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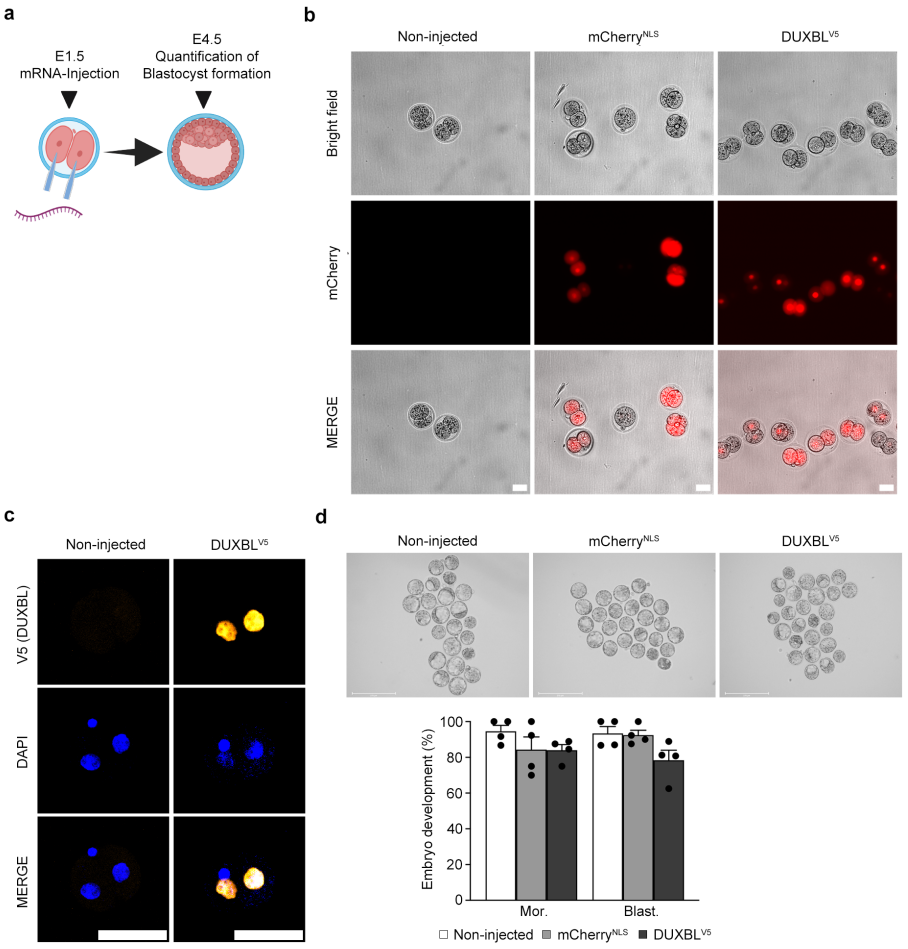
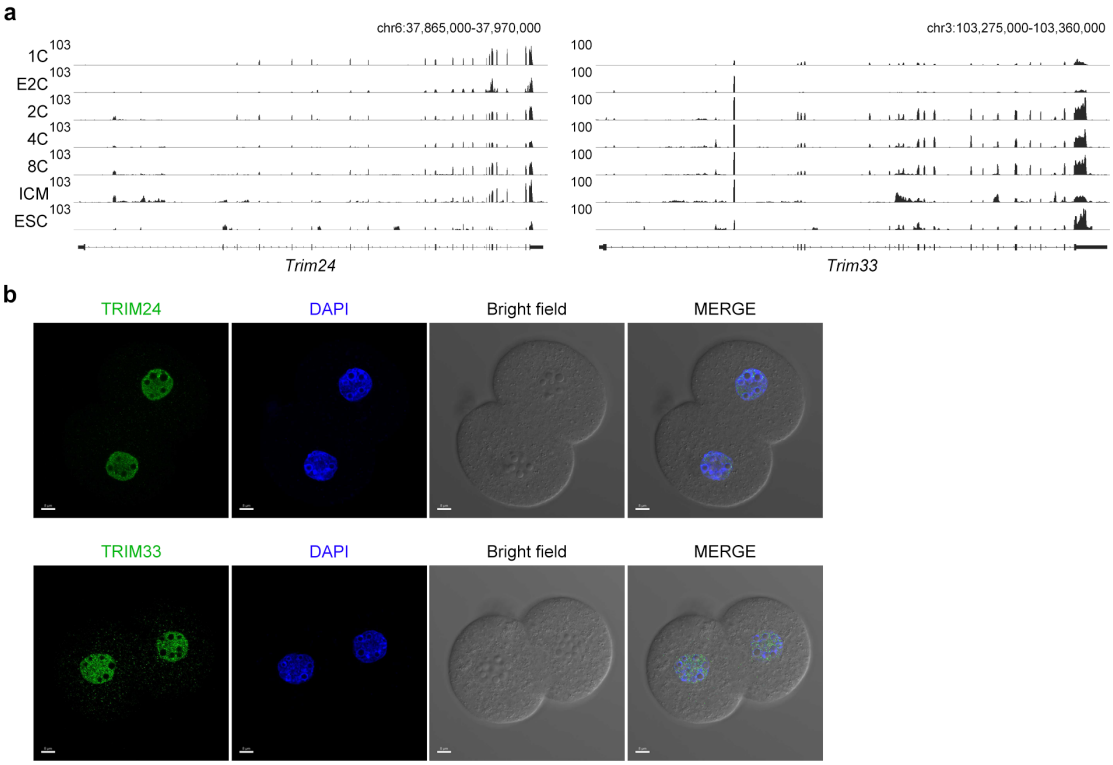
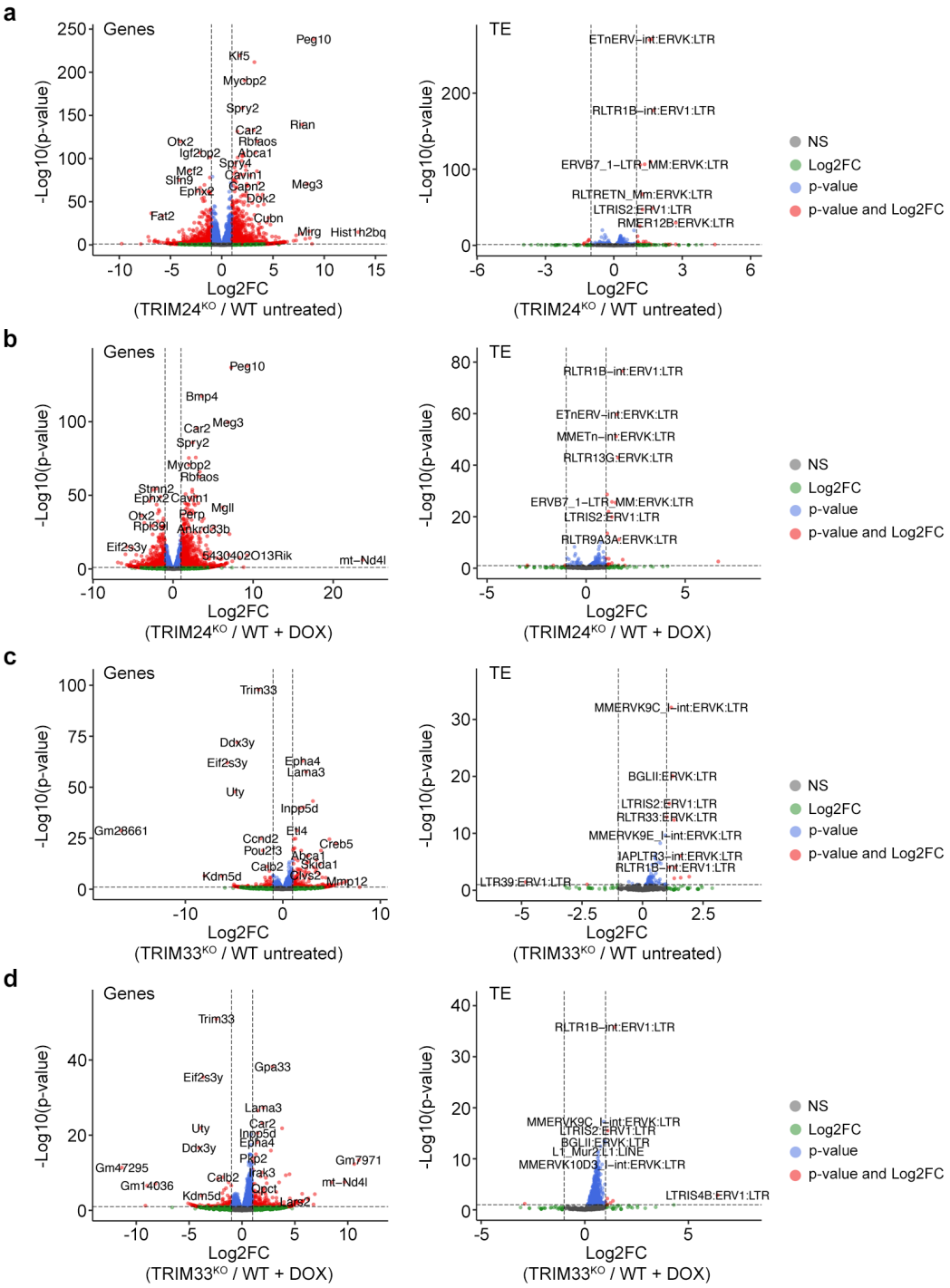
SUPPLEMENTARY FIGURES



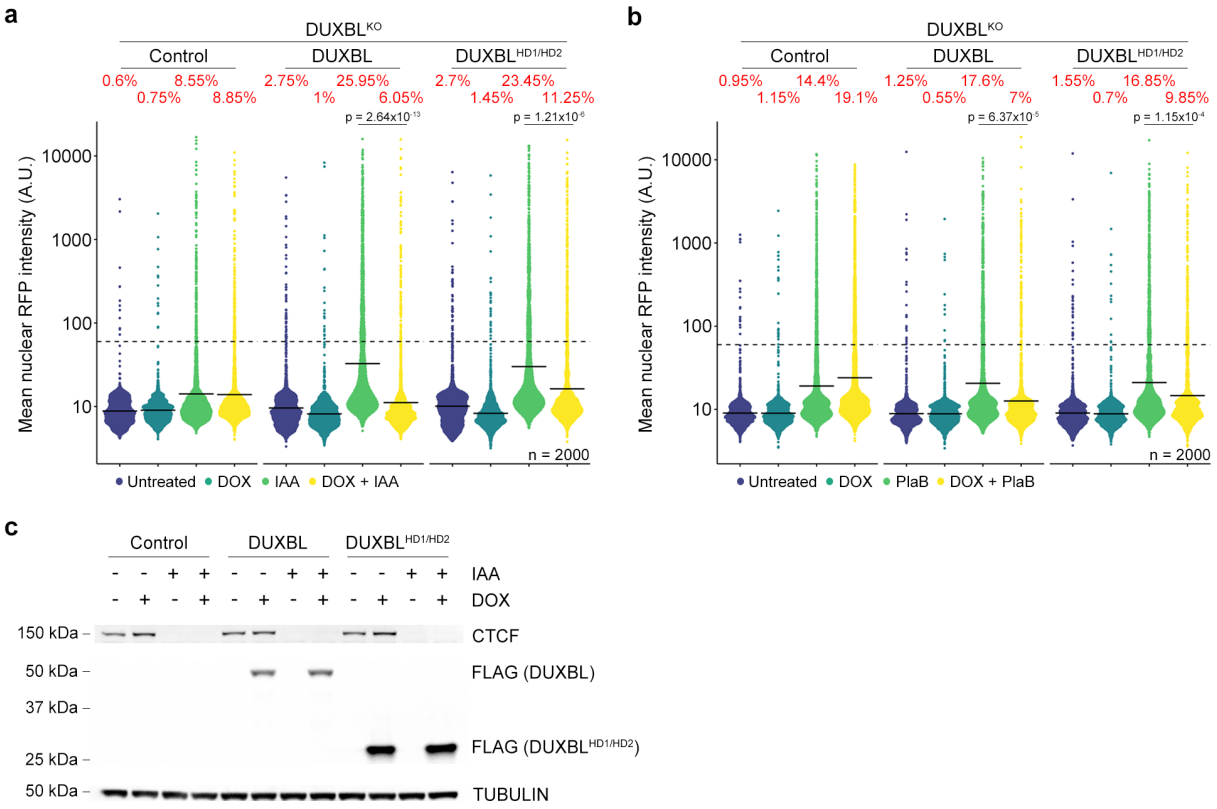
Supplementary Fig. 1: a) Schematic representation of the microinjection experiment. Late 2C-stage embryos (48 hours post hCG injection) were microinjected into each blastomere with mRNA encoding for mCherry^{NLS} or DUXBL^{V5} plus mCherry^{NLS}. Embryos were collected for immunofluorescence analysis or to examine embryonic development at late blastocyst stage (124 hours post hCG injection)(created with BioRender.com). **b)** Representative bright field and fluorescence images of 2C-stage embryos not injected or microinjected with mRNA encoding for mCherry^{NLS} or DUXBL^{V5} plus mCherry^{NLS}. Images were taken 6 hours post microinjection. Scale bars, 50 µm. Four independent experiments were performed but one representative is shown. **c)** Representative immunofluorescence analysis ofthe V5 tag in 2C-stage embryos not injected or microinjected with mRNA encoding DUXBL^{V5} plus mCherry^{NLS}. DAPI was used to visualize the nuclei. Scale bars, 50 µm. At least 10 independent embryos per condition were stained but one representative is shown. **d)** Representative bright field images and plot showing mean ± standard error of the mean (SEM) summarizing four independent experiments with a total of 8-26 microinjected 2C-stage embryos per group [non-microinjected, mCherry^{NLS}-microinjected mRNA and DUXBL^{V5}-microinjected mRNA] per experiment. The percentage of embryos reaching each embryo stage is shown. Embryos correspond to Mor: morula (96 hours post hCG) and Blast: blastocyst (120 hours post hCG). Scale bars, 275 µm.



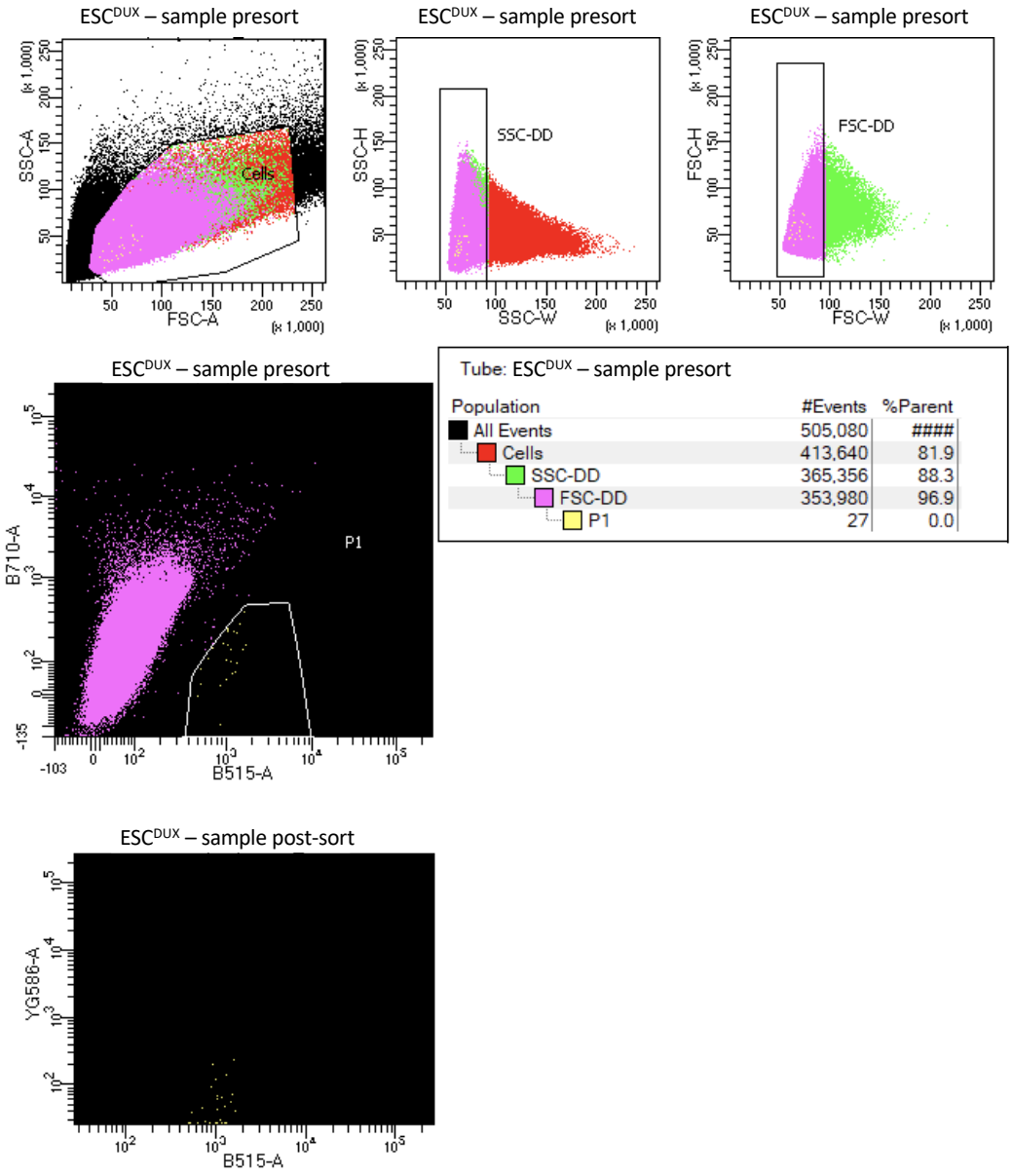
Supplementary Fig. 2: a) Genome browser tracks corresponding to averaged RNAseq samples from[33] showing TRIM24 and TRIM33 expression during embryonic development. **b)** Immunofluorescence analysis of TRIM24 and TRIM33 in late 2C-stage embryos. Scale bars, 8μm. Two independent experiments were performed but only one representative is shown.



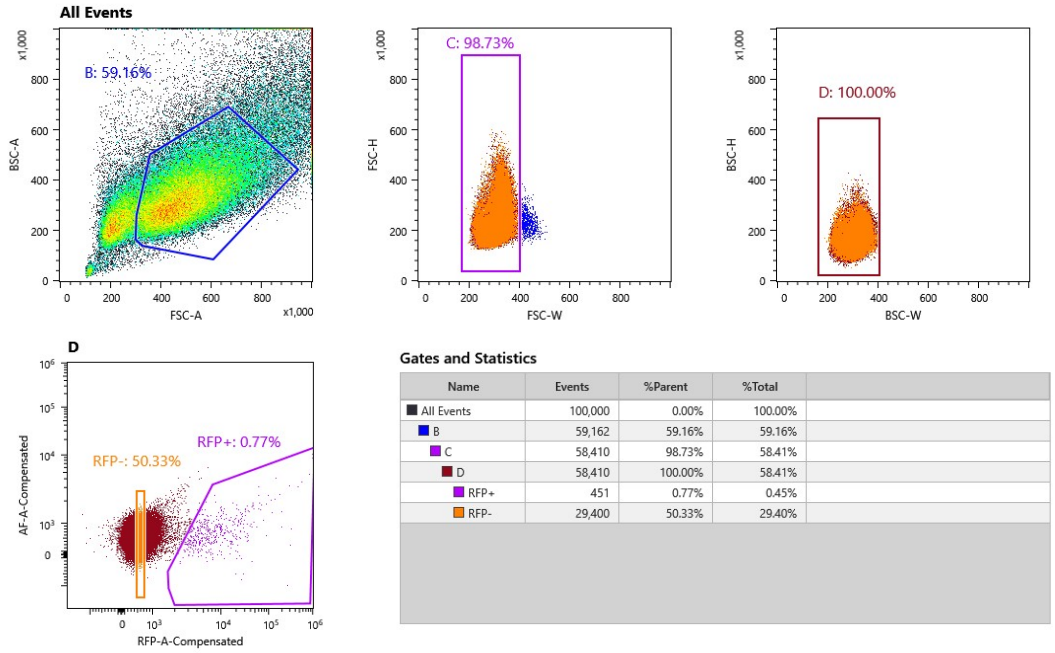
Supplementary Fig. 3: a, b) Volcano plots showing differentially expressed genes (left panels) and TE (right panels) between uninduced TRIM24^{KO} and control ESC lines (**a**) and DOX-treated for 16 hours TRIM24^{KO} and control ESC lines (**b**). **c, d**) Volcano plots showing differentially expressed genes (left panels) and TE (right panels) between uninduced TRIM33^{KO} and control ESC lines (**c**) and DOX-treated for 16 hours TRIM33^{KO} and control ESC lines (**d**). Two independent ESC lines were used per genotype and treatment condition. Log2 fold change>1; pValue<0.01.

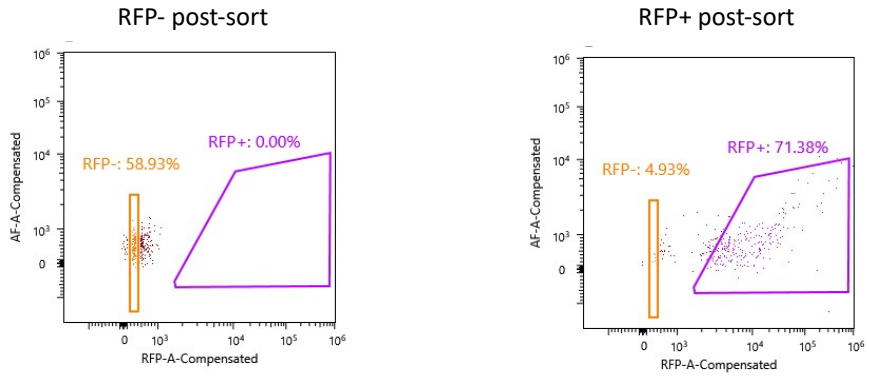


Supplementary Fig. 4: a) High-throughput imaging quantification of RFP+ cells in untreated or IAA/DOX-treated for 48 hours LTR-RFP reporter DUXBL^{KO} ESC$^{CTCF\text{-}AID}$ expressing DUXBL or DUXBL$^{HD1/HD2}$ (see Extended Data Fig. 8h for details). Center lines indicate mean values. Percentages of RFP+ cells above the threshold (dotted line) are indicated. n=2000; p value is shown from one-tailed unpaired *t*-test. Two independent experiments were performed but only one representative is shown. **b)** High-throughput imaging quantification of RFP+ cells in LTR-RFP reporter untreated or DOX-treated DUXBL^{KO} ESC expressing full length DUXBL or DUXBL$^{HD1/HD2}$ and incubated with 2.5 μM PlaB for 24 hours. Center lines indicate mean values. Percentages of RFP+ cells above the threshold (dotted line) are indicated. n=2000; p value is shown from one-tailed unpaired *t*-test. Two independent experiments were performed but only one representative is shown. **c)** Western blot analysis of DUXBL (FLAG) and CTCF performed in lysates from untreated or IAA/DOX-treated DUXBL^{KO} ESC$^{CTCF\text{-}AID}$ expressing DUXBL and DUXBL$^{HD1/HD2}$ FLAG. Tubulin levels are shown as a loading control. Two independent experiments were performed but only one representative is shown.

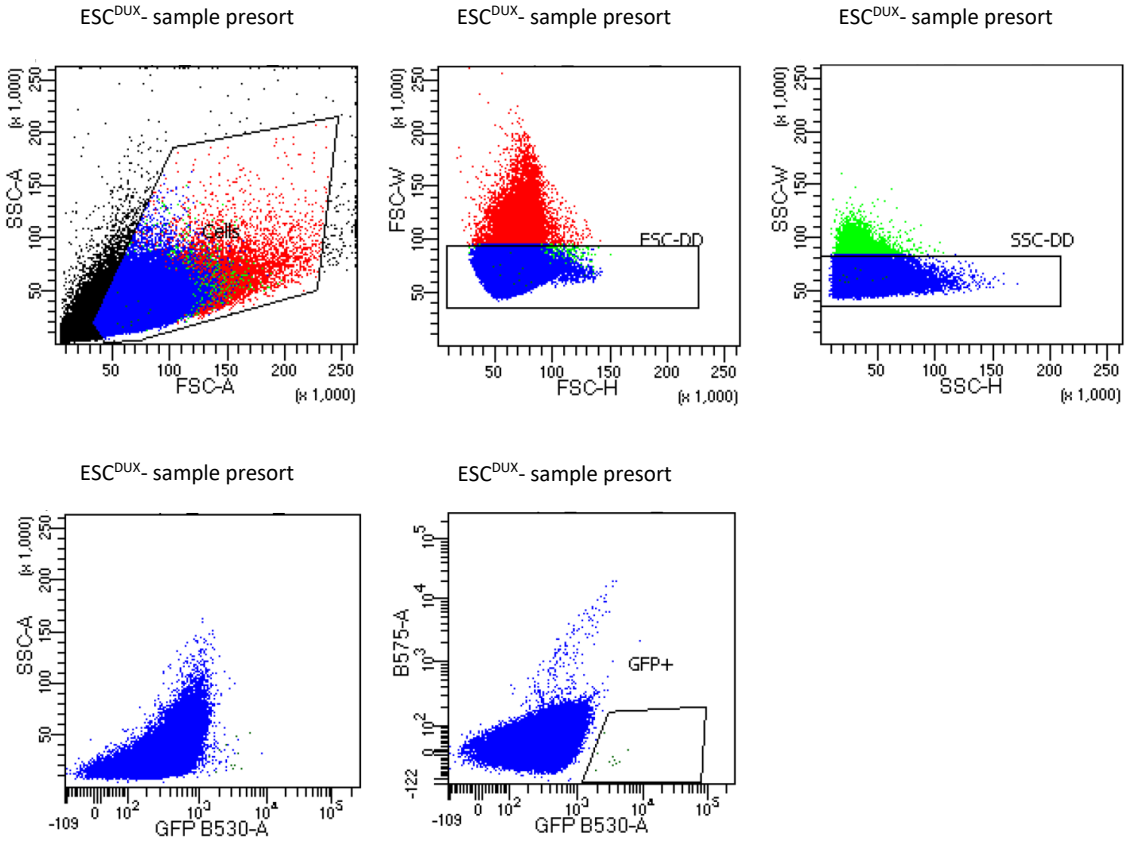


Supplementary Fig. 5: Flow cytometry plots generated from ESC^{DUX} after performing the OCT4-IRES-GFP and DUXBL^{iRFP702}-knock-in targeting as described in Methods. Gating shows the actual GFP+ cells sorted for the experiment. Two independent ESC^{DUX} lines were targeted and subclones isolated from them with similar results, but one representative is shown.





Supplementary Fig. 6: Flow cytometry plots generated from WT and DUXBL^{KO} ESC^{DUX} after performing the sorting of LTR-positive and negative ESC for each cell line. Sorting was performed in a SONY MA900 instrument. Two independent cell lines per condition were used but only one WT representative ESC line is shown as an example for our gating strategy. Post-sort quality control was performed for each sample.



Tube: Sample_TRIM24_CL 7

Population	#Events	%Parent	%Total
All Events	431,066	####	100.0
Cells	392,525	91.1	91.1
FSC-DD	373,893	95.3	86.7
SSC-DD	369,484	98.8	85.7
GFP+	11	0.0	0.0

Supplementary Fig. 7: Flow cytometry plots generated from ESC^{DUX} after performing the OCT4-IRES-GFP and TRIM24^{FKBP}-knock-in targeting as described in Methods. Gating shows the actual GFP+ cells sorted for the experiment. Two independent ESC^{DUX} lines were targeted and subclones isolated from them with similar results, but one representative is shown.

SUPPLEMENTARY METHODS

Collection of mouse embryos for RNAseq

Untreated WT embryos were collected at the late 1C stage (28-30 hours post-hCG), early 2C (32 hours post-hCG injection) and late 2C stage (48 hours post-hCG injection). Embryos microinjected with mRNAs or embryos obtained from *Duxbl*$^{+/\Delta}$ crossings were harvested at late 1C, early 2C, or late 2C stages (30, 36 and 48 hours post-hCG injection, respectively). RNA from single embryos was prepared using the SMART-Seq Stranded Kit (Takara Bio, 634442) and ribosomal cDNA was depleted using AMPure beads (Beckman Coulter, A63880). Alternatively, cDNA was also prepared using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio) following the manufacturer's protocol without RNA extraction. The Nextera XT kit with v2 Full Set indexes (N7-S5) was used for library preparation. Sequencing was performed on a NextSeq500 instrument (Illumina) using v2 chemistry or on a NovaSeq 6000 instrument (Illumina) with 2 x 75 bp paired-end setup.

In vitro transcription of mRNAs for microinjection

Duxbl-V5-2A-NLS-mCherry and *NLS-mCherry* sequences were cloned into pCW57-MCS1-2A-MCS2 (gift from Adam Karpf, Addgene, 71782). Prior to proceed with *in vitro* transcription (IVT), the T7 promoter was added to the constructs by PCR with the corresponding primers (Supplementary Table 14) using a Phanta Max Super-Fidelity DNA Polymerase (Vazyme, P505). Capped and polyadenylated mRNAs were produced using the mMessage mMachine® T7 Ultra Kit (ThermoFisher, AM1345) and cleaned up utilizing the MEGAclear™ Kit (ThermoFisher, AM1345) according to manufacturer's instructions.

Genotyping of *Duxbl*$^{\Delta/\Delta}$ embryos and pups

Genomic DNA of tail biopsies was isolated by Proteinase K treatment followed by 2-propanol precipitation. Embryos were collected at late 2C- (48 hours post hCG), 4C- (56 hours post hCG) and 8C-embryo stage (69 hours post hCG). Genomic DNA was pre-amplified using the Repli-g Single Cell Kit (Qiagen, 150345) according to manufacturer's instructions. Detection of the *Duxbl* wild-type or *Duxbl* deleted locus was performed by PCR using primers PS-416 and PS-417 or PS-412 and PS-413, respectively (Supplementary table 14).

Cell sorting

For cell sorting experiments, trypsinized live OCT4-IRES-GFP ESC with the specific gene targeting (DUXBL) were sorted based on GFP fluorescence levels on a BD FACSAria Violet instrument (Supplementary Fig. 5 and 7). Sorting of LTR-positive and negative ESC from WT and DUXBL^{KO} cell lines was performed in a SONY MA900 instrument (Supplementary Fig. 6). Post-sort quality control was performed for each sample (Supplementary Fig. 16-18).

High throughput imaging (HTI)

A total of 10,000-20,000 ESC (depending on the experiment and on the specific ESC line) were plated on gelatinized μCLEAR bottom 96-well plates (Greiner Bio-One, 655087). ESC were treated with DOX (different concentrations in the range from 10–1000 ng/ml depending on the experiment and on the specific ESC line), 2.5 μM PlaB, 0.53 μM RA or 500 mM IAA as indicated before fixation with 4% PFA in PBS for 10 minutes at room temperature.

Images were automatically acquired either using a CellVoyager CV7000 or a CellVoyager CV8000 high throughput spinning disk confocal microscope (Yokogawa, Japan). Each condition was always performed in triplicate wells and at least 9 different fields of view (FOV) were acquired per well. Image analysis was performed using the Columbus Image Data Storage and Analysis system (PerkinElmer). Nuclei were segmented based on DAPI staining. Detection of nuclear foci was performed on their respective fluorescence channels using the Find Spots module. Various measurements were calculated over the nuclear masks, including mean fluorescence intensities and (when applicable) the number, intensity, area, and colocalization of foci. Cell-level data was exported from Columbus as text files, then analyzed and plotted using R version 4.1.0. When analyzing HTI data, we considered statistically significant those samples that when compared showed an unpaired one or two-tail t-test with a p-value of at least 0.05 or lower.

Live cell imaging

A total of 40,000 ESC^{DUX} stably transfected with LTR-RFP and BR1, BR2 or BR3-eGFP PiggyBAC constructs were plated on gelatin-coated m-Slide 8-well plates (80826, Ibidi) and imaged untreated or DOX-treated every 20 minutes for a total time of 15 hours. To examine 2C-like conversion dynamics, 40,000 WT, TRIM24^{KO}, DUXBL^{KO}; TRIM24^{FKBP}, or DUXBL^{KO} ESC, stably transfected with LTR-RFP construct, were imaged every 30 min for a total time of 48 hours. To visualize DUXBL foci formation, a total of 40,000 000 ESC^{DUX} stably transfected with LTR-RFP and endogenously tagged with a miRFP702 fluorescent protein at the *Duxbl* locus were imaged

in untreated or DOX-treated ESC^{DUX} every 90 minutes for a total time of 14 hours. All images were acquired using the Nikon SoRa spinning disk confocal microscope equipped with 20x plan-apochromat objective lenses (N.A. 0.75 and 0.8, respectively) and stage top incubators to maintain temperature, humidity and CO2 concentration (Tokai Hit STX and Okolab Bold Line, respectively). Image processing was done using Nikon's NIS-Element. In case of the experiments analyzing DUXBL foci or BR-GFP reporter ESC lines, images were denoised using Nikon's NIS-Element denoise AI.

Western blot

Cells were trypsinized and lysed in 50 mM Tris pH 8, 8 M Urea (Sigma) and 1% Chaps (Millipore) followed by 30 min of shaking at 4°C. A total of 20 µg of extracts were run on 4%-12% NuPage Bis-Tris Gel (Invitrogen) and transferred onto Nitrocellulose Blotting Membrane (GE Healthcare). Transferred membranes were incubated with the following primary antibodies overnight at 4°C: DUXBL (1:1000, Custom antibody, GenScript), ZSCAN4C (1:500, AB4340, Millipore Sigma), TRIM24 (1:1000, TA802797, Origene), TRIM33 (1:1000, A301-060A, Bethyl Laboratories), CTCF (1:1000, 07-729, Millipore), HA (1:1000, C29F4, #3724, Cell Signaling), HA (1:1000, 6E2, #2367, Cell Signaling), H2A (1:1000, ab18255, Abcam), FLAG (1:1000, F1804, Sigma Aldrich), Tubulin (1:50000, T9026, Sigma-Aldrich). The next day the membranes were incubated with HRP-conjugated secondary antibodies Goat anti-Rabbit IgG (H+L) (1:5000; Thermo Fisher Scientific, Cat# 31466) or Goat anti-Mouse IgG (H+L) (1:5000; Thermo Fisher Scientific, Cat# 31431) for 1 hour at room temperature. Membranes were developed using SuperSignal West Pico PLUS or SuperSignal West Femto Maximum sensitivity (Thermo Scientific).

RNA extraction and qPCR

Isolation of total RNA and cDNA synthesis were performed by using the Isolate II RNA Mini Kit (Bioline) and SensiFAST cDNA Synthesis Kit (Bioline), respectively. Alternatively, RNA was isolated and cDNA synthesis performed with the Direct-zol™ RNA MiniPrep Plus Kit (Zymo Research, R2072) and PrimeScript RT Reagent Kit with gDNA Eraser (Takara, RR047B), according to the manufacturer's instructions. Quantitative real time PCR was performed with PowerUp SYBR Master mix in a QuantStudio 6 Pro system. Alternatively, Biozym Blue S'Green qPCR Kit Separate ROX (Biozym, 331416S) was also used. Expression levels were normalized to *Gapdh*. For a complete primer list see Supplementary Table 14. When analyzing quantitative real time PCR data, we considered statistically significant those samples that when compared

showed an averaged of two-fold difference in overall gene expression and an unpaired two-tail t-test with a p-value of at least 0.05 or lower.

Immunoprecipitation

To obtain nuclear extracts, ESC were trypsinized, washed with ice-cold phosphate-buffered saline (PBS), resuspended in 1 volume of ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA containing protease inhibitors) and incubated on ice for 10 minutes. Next, 1/10 volume of 1% IGEPAL CA630 (Sigma, Merck) was added to the samples and incubated for 3 additional minutes at room temperature. After that, cells were briefly vortexed, and the cytosolic fraction was obtained by centrifugation for 5 min at 2,500 *g*. The nuclear pellet was resuspended in 1 volume of high-salt-concentration extraction buffer (20 mM HEPES pH 7.9, 0.6 M NaCl, 1 mM EDTA containing protease inhibitors) followed by incubation with shaking at 4°C for 10 min, sonicated briefly (until clear) and incubated with shaking at 4°C for at least 30 min more. The nuclear extract was obtained by collecting the supernatant after centrifugation for 5 min at 16,000 *g*. Protein concentration was determined using the Bradford assay.

A total of 1 or 2 mg of nuclear extract was diluted in binding buffer (25 mM Tris pH 7.9, 200 mM NaCl and 0.5 mM EDTA) and centrifuged for 5 min at 16,000 g at 4°C. Protein G Dynabeads (Invitrogen, Thermo Fisher Scientific) were washed twice with binding buffer and then incubated with 3 μg per milligram of protein of DUXBL antibody, FLAG antibody, HA antibody or a non-specific IgG in the presence of 0.5 μg/ml BSA. Alternatively, anti-FLAG-agarose beads (DYKDDDDK Fab-TrapTM Agarose, FFA, Proteintech) were also used to pull down FLAG-tagged proteins. The antibody-bound beads were washed 5 times with binding buffer and incubated with the cleared supernatant overnight (for Protein G Dynabeads) or for 1 h (for anti-FLAG-agarose beads) at 4°C. The beads were then washed five times with binding buffer with 0.05% IGEPAL CA630 (Sigma, Merck). One tenth of the beads were eluted in loading buffer for Western blot purposes and the rest was processed for Mass Spectrometry analysis.

To analyze the interaction between P300 and DUX or DUXBL, protein extracts from ESC transfected with corresponding expressing vectors and induced by addition of 100 ng/ml Doxycycline (Sigma, D9891-10G) for 24 hours. Briefly, cells were lysed in RIPA buffer with 1 unit/μl Benzonase® (Millipore, E1014) followed by incubation at 4° C for five hours. Subsequently, 1 mg of total protein lysate was incubated with 5 μg anti-V5-antibody (Abcam, ab9116) or normal rabbit IgG control antibody (Cell signaling, 2729) and rotated at 4°C overnight. Samples were then

incubated with protein A beads (Diagenode, C03020002) at 4° C for 3 hours followed by four washes with RIPA buffer. Beads were then incubated with Laemmli Sample Buffer (Bio-Rad, 161-0747) and 50 mM DTT and subjected to gel electrophoresis. Membranes were incubated overnight either with anti-V5-antibody (Abcam, ab9116) or anti-P300-antibody (Invitrogen, RW128).

Mass spectrometry analysis

Samples were solution digested with trypsin using S traps (Protifi), following the manufacturer's instructions. Briefly, proteins were denatured in 5% SDS, 50 mM triethylammonium bicarbonate (TEAB) pH 8.5. They were next reduced with 5 mM Tris(2-carboxyethyl)phosphine (TCEP) and alkylated with 20 mM iodoacetamide. The proteins were acidified to a final concentration of 2.5% phosphoric acid and diluted into 100 mM TEAB pH 7.55 in 90% methanol and loaded onto the S-traps, washed four times with 100 mM TEAB pH 7.55 in 90% methanol, and digested with trypsin overnight at 37 °C. Peptides were eluted from the S-trap using 50 mM TEAB pH 8.5; 0.2% formic acid in water; and 50% acetonitrile in water. These elutions were pooled and dried by lyophilization.

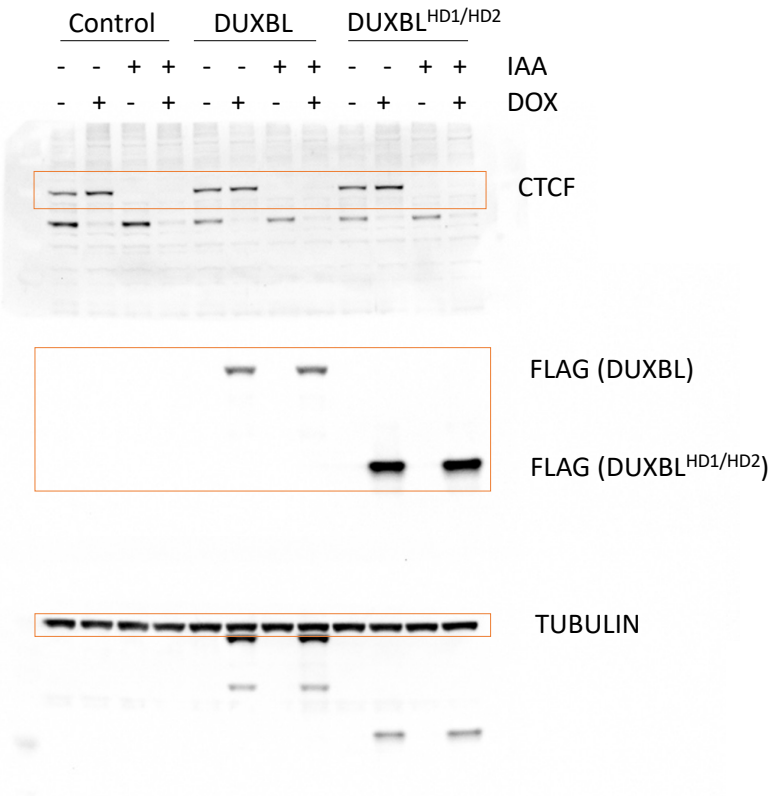
Dried peptides were resuspended in 5% acetonitrile, 0.05% TFA in water for mass spectrometry analysis on either an Obitrap Fusion Tribrid (Thermo Scientific) or an Orbitrap Exploris 480 (Thermo Scientific) mass spectrometer. The peptides were separated on a 75 μm x 15 cm, 3 μm Acclaim PepMap reverse phase column (Thermo Scientific) at 300 nL/min using an UltiMate 3000 RSLCnano HPLC (Thermo Scientific) and eluted directly into the mass spectrometer. For analysis in the Fusion, parent full-scan mass spectra collected in the Orbitrap mass analyzer set to acquire data at 120,000 FWHM resolution and HCD fragment ions detected in the ion trap. For analysis in the Exploris 480, parent full-scan mass spectra acquired at 120,000 FWHM resolution and product ion spectra at 15,000 resolution.

Proteome Discoverer 2.4 (Thermo) was used to search the data against the murine database from Uniprot using SequestHT. The search was limited to tryptic peptides, with maximally two missed cleavages allowed. Cysteine carbamidomethylation was set as a fixed modification, with methionine oxidation as a variable modification. The precursor mass tolerance was 10 ppm, and the fragment mass tolerance was 0.6 Da for data obtained on the Fusion and 0.02 Da for data obtained on the Exploris 480. The Percolator node was used to score and rank peptide matches using a 1% false discovery rate. Label-free quantitation of extracted ion chromatograms from MS1 spectra was performed using the Minora node in Proteome Discoverer.

DUXBL custom antibody

To generate a custom rabbit polyclonal antiserum (GenScript), a poly-histidine tagged DUXBL fragment (amino acids 193-350) was expressed in E. coli and purified by GenScript protein department. Two New Zealand rabbits (rabbit No. R04775 and R04776) were immunized with three injections (200mg/animal) every two weeks by conventional protocol. Freund's adjuvant was added to the immunogen. Seven days following the 3rd immunization, the titer of antiserums was tested by ELISA. Based on in-house testing results, the rabbits were sacrificed according to animal welfare principles and the final antiserum was purified by using antigen affinity column. Total IgG from pre-immune serum was used as negative control. 0.02% sodium azide was added in the final antiserums as preservative.

SOURCE DATA SUPPLEMENTARY FIGURES



Sup. Figure 4c