

Supplementary Figure 1

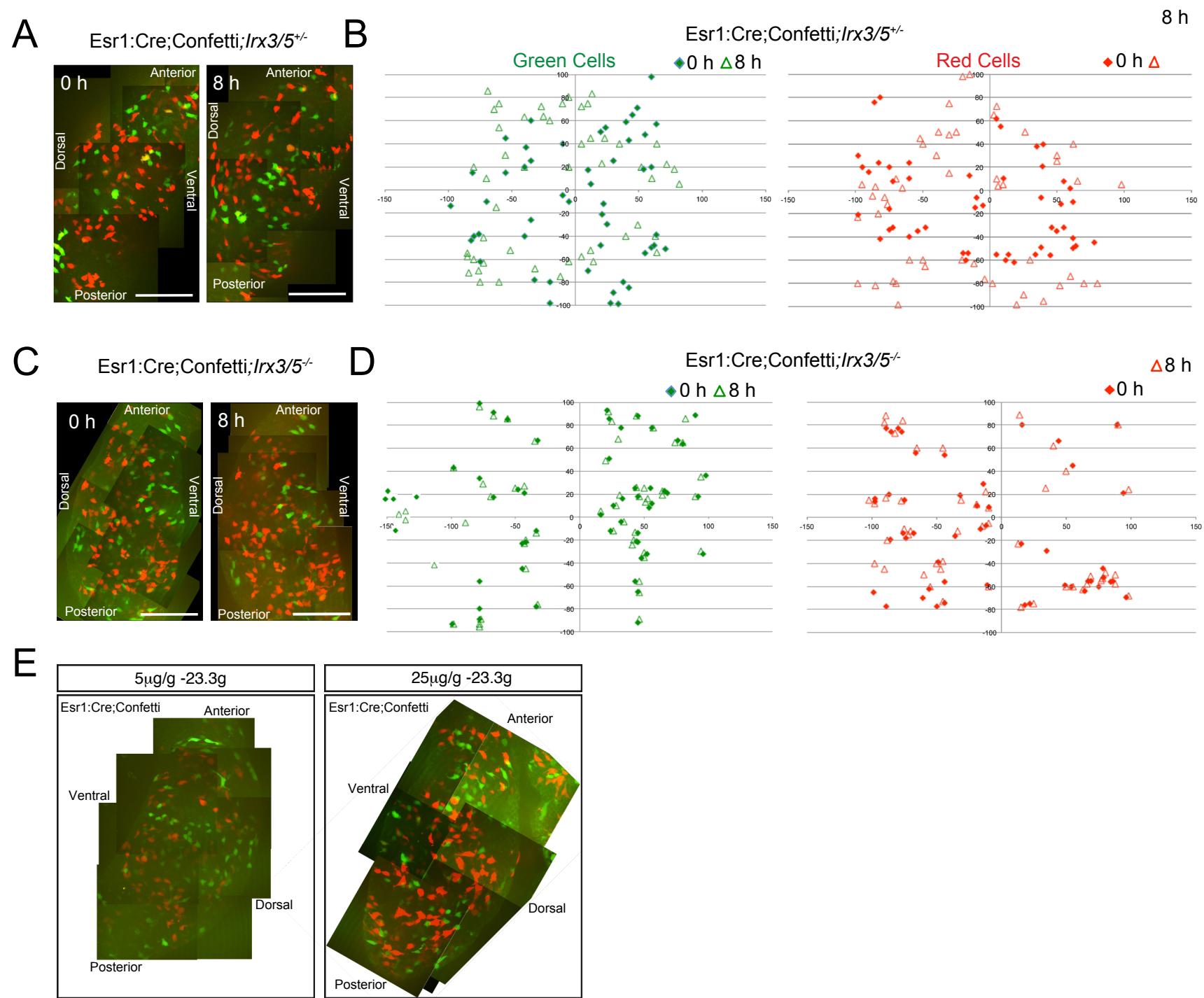


Figure S1

Mesodermal cell displacements are diminished in the early *Irxa3/5^{-/-}* mutant hindlimb bud. **A-D)** Wide field analysis of red and green randomly labelled mesodermal cells in the entire early (29-30 som.) hindlimb field of *Esr1:Cre;Confetti;Irxa3/5^{+/−}* (WT) and *Esr1:Cre;Confetti;Irxa3/5^{-/-}* embryos 24 h following tamoxifen delivery. **B, D)** X-Y plots indicate the position of red cells and green cells relative to an arbitrarily chosen central reference point at 0 h (rhombus) and after 8 h (triangle) in roller culture. *Irxa3/5^{-/-}* mutant mesodermal cells demonstrated a relative lack of displacement compared to *Irxa3/5^{+/−}* cells. Shown are cells tracked from 3 29-30 som. embryos per condition. Scale bars in A and C indicate 100 μ m. **E)** Variation of intraperitoneal tamoxifen dose to pregnant females did not label a recognised subpopulation of limb bud cells, suggesting the labelling was random. Concentration and female weights are noted in the headings.

Supplementary Figure 2

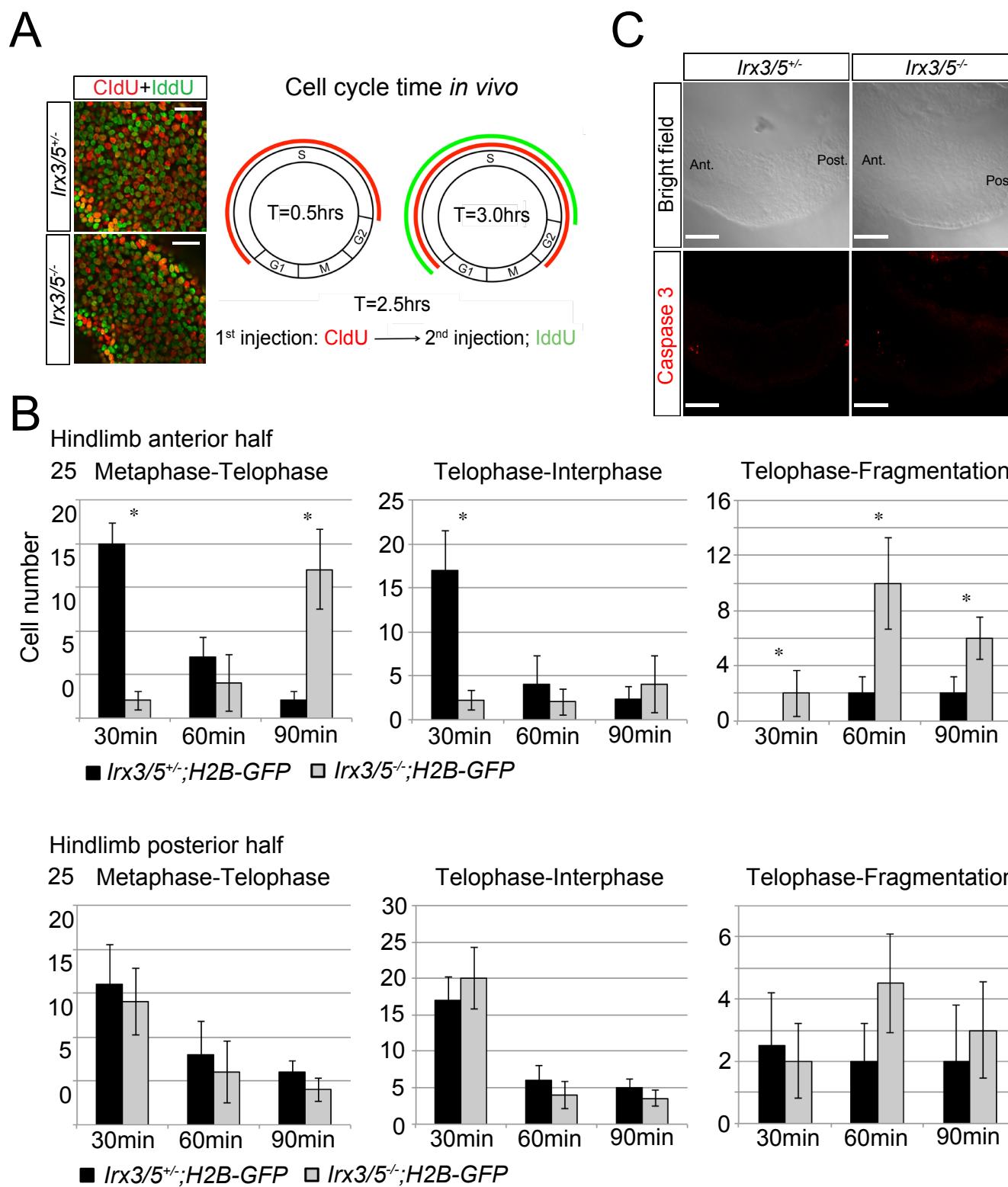


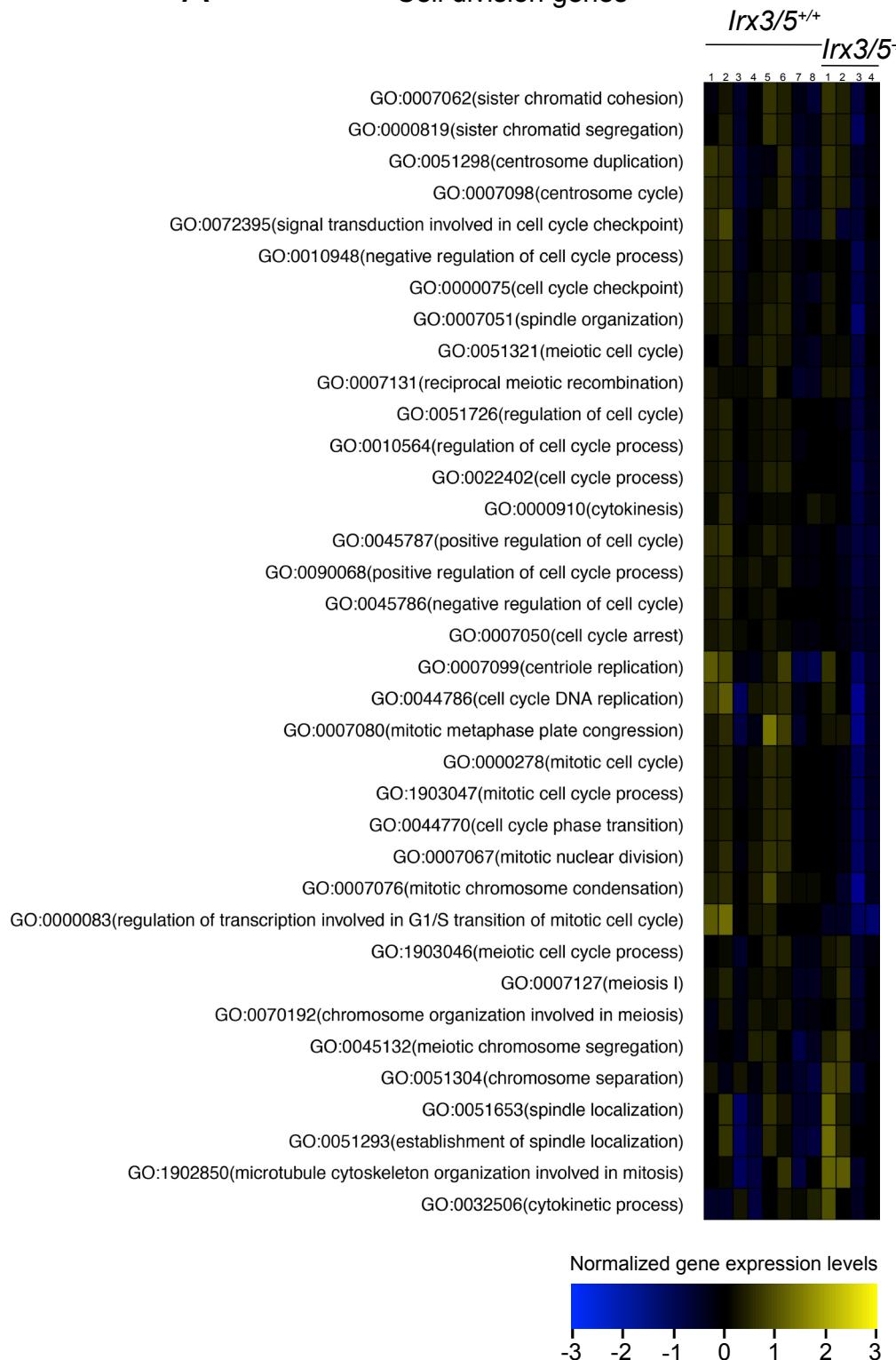
Figure S2

Assessment of cell cycle progression and apoptosis *in vivo*. **A**) Pregnant females were first injected with CldU to label cells in S-phase (left, red bar). After 2.5 h, mice were injected with IddU. Cell cycle time was measured by assessing the proportion of cells that exited S phase between the two injections. Scale bars indicate 40 μ m. **B**) The durations of mitotic phases of individual cells were categorised by live imaging of H2B-GFP reporter embryos *in vivo*. n=3 embryos per condition, error bars denote s.e.m., t test, asterisks indicate p<0.002. **C**) Wholemount immunostaining at 29 som. stage demonstrates minimal increase in the number of caspase 3-positive cells in the anterior region of the *Irx3/5* mutant hindlimb bud. Scale bar indicates 90 μ m.

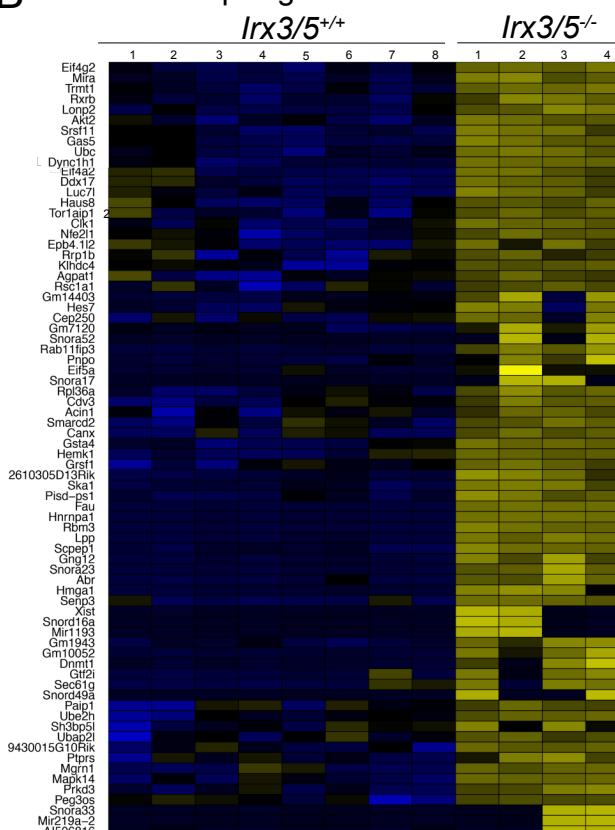
Supplementary Figure 3

A

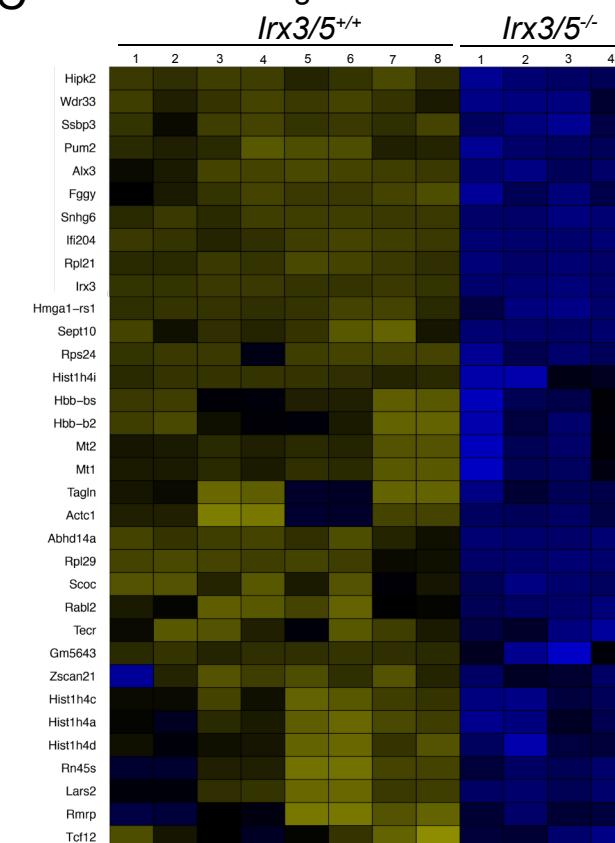
Cell division genes

**B**

Genes up-regulated in *Irx3/5* mutants

**C**

Genes down-regulated in *Irx3/5* mutants

**Figure S3**

Comparison of RNA-seq data among hindlimb fields of 26-27 som. (E9.75) *Irx3/5^{-/-}* mutants. A) None of the cell cycle related genes examined (children terms of GO:0022402 (cell cycle process) and GO:0007049 (cell cycle) were expressed differently in mutants ($p>0.05$). **B)** Significantly up-regulated and **C)** down-regulated genes (false discovery rate (FDR) ≤ 0.05) in *Irx3/5^{-/-}* mutants relative to WT specimens. Differential expression analysis of RNA-seq data was performed by edgeR (Robinson et al., 2010). For each gene, the log transformed Fragments Per Kilobase of transcript per Million mapped reads (FPKM) was scaled to a mean of zero and standard deviation of one. Fisher's exact test was used to determine significance.

Supplementary Figure 4

A Co-IP *in vitro*

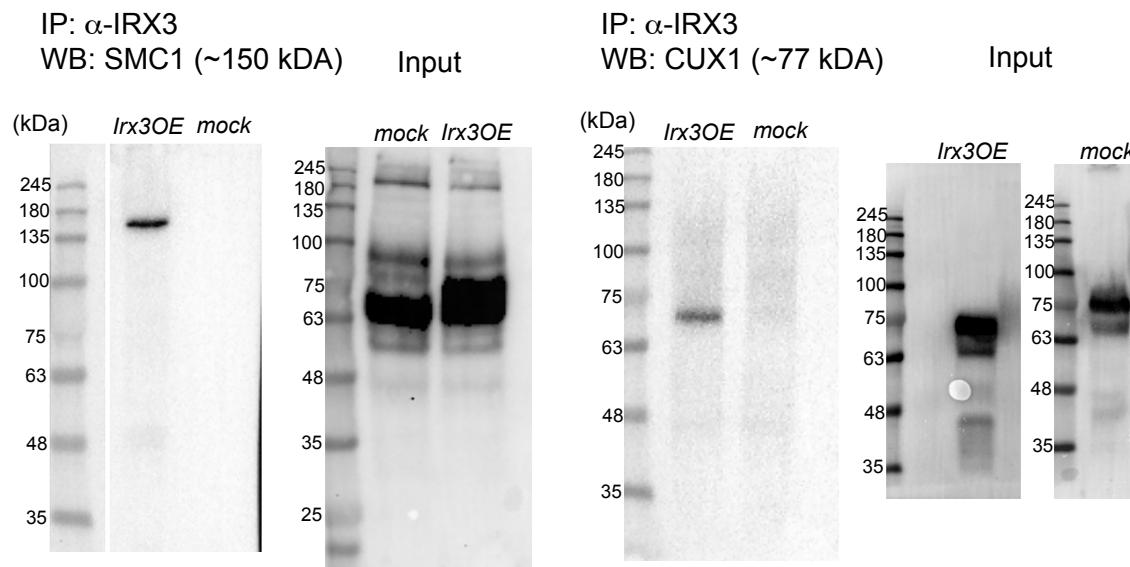


Figure S4

Association of IRX3 with SMC1 and CUX1 *in vitro*. A) HEK 293 cells were transfected with FLAG-Irx3/pDNR-CMV (Irx3OE) or empty pDNR-CMV (mock) and incubated. Lysates were immunoprecipitated using anti-IRX3 antibody and immunoblotted using against SMC1 or against CUX1. Full-length Western blots (WB) are shown.

Supplementary Figure 5

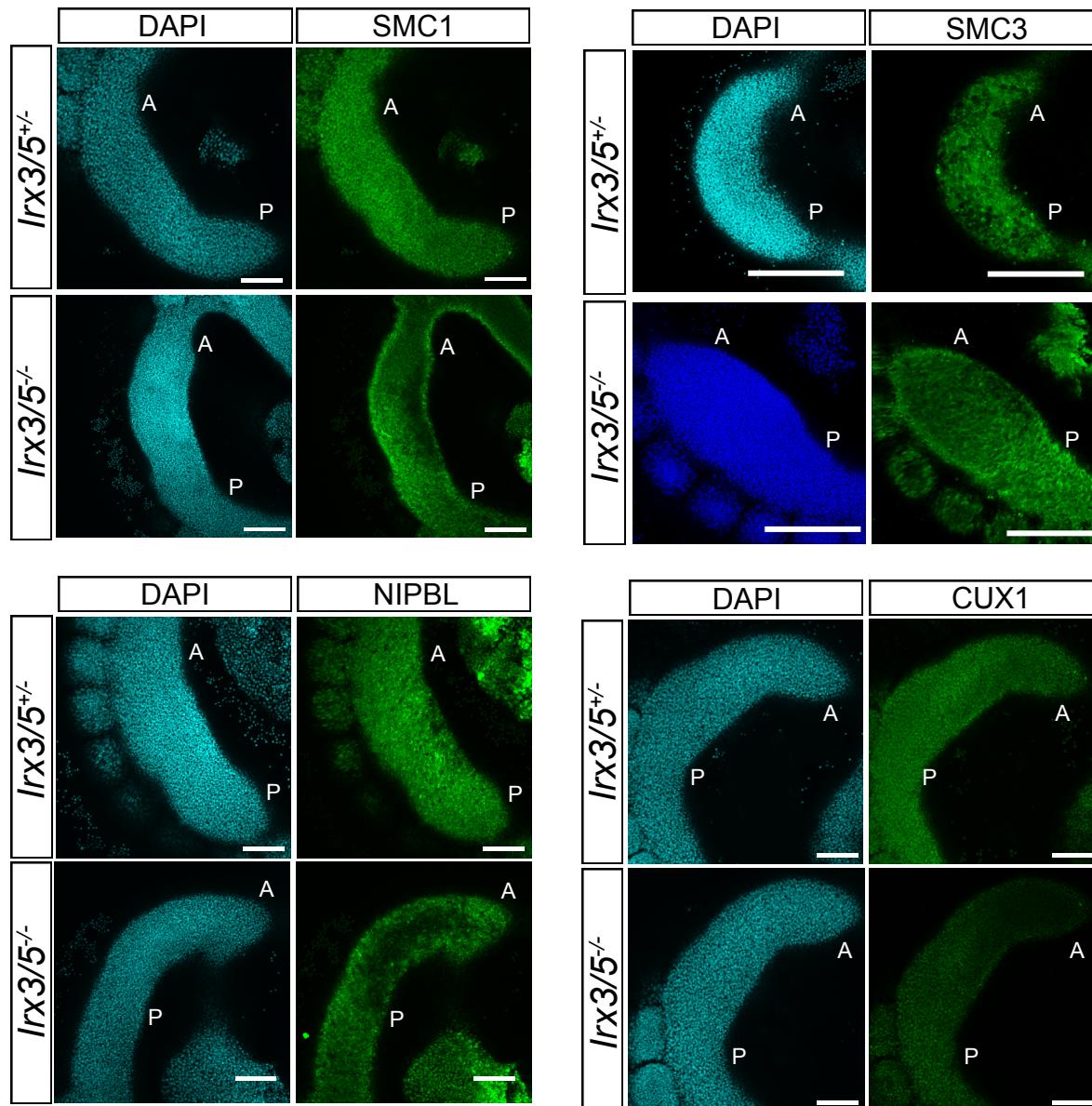


Figure S5

SMC1 and CUX1 require *Irx3/5* *in vitro* and *in vivo*. Low magnification confocal views of the entire 29-32 som. stage hindlimb field show *Irx3/5* are required to maintain immunostain abundance of SMC1, SMC3, NIPBL, and CUX1 predominantly in the anterior (A) half of the limb field. Scale bars denotes 100 µm for SMC1, NIPBL and CUX1, 400 µm for SMC3.

Supplementary Figure 6

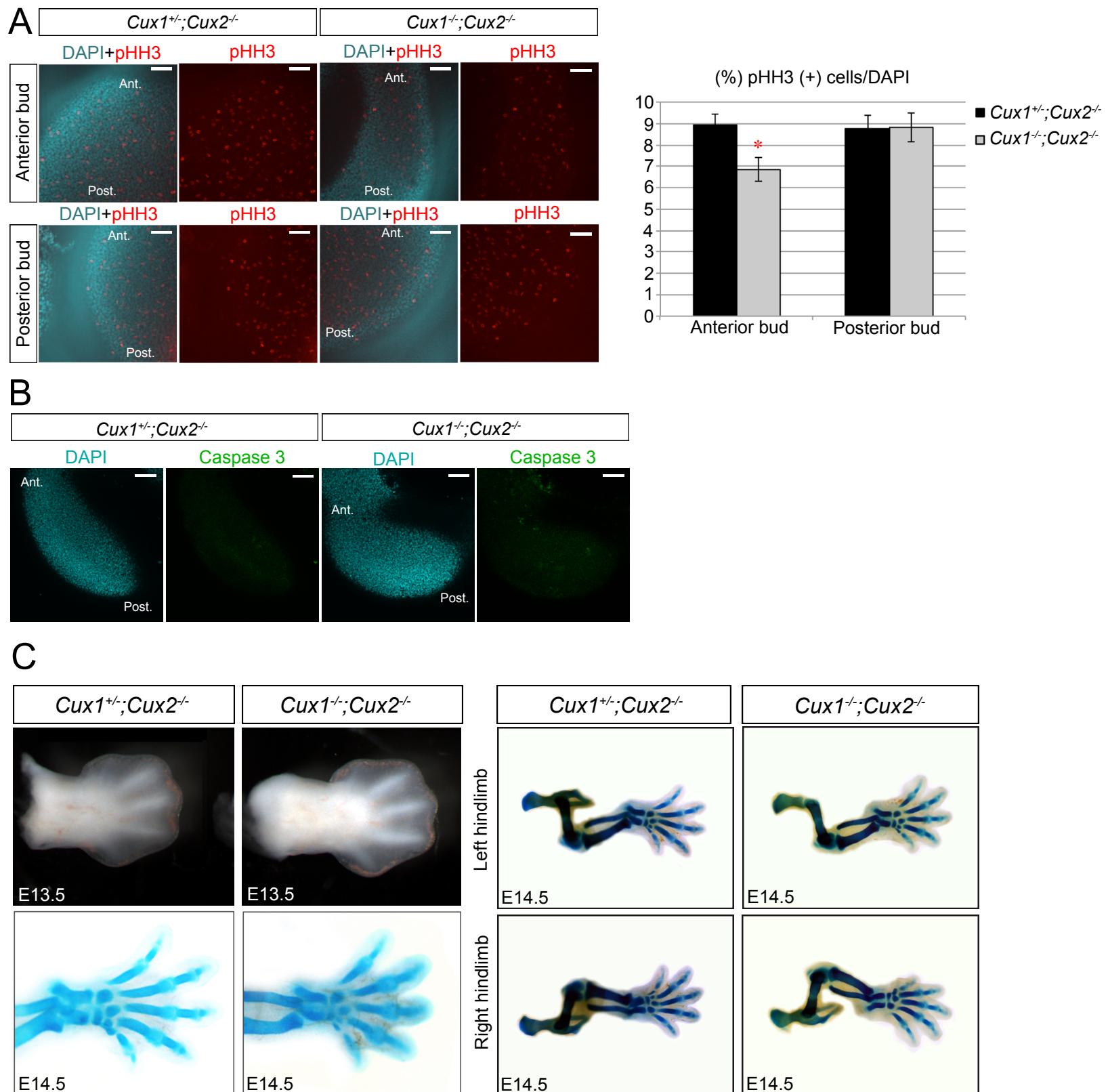


Figure S6

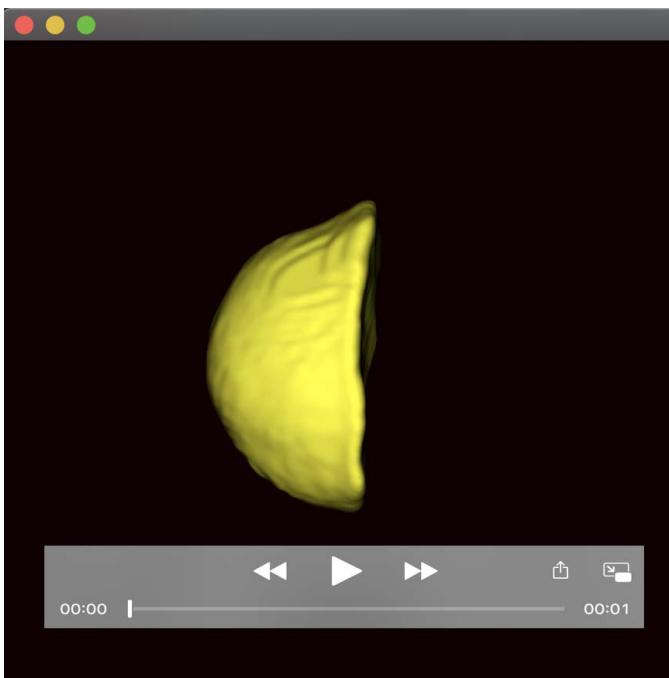
Cux1/2 regulate early limb bud cell division but not skeletal pattern. **A)** The proportion of pHH3-positive cells was diminished in the anterior 29-30 som. hindlimbs of *Cux1/2*-/- mutants. Scale bars denote 50 μ m, $p=0.004$, t test, error bars indicate s.e.m.. **B)** Wholemount caspase 3 immunostaining of 29-30 som. *Cux1/2*-/- mutant hindlimbs. Scale bars denote 100 μ m. **C)** Hindlimb skeletal pattern visualised by alcian blue stain (for cartilage) at E13.5 and E14.5 was not affected by removal of *Cux1/2*.

Table S1. Differentially expressed genes in *lrx3/5^{-/-}* mutants were not significantly associated with cell cycle related Gene Ontology terms (children terms of GO:0022402 (cell cycle process) and GO:0007049 (cell cycle)). Fisher's exact test was used to examined the association between whether genes are significantly up- or down-regulated in *lrx3/5^{-/-}* mutants and whether genes have annotated functions related to cell cycle progression.

Cell cycle related Gene Ontology terms	# genes	# up-regulated genes	# down-regulated genes	Fisher's exact test p-value
GO:0000075(cell cycle checkpoint)	146	1	0	0.695
GO:0000910(cytokinesis)	119	1	0	0.620
GO:0007050(cell cycle arrest)	103	0	0	1.000
GO:0007051(spindle organization)	80	1	0	0.477
GO:0007062(sister chromatid cohesion)	30	0	0	1.000
GO:0007098(centrosome cycle)	46	1	0	0.311
GO:0007099(centriole replication)	17	0	0	1.000
GO:0008608(attachment of spindle microtubules to kinetochore)	20	0	0	1.000
GO:0010564(regulation of cell cycle process)	363	0	0	1.000
GO:0010948(negative regulation of cell cycle process)	158	0	0	1.000
GO:0032506(cytokinetic process)	15	0	0	1.000
GO:0044770(cell cycle phase transition)	267	0	0	1.000
GO:0044786(cell cycle DNA replication)	18	0	0	1.000
GO:0051298(centrosome duplication)	35	0	0	1.000
GO:0051304(chromosome separation)	52	0	0	1.000
GO:0051653(spindle localization)	28	0	0	1.000
GO:0072395(signal transduction involved in cell cycle checkpoint)	12	0	0	1.000
GO:0090068(positive regulation of cell cycle process)	139	0	0	1.000
GO:0098813(nuclear chromosome segregation)	119	0	0	1.000
GO:1903046(meiotic cell cycle process)	115	0	0	1.000
GO:1903047(mitotic cell cycle process)	535	2	0	0.933
GO:0000278(mitotic cell cycle)	593	3	0	0.862
GO:0051726(regulation of cell cycle)	584	0	0	1.000
GO:0022402(cell cycle process)	823	5	0	0.800
GO:0045786(negative regulation of cell cycle)	274	0	0	1.000
GO:0045787(positive regulation of cell cycle)	198	0	0	1.000
GO:0051321(meiotic cell cycle)	120	0	0	1.000

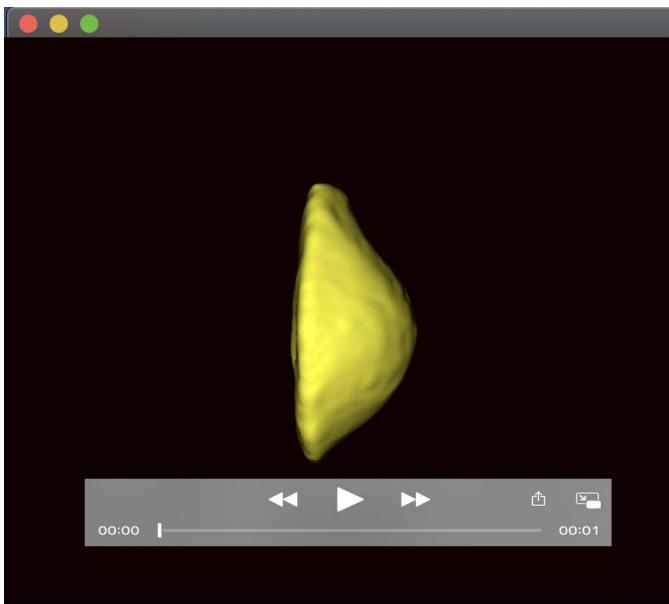
Table S2. NCBI BioSample accession numbers for the full RNA-seq dataset.

Accession	Sample Name and SPUID	Organism	Tax ID	Strain	BioProject
SAMN12877482	WT-4	<i>Mus musculus</i>	10090	mixed	PRJNA575061
SAMN12877483	WT-5	<i>Mus musculus</i>	10090	mixed	PRJNA575061
SAMN12877484	WT-6	<i>Mus musculus</i>	10090	mixed	PRJNA575061
SAMN12877485	WT-7	<i>Mus musculus</i>	10090	mixed	PRJNA575061
SAMN12877486	Irx3-5EM12-3	<i>Mus musculus</i>	10090	mixed	PRJNA575061
SAMN12877487	Irx3-5EM12-6	<i>Mus musculus</i>	10090	mixed	PRJNA575061
SAMN12877488	Irx3-5EM12-5	<i>Mus musculus</i>	10090	mixed	PRJNA575061
SAMN12877489	Irx3-5EM12-4	<i>Mus musculus</i>	10090	mixed	PRJNA575061



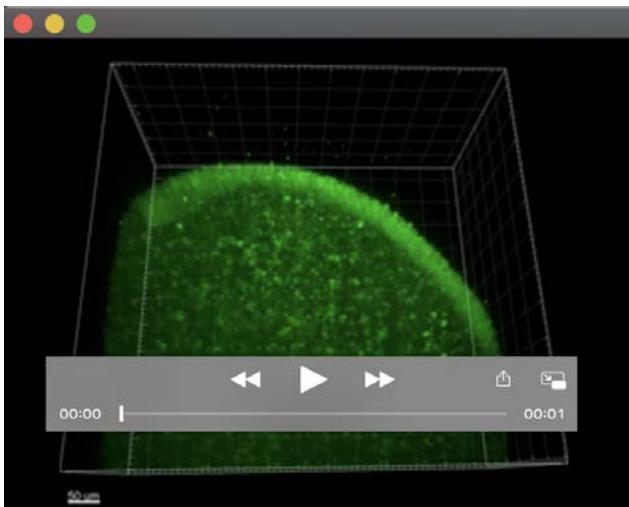
Movie 1

Optical projection tomography of *Irx3/5^{+/-};Esr1-Cre;Confetti* embryonic limb bud at 37 som. Anterior is upward, dorsal view shown at first frame.



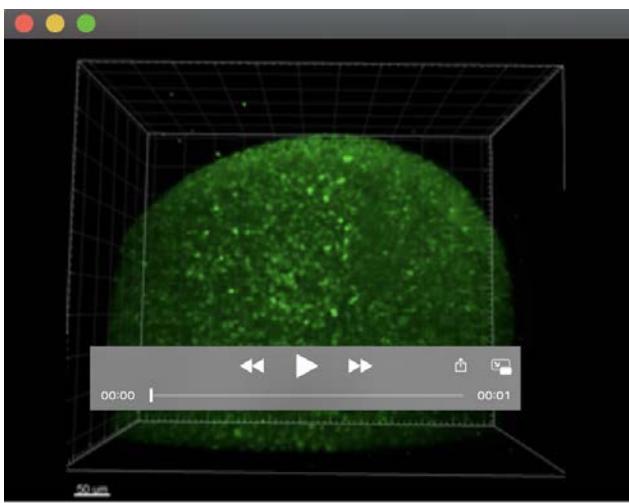
Movie 2

Optical projection tomography demonstrated the smaller and mishapen limb bud of *Irx3/5^{-/-};Esr1-Cre;Confetti* embryos at 37 som. Anterior is upward, dorsal view shown at first frame.



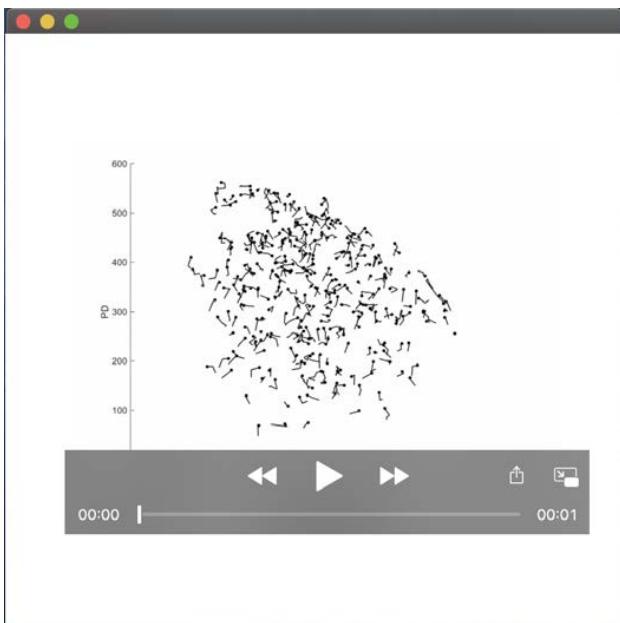
Movie 3

Live light sheet time-lapse movie of *Irx3/5^{+/−}*;H2B-GFP hindlimb at 32 som. Anterior is to the left, and the duration is 2.5 h.



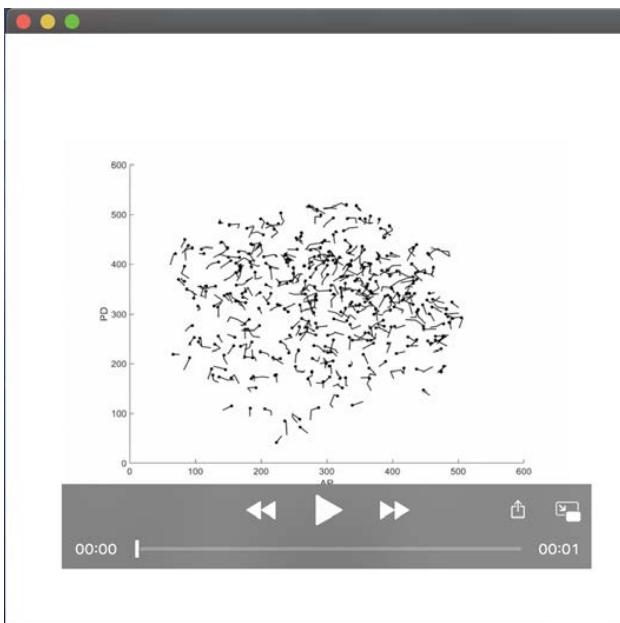
Movie 4

Live light sheet time-lapse movie of *Irx3/5^{−/−}*;H2B-GFP hindlimb at 32 som. Anterior is to the left, and the duration is 2.5 h.



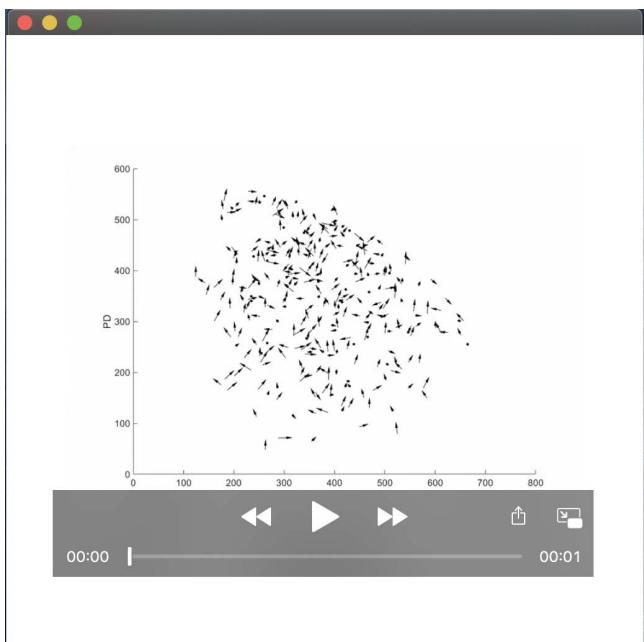
Movie 5

Cell tracks of *Irx3/5^{+/−}*;H2B-GFP hindlimb at 32 som. during 2.5 h light sheet time-lapse. Anterior is to the left.



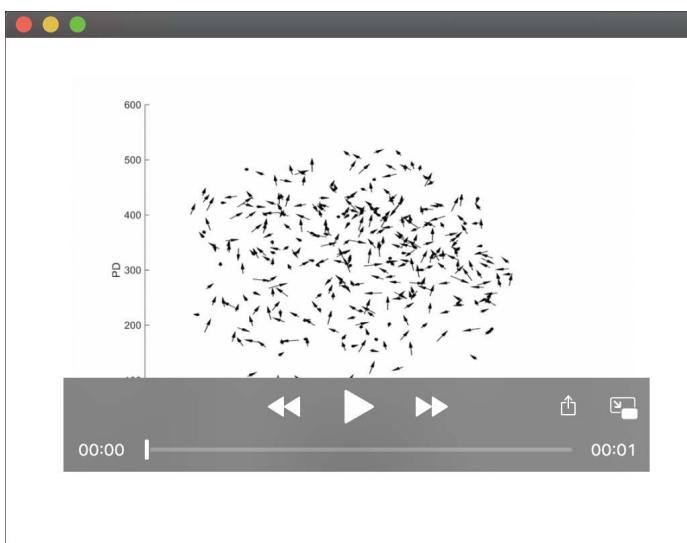
Movie 6

Cell tracks of *Irx3/5^{−/−}*;H2B-GFP hindlimb at 32 som. during 2.5 h light sheet time-lapse. Anterior is to the left.



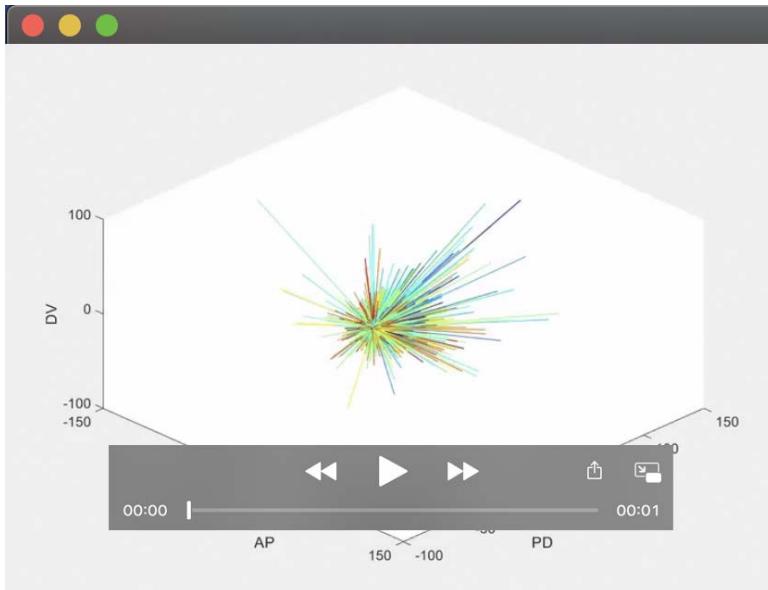
Movie 7

PIV of *Irx3/5^{+/−}*;H2B-GFP hindlimb at 32 som. during 2.5 h light sheet time-lapse. Anterior is to the left.



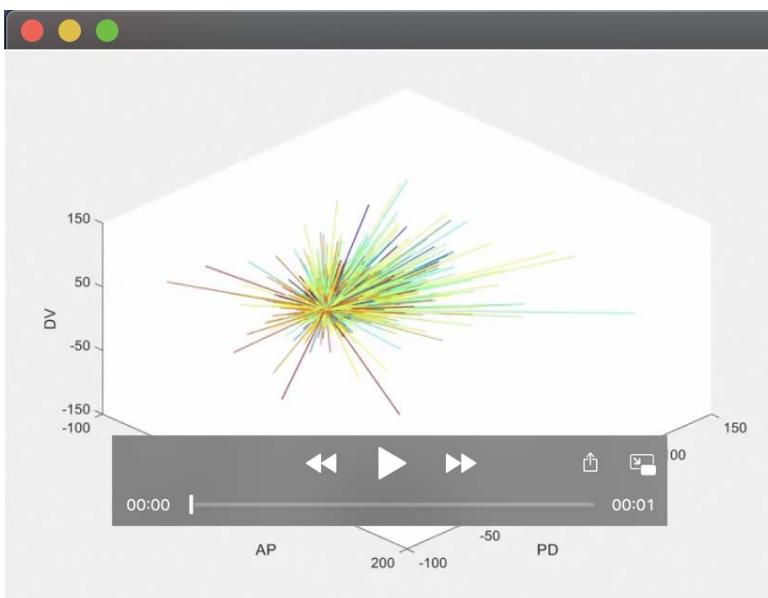
Movie 8

PIV of *Irx3/5^{+/−}*;H2B-GFP hindlimb at 32 som. during 2.5 h light sheet time-lapse. Anterior is to the left.



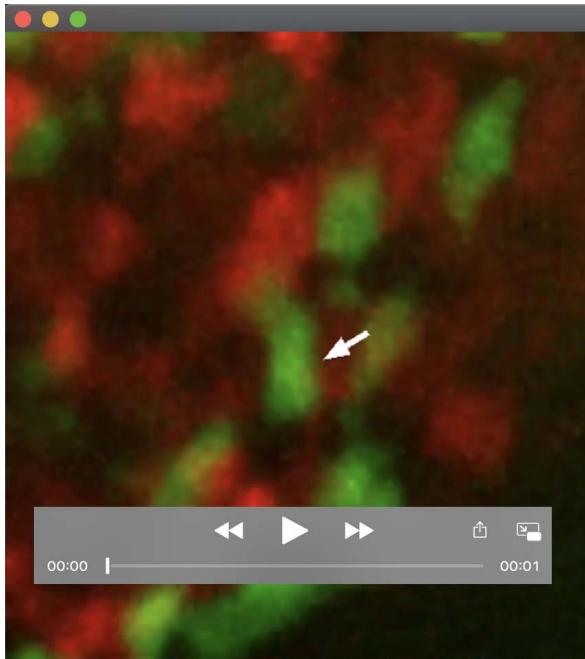
Movie 9

Dandelion plot of cell trajectories of *Irx3/5^{+/-}*;H2B-GFP hindlimb at 32 som. during 2.5 h light sheet time-lapse. Anatomical axes are labelled: AP (anteroposterior), DV (dorsoventral), PD (proximodistal).



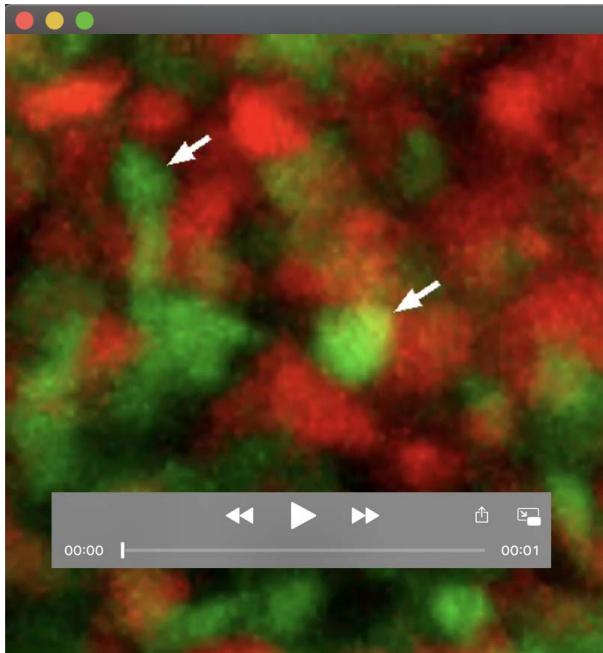
Movie 10

Dandelion plot of cell trajectories of *Irx3/5^{-/-}*;H2B-GFP hindlimb at 32 som. during 2.5 h light sheet time-lapse. Anatomical axes are labelled: AP (anteroposterior), DV (dorsoventral), PD (proximodistal).



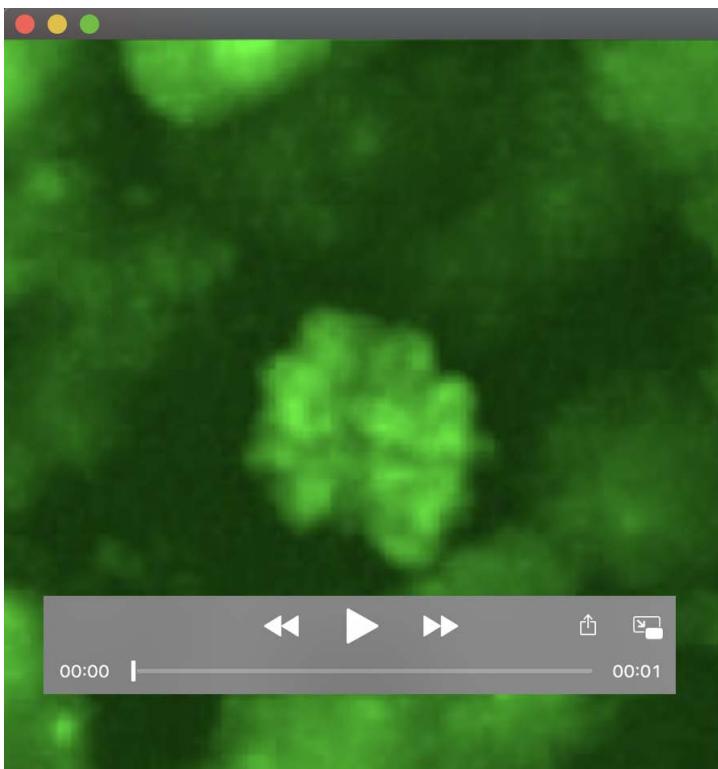
Movie 11

Live confocal time lapse movie of *Irx3/5^{+/−};Esr1-Cre;Confetti* embryo at 29 som. stage revealed that normal daughter cells (arrows) separate completely and intercalate among neighbouring cells in anterior mesoderm. Anterior is upward; dorsal to the left. Time course is 55 min..



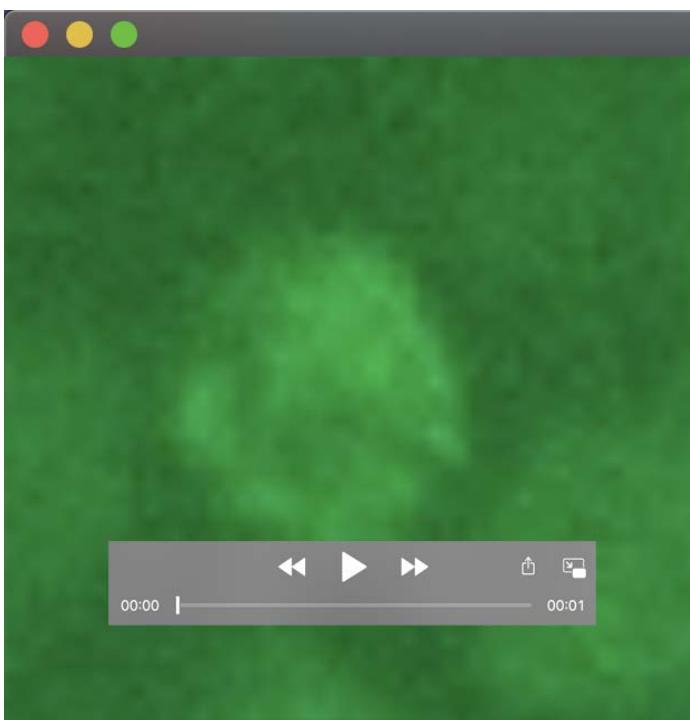
Movie 12

Live confocal time lapse movie of *Irx3/5^{−/−};Esr1-Cre;Confetti* embryo at 29 som demonstrated lack of daughter cell separation and intercalation among neighbours in anterior mesoderm. Anterior is upward; dorsal to the left. Time course is 55 min..



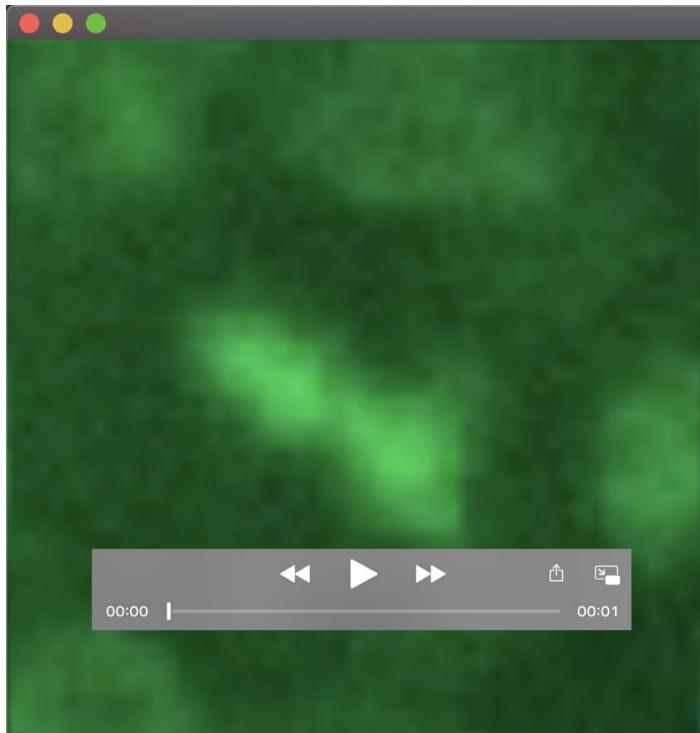
Movie 13

Live light sheet time lapse movie of *Irx3/5^{−/−}*;H2B-GFP anterior hindlimb mesoderm at 29 som. stage showed normal metaphase to anaphase separation of daughter cell chromatin. Time course is 80 min..



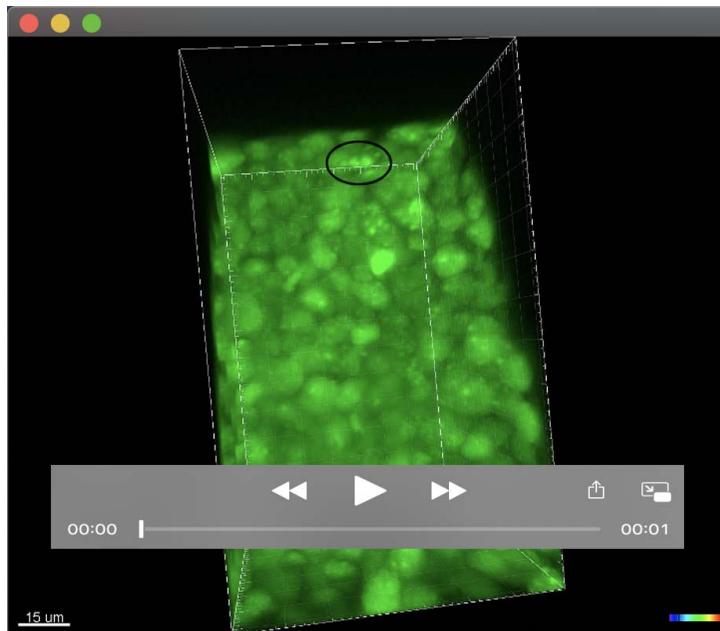
Movie 14

Live confocal time lapse movie *Irx3/5^{−/−}*;H2B-GFP anterior hindlimb mesoderm at 29 som. stage showed anaphase bridge with lagging separation of daughter cell chromatin. Time course is 35 min..



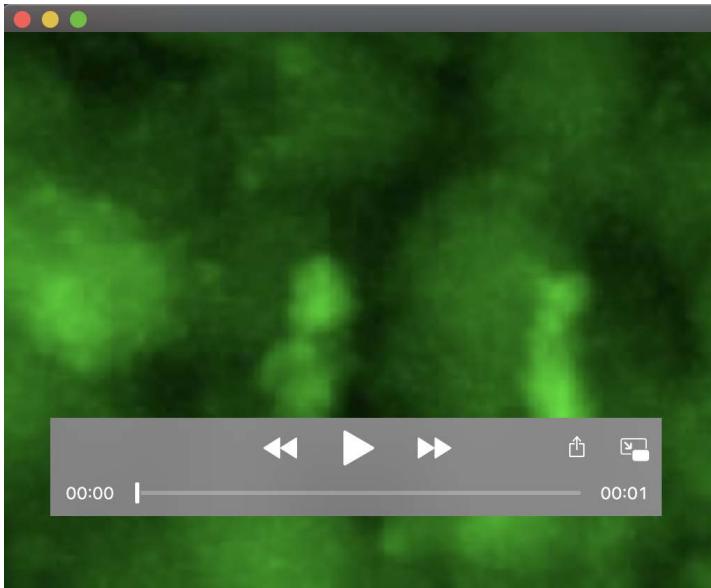
Movie 15

Live confocal time lapse movie of *Irx3/5^{-/-}*;H2B-GFP anterior hindlimb mesoderm at 29 som stage demonstrated persistent chromatin bridge between daughter cells. Time course is 35min.,.



Movie 16

Live light sheet time lapse movie of *Irx3/5^{-/-}*;H2B-GFP anterior hindlimb mesoderm at 29 som. stage showed failed cell division following condensation of chromatin at metaphase and subsequent evidence of chromatin fragmentation. Time course is 80 min..



Movie 17

Live light sheet time lapse movie of *Irx3/5^{-/-}*;H2B-GFP anterior hindlimb mesoderm at 29 som. stage begins at anaphase and demonstrates evidence of fragmentation and loss of both daughter cells. Time course is 80 min..