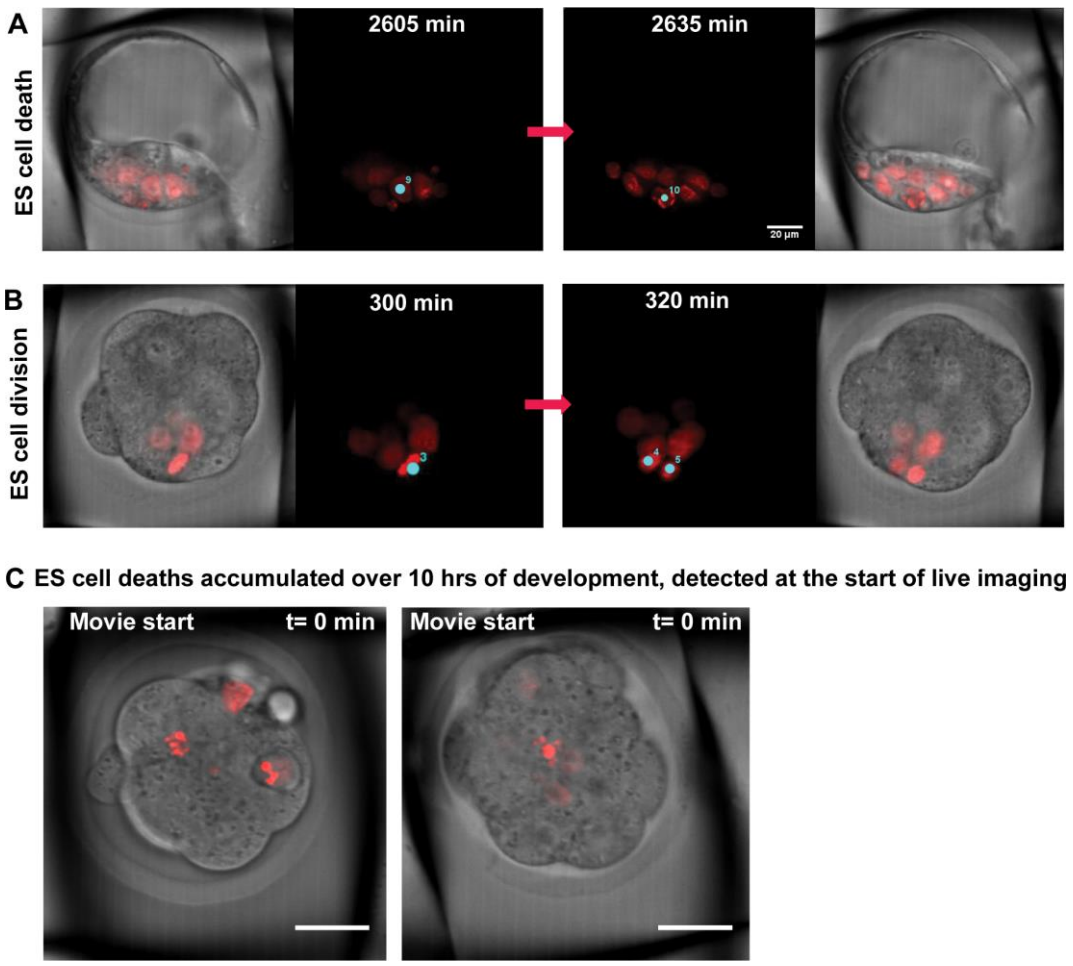

Fig. S1. ESC death and division. **A)** Progressive single plane images from bright field and red channels taken from time-lapse movies of an embryo showing example of an injected ESC undergoing apoptosis. **B)** Progressive single plane images from the Bright Field and red channels taken from time-lapse movies of an embryo showing example of an injected ESC undergoing mitosis. Data was tracked using Fiji (ImageJ) plugin TrackMate, manually marking each division or death event with a numbered circle. Scale bar = 20 µm. **C)** Single plane images from the first time point of recorded development. Going through the z-stack enables visualization of apoptotic ESCs from cell death events that occurred sometime after injection and before imaging started (~10 hrs). Scale bar = 30 µm.

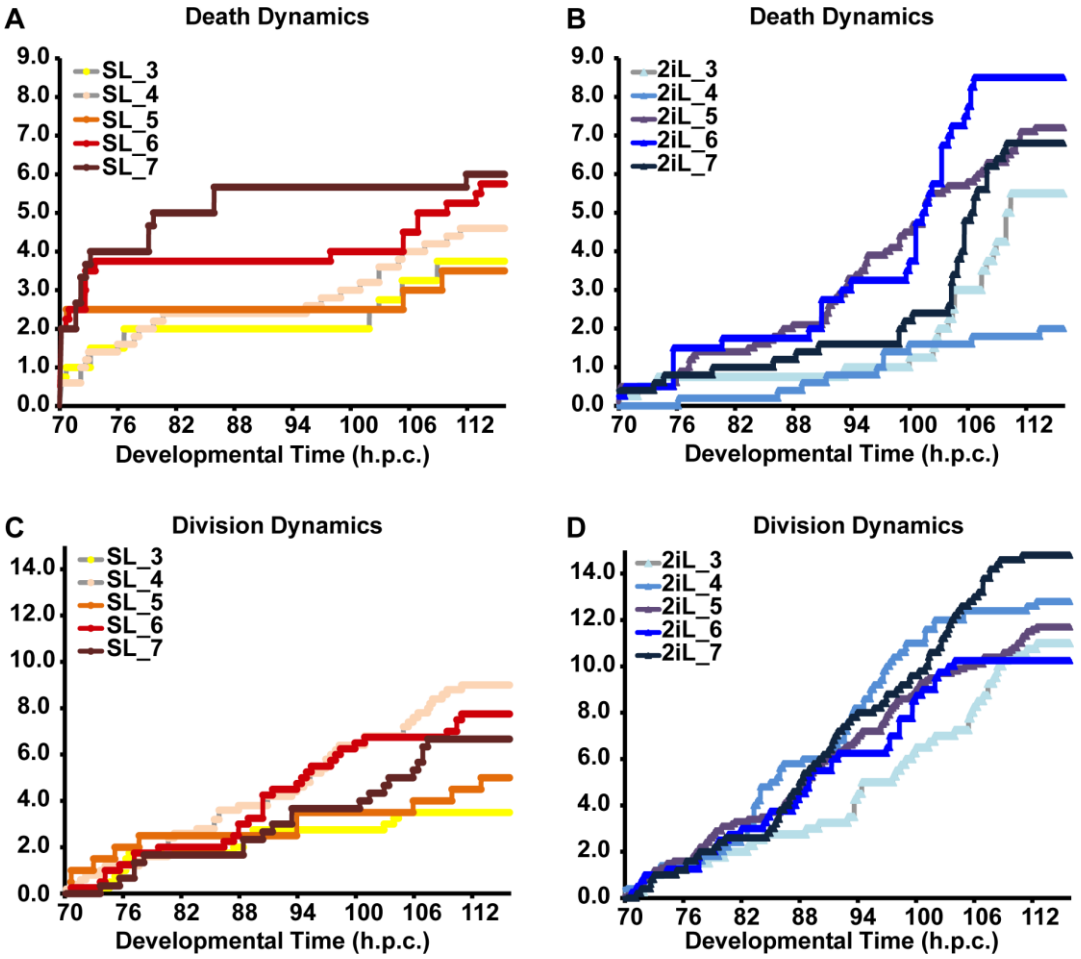

Fig. S2. Dynamics of ESC deaths and divisions per embryo showing groups separated by number of injected ESCs. Individual CDF plots from each embryo group. Cell death dynamics are presented of **A)** SL ESCs **B)** 2iL ESCs, and cell division dynamics of **C)** SL ESCs **D)** 2iL ESCs. No standard deviation is presented since each curve is a single-embryo profile from aggregated data (see Statistical Analysis in Materials and Methods).

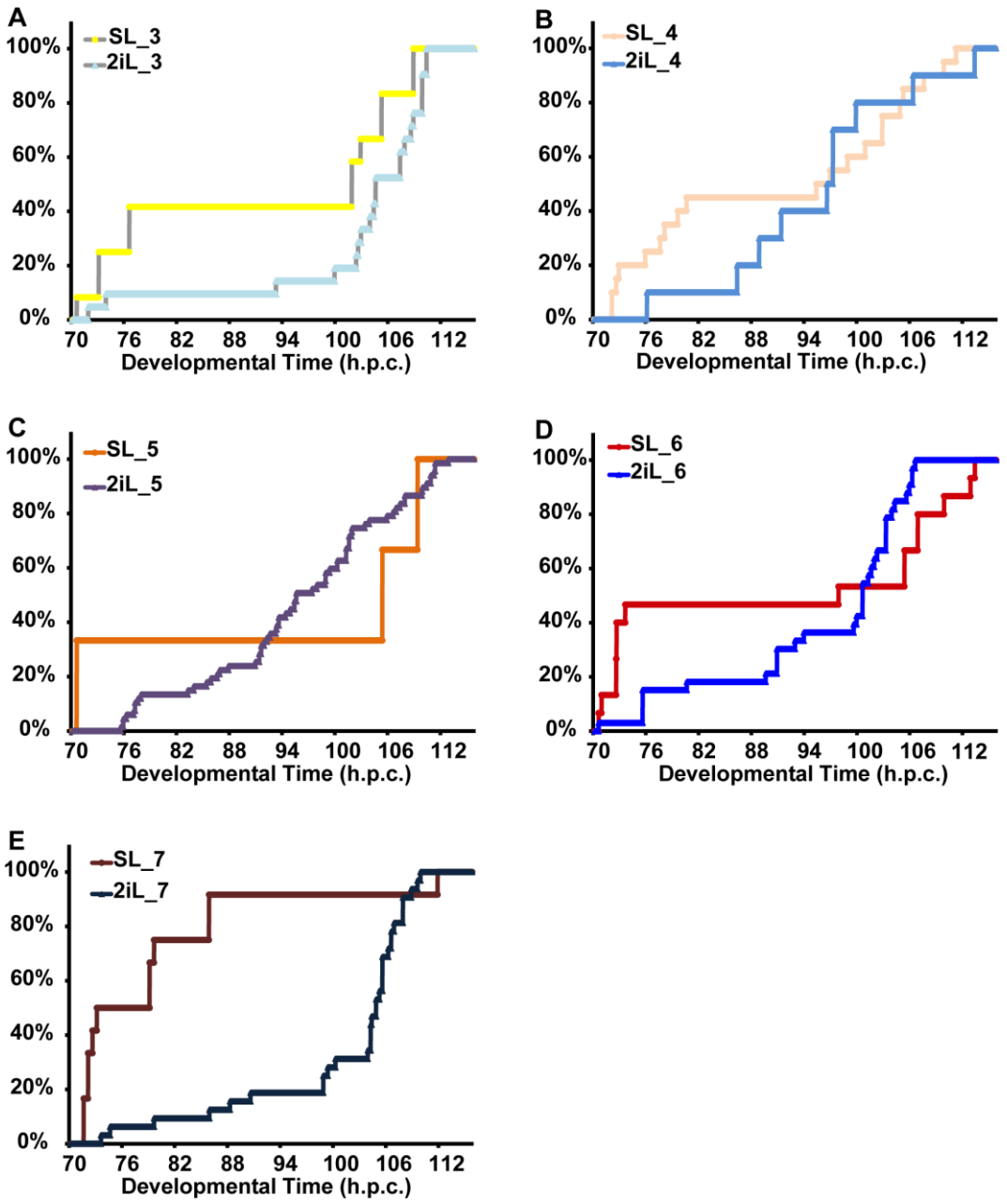

Fig. S3. Dynamics of ESC deaths per embryo with the same number of starting ESCs. Individual profiles representing each embryonic group with initial number of either (**A**) three, (**B**) four, (**C**) five, (**D**) six, or (**E**) seven ESCs. Each curve illustrates the temporal distribution of ESC death accumulation (the CDF) throughout development, per embryo. No standard deviation is presented since each curve is a single-embryo profile from aggregated data (see Statistical Analysis in Materials and Methods). All curves are consistent with the average of the five embryo groups presented in Fig. 2. A higher rate of ESC death is observed at the beginning of development for SL but not for 2iL, regardless of the number of injected ESCs.

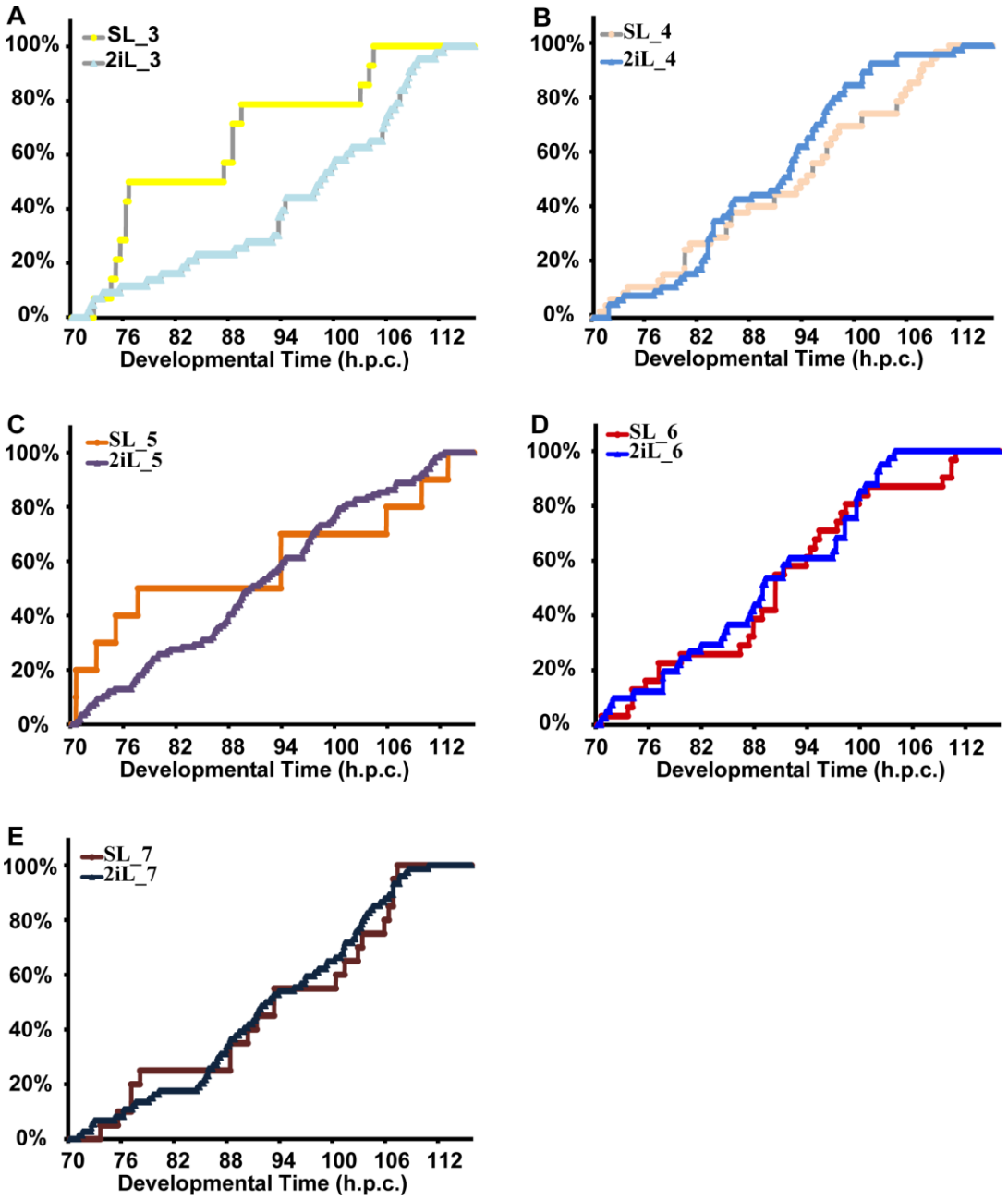

Fig. S4. Dynamics of ESC division per embryo with the same number of starting ESCs. Individual profiles representing each embryonic group with initial number of either (**A**) three, (**B**) four, (**C**) five, (**D**) six, or (**E**) seven ESCs. Each curve illustrates the temporal distribution of ESC division accumulation (the CDF) throughout development per embryo. No standard deviation is presented since each curve is a single-embryo profile from aggregated data (see Statistical Analysis in Materials and Methods). The curves are consistent with the average of the five embryo groups presented in Fig. 3. Comparable rates of ESC division is observed throughout development for SL and 2iL, regardless of the number of injected ESCs, and a peak of ESC division for SL occurs at the beginning of chimaera development.

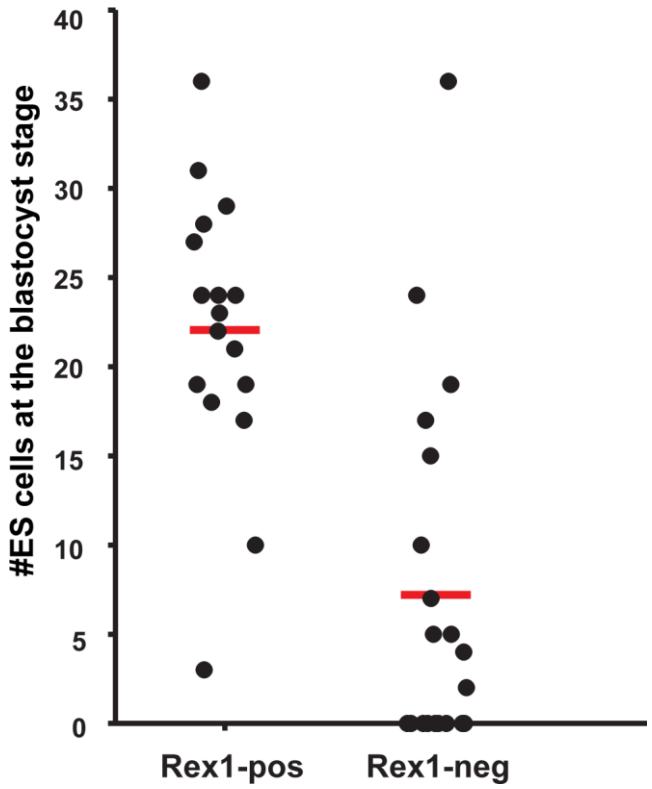

Fig. S5. Embryos injected with Rex1-neg versus Rex1-pos ESCs. The box plot displays the number of ESCs incorporated in chimaeras (113 h. p. c.); 8-cell stage embryos were injected with mKO2 Rex1GFP-pos or neg ESCs (3 to 8 cells) from SL. Each box plot is overlaid with raw data (which are jittered along the x-axis for clarity), where each black dot represents raw data from a single embryo; the red line marks the average value. 11/20 Rex1-neg and 17/17 Rex1-pos chimaeras are displayed as black dots above the baseline.

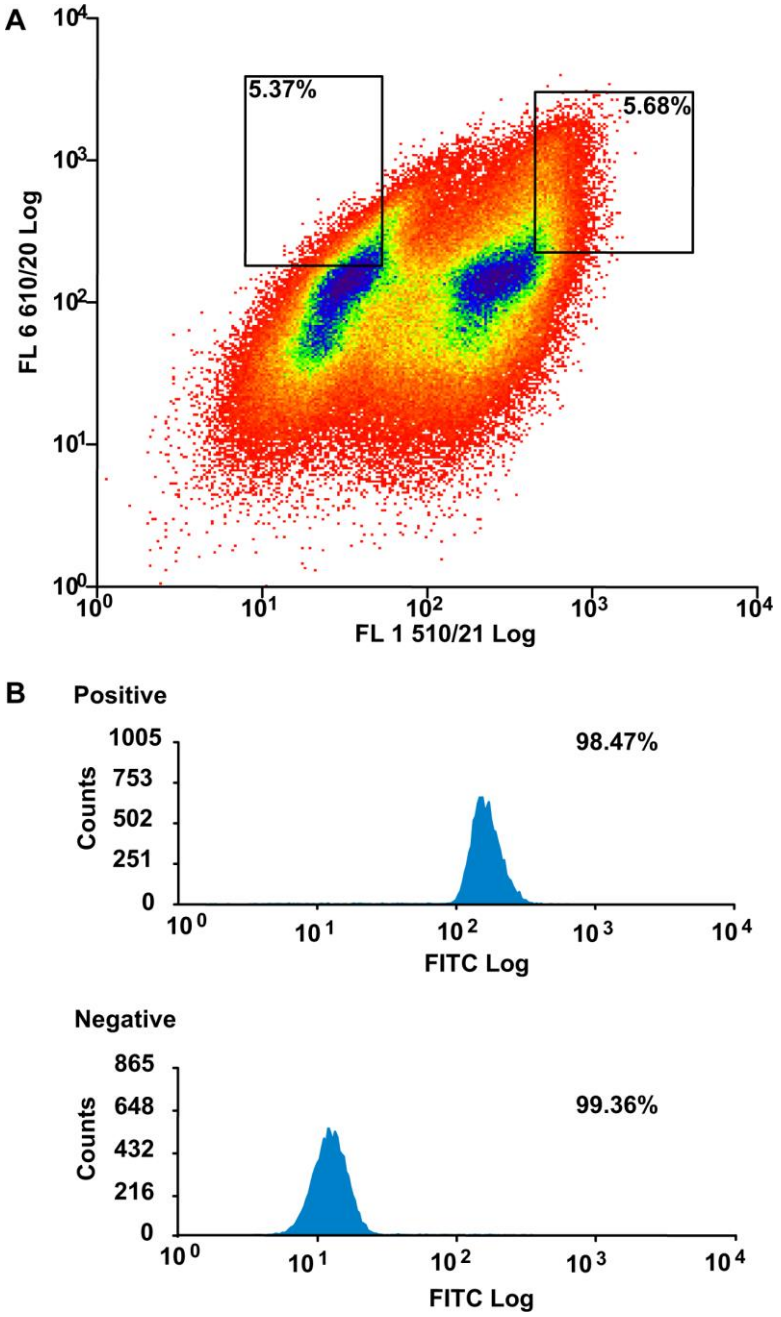

Fig. S6. Fluorescent sorting into Rex1-neg and Rex1-pos ESCs. A) Plot from MoFlo of the sorted sample. On the x-axis is the GFP signal (Log scale) and on the y-axis is red signal from the Kusabira Orange nuclei (Log scale). Approximately 5% of the top and bottom populations were sorted for injection. **B)** From the injection pool, ~10,000 cells were sorted again on the Cyan to test for potential sorting contamination. The x-axis displays the GFP signal (Log scale) and the y-axis is the count of the cells. Upper and lower panels show purity of fluorescent cell sorting.

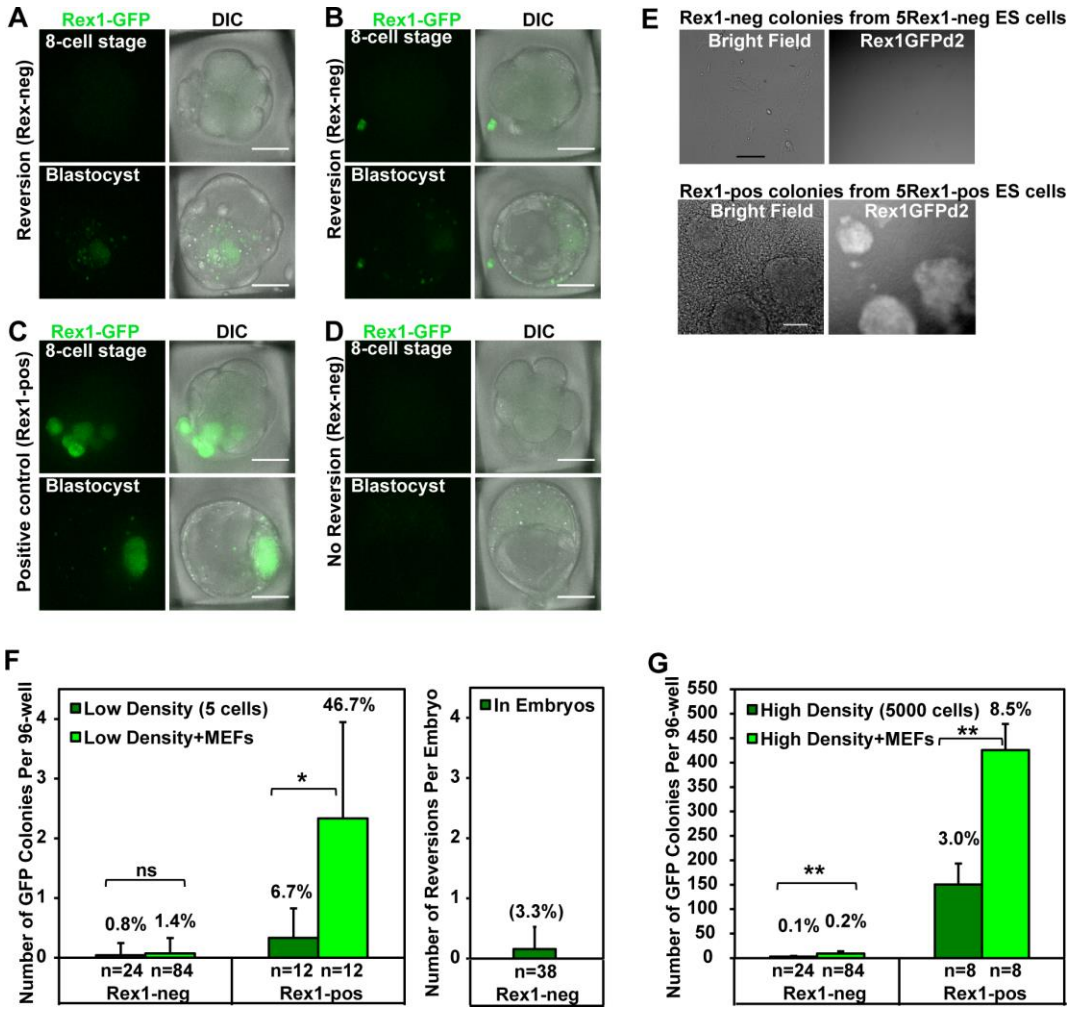

Fig. S7. Rex1 expression dynamics during chimaera development and in vitro. A, **B)** Embryos injected with sorted Rex1-neg ESCs. Images from the first (top) and last (bottom) time points of live imaging are presented. Left-hand panels display Rex1GFP (green) and right-hand panels display the bright field channel overlaid with the green channel (note the GFP positive element in **B** is contaminant outside the 8-cell embryo and remains immobile during imaging). Positive and negative controls are presented in **C** and **D**, respectively; data from the time-lapse experiment is presented in **Table S5**. Scale bar = 30 µm. **E)** In vitro control experiment, where 5 Rex1-neg or Rex1-pos ESCs were placed per gelatinized 96-well, from the pool used for injection, and grown in SL conditions for 12 days. 6/60 Rex1-neg 'colonies' developed (all GFP negative, and only few cells) whereas 21/24 Rex1-pos

colonies developed (all a mixture of GFP positive and negative cells). Left-hand panels: bright field channel, right-hand panels: Rex1GFP. Scale bar = 100 µm. **F, G)** In vitro control experiment, where Rex1-pos or Rex1-neg ESCs were plated in **F)** 'Low Density' (5 cells per well) or **G)** 'High Density' (5000 cells per well) in gelatinized 96-well plates, with or without inactivated murine embryonic fibroblasts (MEFs), and grown in 2iL conditions for 7 days. The number of wells plated for each ESC group is provided at the bottom of each dataset. Plotted data shows the average number of Rex1GFP-positive ESC colonies counted per well on day 7; error bars are standard deviation between wells. The percentages report the fraction of GFP colonies on day 7 from the number of plated ESCs on day 1. Right panel on **F**): Reversion rate in vivo in pooled data from the two chimaera experiments (4/19 and 2/19), where each embryo was injected with 6-7 ESCs in the first experiment, and with 3 in the second experiment. The percentage reports the fraction of Rex1 reversions (within the 2 days of development) from the total number of injected ESCs. In all experiments, approximately 5% of the top and bottom populations were sorted for Rex1-pos and Rex1-neg ESCs as shown in **Fig. S6**. * $P = 0.0046$; ** $P < 0.001$.

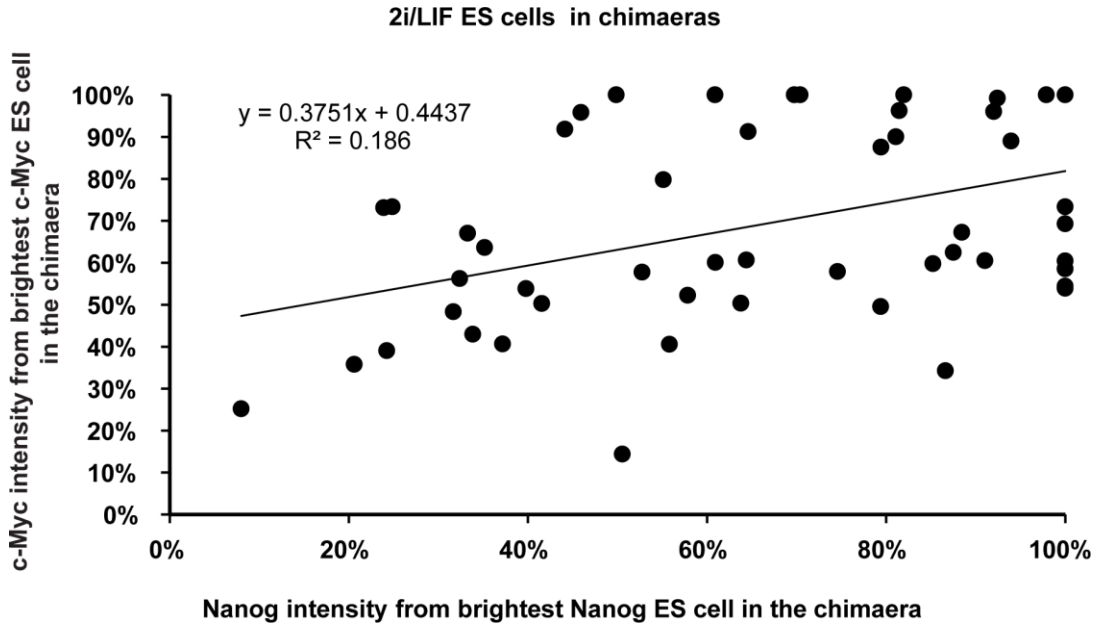

Fig. S8. Weak correlation between Nanog and c-Myc in 2iL chimaeras. Nanog expression appears to correlate weakly with c-Myc expression in 2iL chimaeras (Fig. 6). The x-axis represents ESC Nanog intensity and the y-axis represents ESC c-Myc intensity. Nanog and c-Myc Expression of each ESC is expressed as a percentage from the brightest ESC in the chimaera. The data indicates that there is a weak positive correlation between c-Myc and Nanog. Pearson correlation r = 0.481, P = 0.001.

Table S1. Behaviour of ESCs grown in SL or 2iL conditions and injected into 8-cell embryos

Average data are presented for the embryo groups injected with different numbers (either 3, 4, 5, 6 or 7 cells) of ESCs that were grown in SL or 2iL conditions or sorted into Rex1-neg and Rex1-pos ESC fractions from SL culture. These include numbers of ESC death events and ESC division events accumulated throughout the length of chimaera development. Further, the numbers of total cells (injected ESCs plus newly generated progeny cells) and viable cells are calculated as well as the percentage increase of viable cells from the initially injected ESCs. The tables also provide information about the standard error (s.e.m.) across the embryo groups. Embryos injected with sorted ESCs were grouped according to the number of initial ESCs (see Materials and Methods) and the average for all embryos is provided.

Click here to Download Table S1

Table S2. ESC death events distributed throughout time

	#embryos	#cells at start	# initial deaths	% deaths from injected cells	% deaths from all death events	# total death events
		60 h.p.c.	60 -70 h.p.c.	60 -70 h.p.c.		60-116 h.p.c.
SL all cells	18	87	24	27.6	27.9	86
SL per embryo		4.8 ± 0.3	2.0 ± 0.3	26.3 ± 5.5	26.6 ± 6.0	4.7 ± 0.5
2iL all cells	28	141	6	4.3	3.6	169
2iL per embryo		5.0 ± 0.2	1.2 ± 0.1	4.2 ± 1.8	6.0 ± 3.7	6.0 ± 1.1
	#embryos	60 h.p.c.	60-66.6 h.p.c.	60-66.6 h.p.c.		60-114 h.p.c.
Rex1- all cells	33	166	76	45.8	52.1	146
Rex1- per embryo		5.0 ± 0.2	2.3 ± 0.2	40.9 ± 4.7	60.17 ± 6.64	4.0 ± 0.4
Rex1+ all cells	17	99	14	14.1	17.9	78
Rex1+ per embryo		5.8 ± 0.3	1.7 ± 0.2	25.6 ± 4.4	35.2 ± 7.3	4.6 ± 0.4

Injections were conducted at ~60 h.p.c, and imaging started at ~70 h.p.c. for unsorted cells (SL or 2iL), or at ~66.6 for sorted cells (Rex1-neg or Rex1-pos). Average values ± standard error (s.e.m.) are presented.

Table S3. Cell death and division events accumulated in time

	# embryos	% Deaths from total cells	% Divisions from total cells	new cells:dead cells
SL	18	49.1 ± 5.8	48.7 ± 5.0 *	1.7 ± 0.4
2iL	28	36.7 ± 6.1	67.2 ± 4.0 *	3.7 ± 0.8
Rex1-	33	64.8 ± 5.7	16.3 ± 4.4 †	0.32 ± 0.11
Rex1+	17	52.6 ± 6.9	40.2 ± 7.2 †	1.42 ± 0.46

Average values ± standard errors (s.e.m.) are presented. Fractions of the accumulated ESC deaths or divisions are calculated from the total number of cells at the end of imaging (injected ESCs plus newly generated progeny cells). Two-way t-test between the 2iL fractions and SL fractions of dead cells yielded a P-value of 0.1302, indicating that these fractions are not significantly different. Two-way t-test between the 2iL fractions versus SL fractions of new cells per embryo yielded P-value of 0.0056, indicating that the distributions of these fractions are statistically different (*). Sorted cells were previously grown in SL culture. Two-way t-test comparing the fractions of dead Rex1-pos ESCs per embryo with the fractions of dead Rex1-neg yielded a P-value of 0.2068. Two-way t-test between the fractions of new Rex1-pos ESCs per embryo and the fractions of new Rex1-neg yielded a statistically significant P-value of 0.0043 (†).

Table S4. Comparison between epiblasts from Rex1-pos and Rex1-neg ESCs

Dataset	Embryo group	Average ± s.d.	P-value
Fig. 5A All embryos	Rex^- ES cells	3 ± 5	**0.0010**
	Rex^+ ES cells	17 ± 17	
Fig. S5 All embryos	Rex^- ES cells	7 ± 10	**<0.0001**
	Rex^+ ES cells	22 ± 8	
Fig. 5A chimaeras only	Rex^- ES cells	7 ± 7	**0.0600**
	Rex^+ ES cells	20 ± 17	
Fig. S5 chimaeras only	Rex^- ES cells	13 ± 10	**0.0100**
	Rex^+ ES cells	22 ± 8	

The numbers of progeny ESCs in chimaeras were compared between experiments following injection of Rex1-GFP sorted cells to determine whether there are statistically significant differences between Rex1-neg and Rex1-pos ESC survival. P-value < 0.05 indicates that the values are from different distributions at the 5% significance level. The table provides average values ± standard deviation (s.d.) for each cell type and experiment.

Table S5. Live imaging of 8-cell embryos injected with Rex1-neg ESCs

# total embryos	Reversion	Reversion then death of ES cell	Contamination*	No GFP signal
19	2	2	3	12

Data is presented for the occurrence of Rex1GFP-neg ESC up-regulating Rex1GFP during development, termed 'reversion' of Rex1 state. 19 embryos were injected at the 8-cell stage with 3 ESCs each and imaged until late blastocyst stage on the spinning disk microscope. We also observed 'reversion' followed by ESC death of the reverted cells in 2/19 chimaeras.

*Contamination in Rex1-neg injected embryos (3/19) was defined as GFP positive cell observed at the start of live imaging; however we cannot exclude the possibility that these Rex1-pos cells represent reversion events that occurred before imaging started (about 4-5 hours after injection). Furthermore, GFP signal from the Rex1-pos ESCs in the 'contaminated' embryos (3 ESCs in one embryo, and 1 ESC in each of two embryos) disappeared soon after imaging started. Hence these cells either down-regulated Rex1GFP or died within a few hours during the morula stage. Taking into consideration the high purity of fluorescent cell sorting (Fig. S6), it is unlikely that all the Rex1-pos ESCs at the start of imaging originated from cell sorting contamination.