesin has been shown to result in aneuploidy in vivo that corresponds to anaphase chromatid bridge formation in vitro (Remeseiro et al., 2012) and to lagging chromosomes in plant cells (He et al., 2019). The mutant phenotypes we observed may be the result of overlapping functions of cohesin and possibly of other regulators, such as condensin and CUX1. The mechanism by which multiple cohesin subunits are lost in the absence of Irx3/5 is not clear, especially as only a small fraction of those proteins appear to be associated with IRX3/5 at any one time. One possibility is that IRX3/5 are intermittently required as accessory subunits which contribute to the formation or stabilisation of the cohesin complex. The Iro box is a poorly characterised IRX domain that may bind to unexpected partners (Barrios et al., 2015) to mediate such a function. IRX proteins may modify sister chromatid segregation and transcriptional activation as do other accessory subunits of cohesin such as BRCA2, PDS5, OCT4, and SALL4 modify (Abboud et al., 2015; Misulovin et al., 2018). Other potential mechanisms involve control over the translation of cell division-related transcripts, possibly in conjunction with cohesin itself (Xu et al., 2015), and the intriguing possibility that IRX3/5 function as pioneer transcription factors which make chromatin accessible for cohesin or CUX1 accumulation at

![Fig. 5. IRX3/5 maintain cohesin subunits and CUX1 in vivo.](image)

(A,B) Immunofluorescence intensities of SMC1, NIPBL, SMC3 and CUX1 were diminished in the absence of Irx3/5 in anterior (A), but not posterior (B), limb bud mesoderm. (C) Quantification of A and B (29-32 som.; n=3 embryos per condition; *P=0.008 for anterior SMC1, *P=0.003 for anterior NIPBL, *P=0.004 for anterior SMC3, *P=0.004 for anterior CUX1; unpaired, two-tailed t-test, error bars indicate s.e.m.). (D,E) Transcription of Smc1a, Smc1b and Cux1 were unchanged by real time RT-PCR in anterior and posterior limb bud tissue in Irx3/5−/− and Irx3/5+/− embryos (29-30 som., n=3 embryos for each of three independent experiments). Error bars indicate s.e.m. Scale bars: 20 µm.
transcriptionally active sites. Substantial further investigation is required to test these speculations. A somewhat puzzling observation in this study is that the fundamental process of mitotic chromatid segregation is regulated in a regional fashion. An upstream signalling or indirect mechanism may help to explain this observation, although other organ- and maturation-specific functions of cohesin and other fundamental cell division processes have been identified in Drosophila and mouse embryos (Song et al., 2017; Smith et al., 2014; Kim et al., 2019). Developmental complexity may have evolved in concert with the expression domains of Irx genes. Overall, evidence of interdependent protein expression and functional overlap shown by our mutant analyses suggests cooperation between these regulators, a concept that is supported by the occurrence of tibial deficiency in cohesinopathies (Pfeiffer and Correll, 1993) and in Irx3/5 mutant mice (Li et al., 2014). Cell death, especially at later stages of limb bud development (Li et al., 2014), likely partially underlies undergrowth, dysmorphology and skeletal defects in Irx3/5 mutants. However, the majority of cells that managed to separate despite a chromatin bridge exhibited disoriented planes of division and deficient intercalations that were associated with altered trajectories of mesodermal cells, suggesting those processes also contribute to the dysmorphology observed in mutants. Basal mechanisms of pattern formation, such as reaction-diffusion (Raspovic et al., 2014) and morphogen gradients (Woolley et al., 2014), invoke distinct modes of

**Fig. 6. Cux1/2 share overlapping functions with Irx3/5.** (A) Cux1/2−/− double mutant embryos at 32-38 som. have smaller hindlimb buds partly owing to PD undergrowth that is most pronounced in the anterior region by 38 som. (B) Static confocal imaging of anti-pHH3 immunostained embryos show chromatin bridges were relatively abundant among Cux1/2−/− mutant mesodermal cells in the anterior hindlimb field at 29-30 som. (*P=0.018, unpaired, two-tailed t-test). (C) Section immunostaining revealed diminished anterior hindlimb SMC1 (n=3, *P=0.002, unpaired, two-tailed t-test) and NIPBL (n=3, *P=0.002, unpaired, two-tailed t-test) in 29-30 som. Cux1/2−/− mutants. AP, anteroposterior; DV, dorsoventral; PD, proximodistal. Error bars denote s.e.m. Scale bars: 200 µm (A); 20 µm (C).
molecular interaction but are each affected by the volume and shape of the tissue in which they function (Dekanty and Milán, 2011). It is therefore conceivable that IRX3/5 influence skeletal pattern by coordinating the form of the early hindlimb bud with the expression of patterning molecules such as GLI3 (Li et al., 2014). In *Drosophila*, the Iroquois protein Cauolian was also shown to regulate the size of developmental fields by binding to Cyclin-E-containing complexes (Barrios et al., 2015). Therefore, a dual transcriptional/nontranscriptional role for Iroquois is potentially conserved and may be common to other transcription factors in order to coordinate early tissue morphogenesis with subsequent pattern formation.

**MATERIALS AND METHODS**

**Mouse strains**

Analysis was performed using the following mouse strains: *Irx3*+/−∗/∗ (Li et al., 2014) *Irx3* and *Irx5* were targeted separately in embryonic stem cells without affecting the intervening region as described previously (Li et al., 2014). *Irx3-myC*, CAG: H2B-GFP [Jackson Laboratory, B6.Cg-Tg(HIS112BB/EGFP) 1 PuJ; Hadjantonakis and Papaioannou, 2004]. Confetti (Snippet et al., 2010), Esr1:Cre [Jackson Laboratory, B6.N129S(Cg)-Esr1tm1lccge/(Ardj). Lee et al., 2014], *Cux2+/−* (Luong et al., 2002) and *Cux2+/−* (Iulianella et al., 2008). To generate conditional mutant embryos, flox/flox females carrying the appropriate fluorescent reporter were bred to flox/+;Cre males. All animal experiments were performed in accordance with protocols approved by the Hospital for Sick Children Animal Care Committee and the Canadian Council on Animal Care.

**Optical projection tomography and limb bud morphology analysis**

E9.5 mouse embryos were harvested and fixed in 4% paraformaldehyde overnight at 4°C. The OPT system was custom-built and has been fully described previously (Wong et al., 2013). The 3D datasets were reconstructed from auto-fluorescence projection images acquired over a 10 min scan time at an isotropic voxel size of 3.85 µm. The 3D surface renderings of the OPT data were generated by Amira software, version 5.3.3 (Visualization Sciences Group).

**Live imaging and cell tracking**

Three-dimensional time-lapse microscopy was performed on a Zeiss Lightsheet Z.1 microscope. Embryos were suspended in a solution of DMEM without phenol red containing 12.5% rat serum and 1% low-melt agarose (Invitrogen) in a glass capillary tube. Once the agarose had solidified, the capillary until the portion containing the embryo was completely outside of the capillary. The temperature of the imaging chamber was maintained at 37°C with 5% CO2. Images were acquired using a 20×/1.0 objective with dual-side illumination, and a ∼90° interval of 0.479 µm. All experiments were imaged in multi-view mode with three evenly spaced views spanning the TruSeq RNA v2 reagents. The library was size-selected to produce insert sizes of ~200 bp, and a 50 bp pair-end run was performed for >20 million reads for each library. The raw RNA-seq reads were mapped to the mouse genome (mm10) using TopHat (v2.0.13) and Cufflinks (v2.2.1) pipeline. Differential expression analysis was performed by edgeR (Robinson et al., 2010; Fisher’s exact test was used to determine significance. NCBI BioSample accession numbers for the full dataset are in Table S2.

**Proximity biotinylation coupled to mass spectrometry**

Constructs for BioID experiments were generated via Gateway cloning into pDEST 5’ BirA*-FLAG-pcDNA5-FRT-TO (Lambert et al., 2015). Entry clones for *Irx3* and *Irx5* were first generated by performing PCR reaction from plasmids with full length cDNA (Flag-Irx3 in pENTR2B and Flag-Irx5 in pENTR2B). Primers for *Irx3* were ggggacaactttctgacaaataaggggacccAT-GTCCTTCCCCCACGCTG and ggggacaactttctgacaaataaggggacccAT-GTCCTTCCCCCACGCTG and ggggacaactttctgacaaataaggggacccAT-GTCCTTCCCCCACGCTG. Primers for *Irx5* were ggggacaactttctgacaaataaggggacccAT-GTCCTTCCCCCACGCTG and ggggacaactttctgacaaataaggggacccAT-GTCCTTCCCCCACGCTG.
HEK293 cell pellets harvested from two 15 cm plates were thawed in 1.5 ml ice cold RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 0.1% SDS and 0.5% sodium deoxycholate. PSMF (1 mM), DTT (1 mM) and Sigma protease inhibitor cocktail (P8340, Sigma-Aldrich, 1:500) were added immediately before use. The samples were sonicated at 4°C using three 0 s bursts with 2 s pauses at 35% amplitude. Then, 100 units of benzonase was added and the lysates were incubated at 4°C for an hour with rotation. For each sample, 60 μl of streptavidin-sepharose bead slurry (17-5113-01, GE Healthcare) were pre-washed three times with 1 ml of lysis buffer by pelleting the beads with gentle centrifugation (~500 g) and aspirating off the supernatant before adding the wash. Biotinylated proteins were captured on pre-washed streptavidin beads for 3 h at 4°C with rotation. The beads were then gently pelleted and the unbound supernatant was saved for further analysis. The beads were then washed twice with 1 ml RIPA buffer and three times with 1 ml 50 mM ammonium bicarbonate (pH 8.0). Following the final wash, the beads were pelleted and any excess liquid was aspirated off. Beads were then resuspended in 100 μl of 50 mM ammonium bicarbonate, and 1 μg of trypsin solution was added. The samples were incubated overnight at 37°C with rotation and then an additional 1 μg of trypsin was added, followed by a further incubation for 2-4 h. The beads were pelleted and the supernatant was transferred to a fresh tube. The beads were rinsed twice with 100 μl HPLC grade water and the wash fraction was combined with the supernatant. The peptide solution was acidified with 50% formic acid to a final concentration of 2% and the samples were placed in a speedvac to dry. Tryptic peptides were resuspended in 25 μl 5% formic acid and stored at −80°C until analysed by mass spectrometry.

**Mass spectrometry analysis**

BioID samples and controls were analysed by mass spectrometry (MS) in two biological replicates. We loaded 5 μl of each sample at 400 nl/min onto a 75 μm × 12 cm emitter packed with 3 μm ReproSil-Pur C18-AQ (Dr Maisch HPLC GmbH). The peptides were eluted from the column over a 90 min gradient generated by a NanoLC-Ultra 1D plus (Eksigent) nano-pump and HPLC grade water and the wash fraction was combined with the supernatant. The peptide solution was acidified with 50% formic acid to a final concentration of 2% and the samples were placed in a speedvac to dry. Tryptic peptides were resuspended in 25 μl 5% formic acid and stored at −80°C until analysed by mass spectrometry.

The LTQ Orbitrap Velos instrument (Thermo Electron) was operated with Xcalibur 2.0 in data-dependent acquisition mode with the following parameters: one centroid MS (mass range, 400 to 2000) followed by MS-MS on the 10 most abundant ions. General parameters were as follows: activation type=CID, isolation width=1 mass/charge ratio (m/z), normalised collision energy=35, activation Q=0.25, activation time=10 ms. For data-dependent acquisition, the minimum threshold was 500, the repeat count=1, repeat duration=30 s, exclusion score list=500, exclusion duration=30 s and exclusion mass width (by mass)=low 0.6, high 1.2.

**MS data analysis**

Two biological replicates of each bait were analysed by BioID-MS against a series of controls which included BirA alone, BirA fused to GFP, and cells expressing different nuclear proteins (KATs, BRPF3, BRD4, CBX3). Mass spectrometry data generated using a LTQ Orbitrap Velos were stored, searched and analysed using the ProHits laboratory information management system (LIMS) platform (Liu et al., 2010). Within ProHits, the resulting RAW files were converted to mzXML using ProteoWizard (v3.0.4468) and then searched using Mascot (v2.3.02). The spectra were searched with the RefSeq database (version 53, May 23th, 2013) acquired from NCBI against a total of 34,374 human and adenovirus sequences supplemented with ‘common contaminants’ from the Max Planck Institute (http://lotus1.gwdg.de/mpg/mmbc/maxquant_input.nsf/7994124a2498328fc125748d0048feefc/SFILE/contaminants.fasta) and the Global Proteome Machine (GPM; http://www.thegpm.org/crap/index.html). The database parameters were set to search for tryptic cleavages, allowing up to two missed cleavage sites per peptide with a mass tolerance of 50 ppm for precursors with charges of 2+ to 4+ and a tolerance of ±0.6 amu for fragment ions. Variable modifications were selected for deamidated asparagine and glutamine and oxidised methionine. Significance analysis of interaction (SAINT) express version 3.3 (Teo et al., 2014) was used as a statistical tool to calculate the probability value of each potential protein-protein interaction from background contaminants using default parameters. Dot plots of Irx3 and Irx5 interactions obtained by BioID were generated using custom built proteomics data visualisation tools (Knight et al., 2015). All MS files used in this study were deposited at Proteome Exchange (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD004309).

**Immunofluorescence**

E9.5-10.5 mouse embryos were fixed overnight in 4% paraformaldehyde in PBS followed by three washes in PBS. Embryos were permeabilised in 0.1% Triton X-100 in PBS for 20 min and blocked in 3% normal donkey serum (in 0.05% Triton X-100 in PBS) for 1 h. Embryos were incubated in primary antibody for 5 h at room temperature, followed by overnight incubation at 4°C. Embryos were washed (four washes, 20 min each), and then incubated in secondary antibody for 3-5 h at room temperature. Embryos were washed (four washes, 20 min each), followed by a final wash overnight at 4°C, and stored in PBS. Images were acquired using a Quorum spinning disk confocal microscope and Nikon A1 confocal microscope at 10×, 20×, or 40× magnification or Leica TCS SP8 STED 3× microscopy at 100× magnification, and image analysis was performed using Velocity software and ImageJ. Relative fluorescence intensity was derived by normalising intensity of immunostaining to the corresponding DAPI stain intensity (set at 1) within a field of 15 mesodermal cells and averaged over three embryos, similar to a procedure we described previously (Lau et al., 2015).

**Antibodies company and catalogue number**

**Irx3** [Novus Biologicals, IDT, H0079191-M05, mouse, 1:250 – used for immunofluorescence (IF)]; IRX3 (Santa Cruz Biotechnology, G-6, 166877, mouse, 1:100 – used for co-IP in vivo); SMC1 (Abcam, ab21583, rabbit, 1:250 IF, 1:1000 – used for immunoblotting (IB); SMC3 (Novus Biological, NB100-207 IF, in vitro IB; Abcam, ab9263, 1:1000 in vitro IB); NIPBL (Santa Cruz Biotechnology, C-9, sc-374625, 1:1000); CUX1 (Abcam, ab140042, mouse, 1:250 IF, in vitro IB; Abcam, ab230844, rabbit, 1:2500 in vivo IB); IgG (NA931V, GE Healthcare, 1:100) or donkey anti-rabbit IgG (NA934V, GE Healthcare, 1:100) co-IP in vitro, IgG (Santa Cruz Biotechnology, 2343, 1:100) co-IP in vivo; Caspase-3 (BD Biosciences, 559565, 1:250); F-Actin (Alexa Fluor 633 Phalloidin, Thermo Fisher Scientific, A22284, 1:1000).

**Co-immunoprecipitation in vitro**

HEK293T cells were transfected with full-length FLAG-Irx3 that was cloned into the pDNR-CMV expression construct (Zhang et al., 2011) (Clontech) using Lipofectamine 2000 (11668027, Life Technologies). Cell lysates were prepared after 2 days and co-IP assays were performed using Protein G PLUS-Agarose (sc-2002, Santa Cruz Biotechnology) and anti-Irx3 antibody (homemade). Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (IPVH00010, Millipore) and detected by HRP sheep anti-mouse IgG (NA931V, GE Healthcare, 1:1000) or donkey anti-rabbit IgG (NA934V, GE Healthcare, 1:100) and a chemiluminescence system. Input lanes represent immunoprecipitated lysate that was run on separate gels without western blot antibody or further treatment.
In vivo
We lysed 30 E10.5 hindlimb buds in PLC lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% TritonX-100, protease inhibitors (11836153001, Roche)] using the Precellys Lysing kit (KT03961-I-003.2) using 200-250 μg cell lysate per sample. Then 50% protein A/G-agarose was prepared with cold PBS, and 50% A/G-agarose working fluid was added to the sample (100 μg/mL). The concentration of total sample protein was determined by bicinchoninic acid (BCA) at 4°C for 10 min. Cell lysates were prepared after 2 days and co-IP assays were performed by using Protein G PLUS-Agarose (sc-2002, Santa Cruz Biotechnology) and anti-IRX3 (Santa Cruz Biotechnology) or anti-IgG antibody.

Western blotting

In vitro
HEK293 cell were lysed in PLC lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% TritonX-100, protease inhibitors (11836153001, Roche)]. Proteins were separated by SDS-PAGE, transferred to PVDF membranes (PerkinElmer), and incubated with primary antibodies overnight at 4°C. Immunoblots were developed using HRP-conjugated secondary antibodies and Western lighting Plus- ECL (NEL104001EA, PerkinElmer). Lysing kit (KT03961-I-003.2) using 200-250 μg cell lysate per sample. Then 50% protein A/G-agarose was prepared with cold PBS, and 50% A/G-agarose working fluid was added to the sample (100 μg/mL). The concentration of total sample protein was determined by bicinchoninic acid (BCA) at 4°C for 10 min. Cell lysates were prepared after 2 days and co-IP assays were performed by using Protein G PLUS-Agarose (sc-2002, Santa Cruz Biotechnology) and anti-IRX3 (Santa Cruz Biotechnology) or anti-IgG antibody.

In vivo
Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (IPVH00010, Millipore) and detected by HRP sheep anti-mouse IgG (NA931V, GE Healthcare, 1:100) or donkey anti-rabbit IgG (NA934V, GE Healthcare, 1:100), and a chemi-luminescence system. The membranes were cleaned three times with Tris buffered saline with Tween (TBST) solution for 10 min each time. The skinned milk was sealed, and 3 ml added to the antibody incubator. The membranes were sealed for 1 h at room temperature. Membranes were cleaned with TBST solution three times, for 10 min each time; incubation with the primary antibody – anti-SMC1 antibody (ab21583, Abcam, rabbit, 1:1000), anti-SCM3 antibody (ab9263, Abcam, rabbit, 1:1000), anti-CUX1 antibody (ab230844, Abcam, rabbit, 1:2500) or anti-IgG (Santa Cruz Biotechnology, 2343, 1:100) – was carried out overnight at 4°C. Incubation with the secondary antibody was at room temperature for 1 h. Finally, an appropriate amount of luminescent solution (A and B liquid volumes were mixed) was used to incubate the membrane for 3 min; the gel imaging system was exposed and Quantity-One software was used to analyse the protein content.

Quantitative real-time PCR
RNA was isolated from E10.5 hindlimbs. At least three independent biological replicates were used, and each experiment was repeated at least twice. Reactions utilised TaqMan Universal PCR master mix (Applied Biosystems), and TaqMan Gene Expression Assay probes for mouse Cux1 (Forward, CGGGGGAACAGTGTTACAT; Reverse, GCATTAGCTGC-CGCTGGAGA), Smc1a (Forward, ATGGACCCATGCTGTCG; Reverse, CTCCATGTACGTCCGCG), Smc1b (Forward, GGTCAT-CGGCCCTTCCAGA; Reverse, CAGGGTCAGATGGTCTCC), beta-actin (PrimeBank ID 6671509a1), Gapdh (Mm03302249_g1) was used as the endogenous control for target gene normalisation. Gene expression data are available at NCBI Gene Expression Omnibus (GEO) under the accession number GSE211986.

In situ proximity ligation assay (PLA)
PLA (DUO92101, Sigma-Aldrich) was performed in a similar manner as standard immunohistochemistry until the secondary antibody incubation step. PLA probe dilution/incubation time, rolling circle amplification times and polymerase concentrations were optimised for frozen tissue sections. For detection of IRX3 in close proximity to either SMC1 or CUX1, two primary antibodies raised in different species were used. Secondary anti-mouse (PLUS) and anti-rabbit (MINUS) antibodies conjugated to complementary oligonucleotides provided in the kit were used. Slides were incubated overnight at 4°C for primary antibody reaction and then 2 h at 37°C in blocking solution containing PLUS and MINUS probes. A polymerase dilution of 1/80 was optimal for single recognition; incubation time was 2 h under gentle agitation.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Data availability
All mass spectrometry files used in this study were deposited at MassIVE (http://massive.ucsd.edu) under accession number MSV00079812 as well as to the ProteomeXchange (PXD004309). NCBI BioSample accession numbers for the full RNA-seq datasets generated in this study are in Table S2.

Supplementary information
Supplementary information available online at https://dev.biologists.org/lookup/doi/10.1242/dev.180042.supplemental

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