**Supplementary Figure 1: Validation of knock-in cell lines**
a General knock-in strategy used in this study. **b** PCRs to verify knocked-in reporters in the SNSF and SBROS cell lines. **c** Western blot confirming the tagging of endogenous SOX2 and OCT4. **d** Example images used for the quantification of total OCT4 (aOCT4) and correlation with heterozygous OCT4-HALO expression.
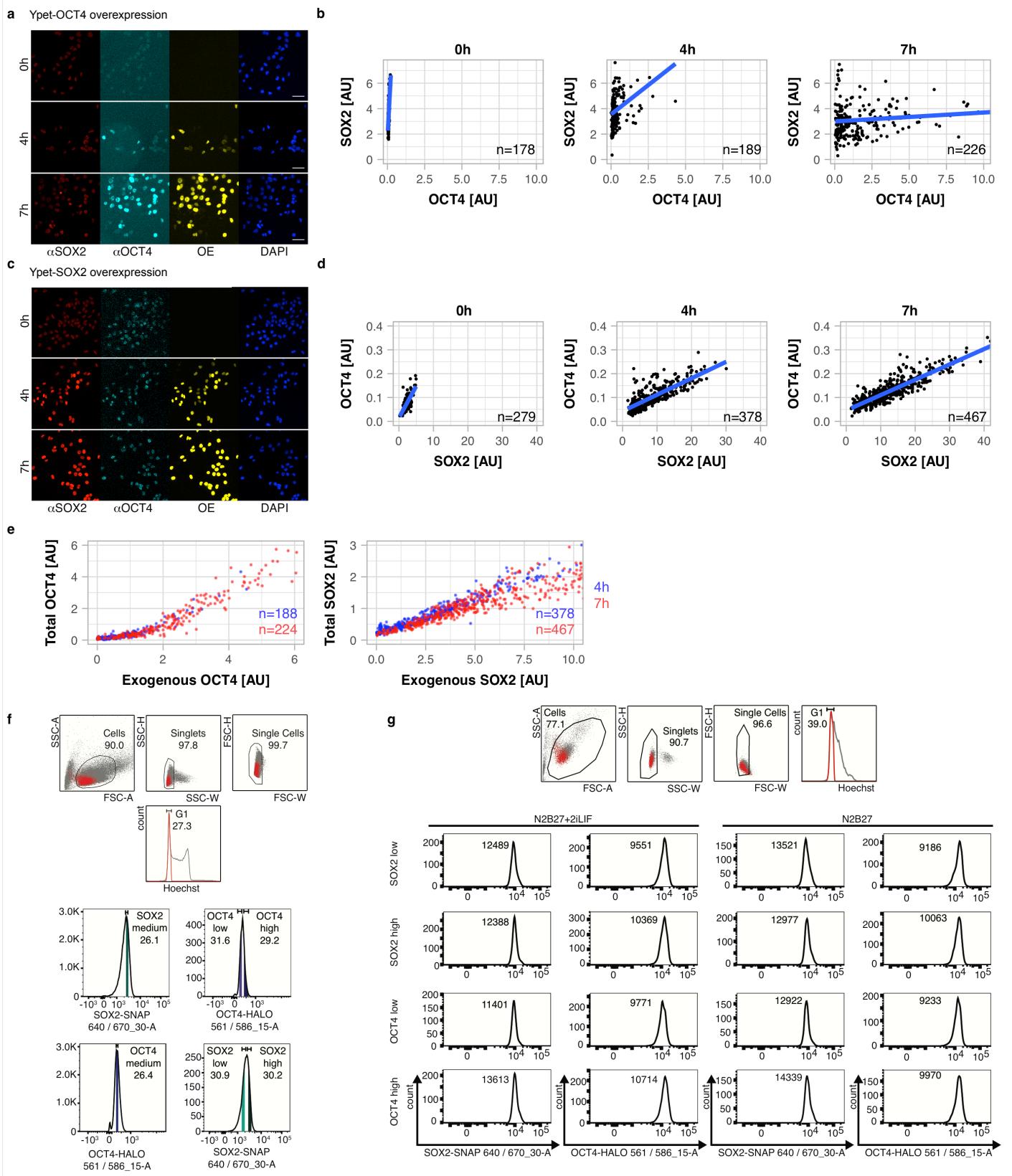
Supplementary Figure 2: Cross regulation of SOX2 and OCT4
a-b YPET-OCT4 overexpression time-course; **a** fluorescence microscopy images after immunofluorescence staining of SOX2 and OCT4. **b** quantification of total SOX2 and OCT4 levels in single cells. **c-d** YPET-SOX2 overexpression time-course; **c** fluorescence microscopy images after immunofluorescence staining of SOX2 and OCT4. **d** quantification of total SOX2 and OCT4 levels in single cells (0h: R=0.74; 4h: R=0.80; 7h: R=0.89). **e** Total OCT4 or SOX2 as a function of overexpression of YPET-OCT4 or YPET-SOX2, respectively, at 4 hours (blue) and 7 hours (red) after addition of dox. **f** Sorting strategy to sort for endogenous medium levels of SOX2 or OCT4. These populations were then sorted in OCT4 or SOX2 high and low, respectively. **g** Raw data of the determination of SOX2 and OCT4 levels after sorting intermediate SOX2 or OCT4 expressing cells for high or low OCT4 or SOX2 levels, respectively.
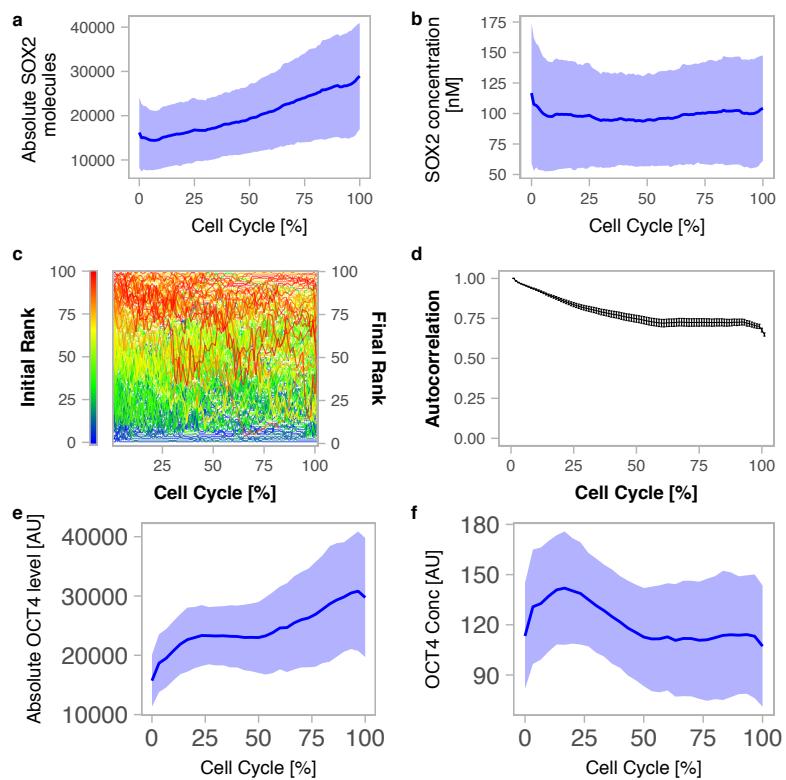
Supplementary Figure 3: Average SOX2 and OCT4 levels and concentration in pluripotency and SOX2 mixing time using data from one cell cycle
a-b Average of absolute SOX2 protein levels (**a**) and concentration (**b**) in pluripotency (N=164 cells, shaded area: SD). **c** Changes of single cell ranks of SOX2 levels over time using data from one cell cycle (N=100 cells). Red: initially high expressing cells; Blue: initially low expressing cells. **d** Rank-based autocorrelation function of the SOX2 ranks. Error bars denote standard error estimated by bootstrapping. **e-f** Average of OCT4 protein levels (**e**) and concentration (**f**) in pluripotency (N=48 cells, shaded area: SD).
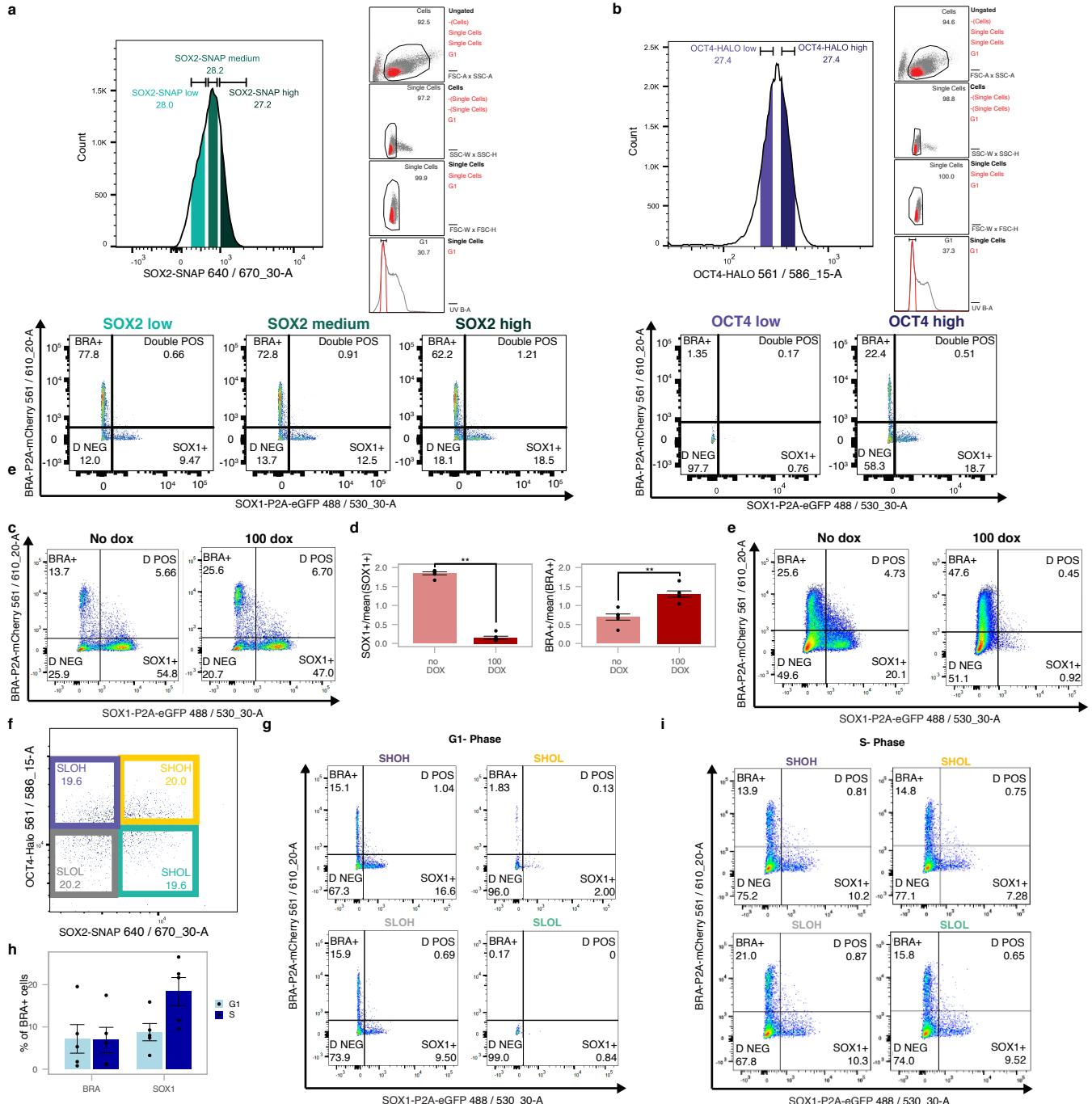
Supplementary Figure 4: Impact of initial SOX2 and OCT4 levels on differentiation outcomes
a,b Sorting strategies to evaluate the impact of SOX2 (**a**) or OCT4 (**b**) levels on differentiation outcomes and example of flow cytometry data. **c** Example of flow cytometry data after four days of differentiation with or without OCT4 overexpression for 12 hours before differentiation (see Fig.3g). **d** Outcome after four days of differentiation with or without 100ng/ml dox throughout differentiation. % of SOX1+ and BRA+ cells are normalized on the average for each experiment; N=6; ** p<0.01. **e** Example of flow cytometry data after four days of differentiation with or without dox throughout differentiation (see Supplementary Fig.4d). **f** Sorting strategy for SHOH, SHOL, SLOH and SLOL populations in G1-phase. **g** Example of flow cytometry data of SHOH, SHOL, SLOH and SLOL sorted in G1 phase (see Fig. 3i). **h** Average fraction of BRA+ and SOX1+ cells after four days of differentiation, when sorted for G1 or S-phase, respectively. Error bars: SE. N=5 experiments. **i** Example of flow cytometry data of SHOH, SHOL, SLOH and SLOL sorted in S phase (see Fig. 3j).
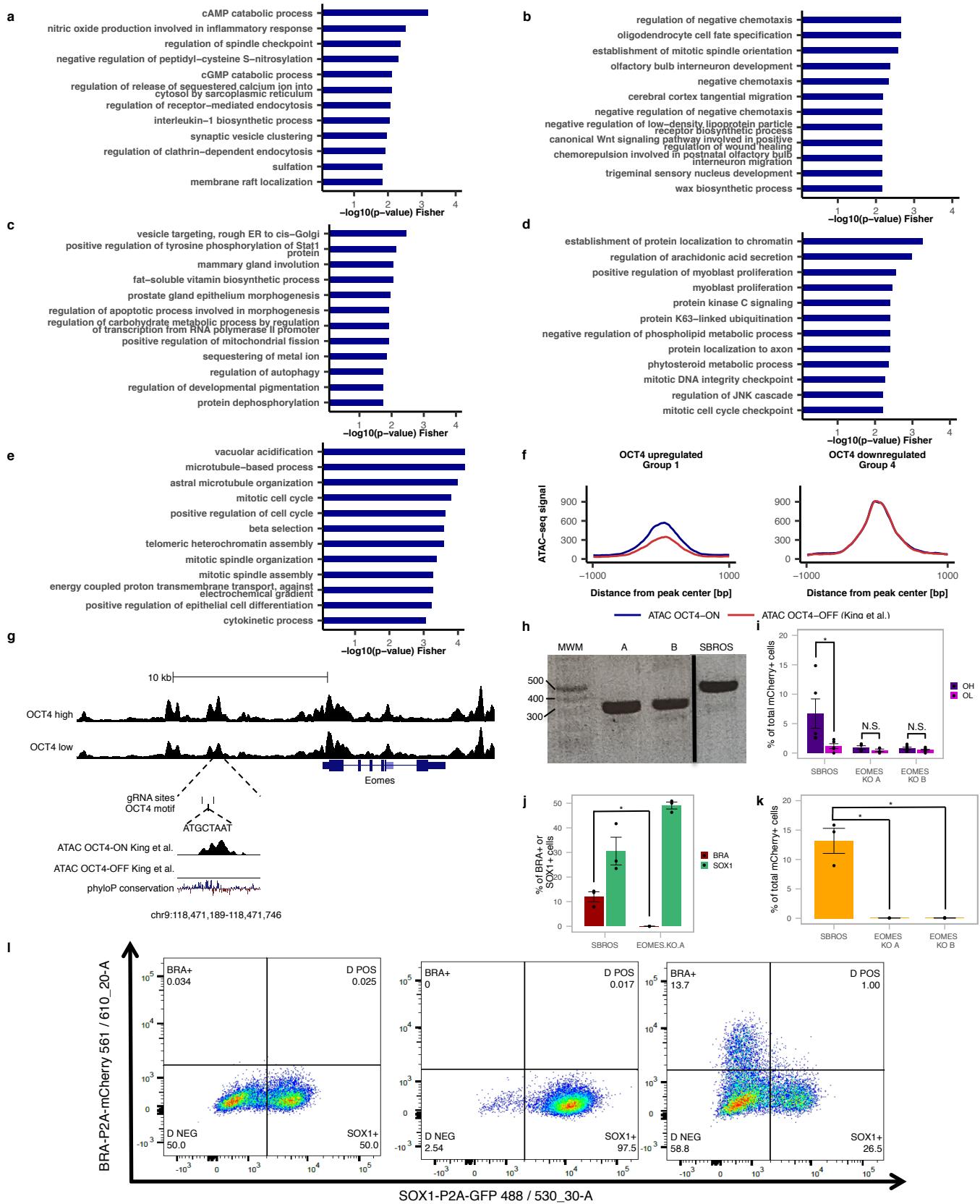
Supplementary Figure 5: Gene Ontology Enrichment Analysis in groups 2 to 6 from the ATAC-seq analysis and phenotype of *Eomes* enhancer mutants

a-e Top 10 GO terms in group 2, 3, 4, 5 and 6, respectively (see Fig.4). **f** Changes in accessibility upon OCT4 knock-down (data from [19]) of regions up or down-regulated in OH cells. **g** *Eomes* enhancer depending on OCT4, showing the position and sequence of the OCT4 motif, the ATAC-seq signal in the presence or absence of OCT4 (data from [19]), and the PhyloP conservation score for placental mammals. **h** PCR on genomic DNA of the modified *Eomes* enhancer in the *Eomes* KO A and B clone and the parental SBROS cell line. **i** % of total mCherry + cells (GFP- and GFP+) after four days of differentiation for cells sorted in G1-phase for SBROS (N=5 experiments), *Eomes* KO A (N=4 experiments) and *Eomes* KO B (N=6 experiments) cells. OL: OCT4-low cells; OH: OCT4-high cells. **j** Outcome after four days of differentiation for unsorted SBROS and *Eomes* ES KO A cells (N= 3 experiments). **k** % of total mCherry + cells (GFP- and GFP+) after four days of differentiation for unsorted SBROS, *Eomes* KO A and *Eomes* KO B cells (N= 3 experiments). * p<0.05. N.S: non-significant. **l** Example of flow cytometry analysis for experiments shown in panel j and k.

Supplementary Tables

SOX2-half-life	OCT4-half-life
7.66	8.63
7.44	6.41
9.69	8.29
7.70	7.17
7.07	7.07
7.65	7.64
8.61	7.39
7.60	7.59
9.45	10.60
9.55	8.88
9.63	5.36
8.12	9.68
7.66	7.29
6.67	8.13
9.82	7.22
8.37	9.52
7.18	8.27
8.80	7.12
6.70	7.35
7.27	5.72

Table 1: Raw data showing the protein half-life for SOX2-SNAP and OCT4-HALO in hours in each single cell measured. Figure 1g

WT	SNSF	SBROS
14.03	17.96	14.24
15.45	18.93	15.70
16.38	16.76	14.67
19.73	16.59	16.17
17.04	16.73	14.13
16.42	16.39	14.99
		15.45
		16.82

Table 2: Raw data showing the cell cycle duration of the different cell lines in hours. Figure 1h

YPet-SOX2-delDBD		
time	*Oct4*	*Sox2*
0	1	1
0	1	1
0	1	1
2	1.07	0.88
2	0.92	1.13
2	0.71	0.59
6	1.12	0.62
6	0.92	1.00
6	0.67	0.62
SOX2-SNAP		
time	*Oct4*	*Sox2*
0	1	1
0	1	1
0	1	1
2	0.96	0.58
2	1.60	0.72
2	1.09	0.96
6	0.84	0.26
6	1.28	0.19
6	0.83	0.23

Table 3: Normalized qPCR data for *Sox2* and *Oct4* mRNA upon YPet-SOX2-delDBD or SOX2-SNAP overexpression. Figure 2b

Ctrl	Sox2OE
5.59	10.78
4.36	10.12
4.04	6.81
4.39	5.35
3.79	3.89
3.83	4.93
3.65	14.47
5.66	2.95
4.70	8.42
3.70	3.79
4.25	4.63
4.53	6.52
5.74	5.52
6.24	4.68
5.12	10.96
4.47	5.67
5.17	6.04
3.88	6.15
3.12	6.32
5.53	8.19

Table 4: Raw data of OCT4 half-life (in hours) with or without SOX2-SNAP overexpression (Sox2OE) in individual cells. Figure 2c

	SOX2 high			SOX2 low			OCT4 high			OCT4 low	
Initial		SOX2	OCT4		SOX2	OCT4		SOX2	OCT4		SOX2	OCT4
1		4024	271		2340	200		3268	328		3222	159
2		4716	516		2947	509		3771	657		3723	385
3		5312	360		3000	354		4283	481		4240	242
4		2904	363		1488	416		2476	553		2488	213
	SOX2 high			SOX2 low			OCT4 high			OCT4 low	
8h 2iLIF	n	SOX2	OCT4	n	SOX2	OCT4	n	SOX2	OCT4	n	SOX2	OCT4
1	4017	18570	10677	1764	12437	6769	3014	17800	11809	1251	14752	6425
2	1171	37134	22004	967	32140	20656	720	37848	21981	533	34152	19830
3	522	38511	17281	3155	29544	15502	2076	35557	20283	1211	36378	21418
4	3186	8376	11430	2458	5589	12097	1394	6160	12462	4788	7514	8435
	SOX2 high			SOX2 low			OCT4 high			OCT4 low	
8h Diff	n	SOX2	OCT4	n	SOX2	OCT4	n	SOX2	OCT4	n	SOX2	OCT4
1	5371	19255	10649	2224	12433	6782	2077	18107	12575	2039	14777	7247
2	1529	39463	21126	936	27365	18453	1260	31363	19771	567	37070	18334
3	567	34713	15074	2650	25516	15642	1644	32022	18668	1312	31604	15006
4	845	7714	12914	2021	5275	12232	1171	5698	13029	3549	7100.00	9614

Table 5: Raw data from the flow cytometry experiment to test the cross-regulation of endogenous SOX2 and endogenous OCT4. The reported values represent the mean fluorescence intensity of SOX2-SNAP (stained with SiR-647) and OCT4-HALO (stained with TMR dye). n represents the number of cells analysed. Figure 2e,f

1	BRA+	DPOS	DNEG	SOX1+
Low	76.85	0.73	12.30	10.09
Med	72.75	0.87	13.15	13.20
High	64.85	1.03	17.90	16.25
2	BRA+	DPOS	DNEG	SOX1+
Low	86.75	0.01	12.85	0.40
Med	79.80	0.04	19.30	0.89
High	68.75	0.10	29.60	1.53
3	BRA+	DPOS	DNEG	SOX1+
Low	77.90	0.47	15.30	6.36
Med	76.45	0.37	15.70	7.52
High	76.20	0.33	15.80	7.71
4	BRA+	DPOS	DNEG	SOX1+
Low	87.50	0.07	10.50	1.93
Med	86.90	0.21	9.26	3.63
High	83.60	0.27	11.80	4.37
5	BRA+	DPOS	DNEG	SOX1+
Low	6.72	0.24	84.00	9.07
High	5.62	0.16	82.95	11.30

Table 6: Raw data from the flow cytometry experiment to test the differentiation outcome after sorting for different SOX2 levels. The values represent the percentage of the marker in the population. Figure 3d

1	BRA+	DPOS	DNEG	SOX1+
Low	1.22	0.03	95.55	3.19
High	3.34	0.14	89.35	7.18
2	BRA+	DPOS	DNEG	SOX1+
Low	1.13	0.25	95.10	3.51
High	12.50	0.51	63.90	23.15
3	BRA+	DPOS	DNEG	SOX1+
Low	0.85	0.09	98.35	0.72
High	16.01	0.46	66.90	16.60
4	BRA+	DPOS	DNEG	SOX1+
Low	0.62	0.03	88.80	10.55
High	0.72	0.09	76.55	22.65

Table 7: Raw data from the flow cytometry experiment to test the differentiation outcome after sorting for different OCT4 levels. The values represent the percentage of the marker in the population. Figure 3e

SOX1	
no DOX	100ng/ml DOX
57.45	55.6
62.85	51.75
53	42.65
69.35	72.9
69.45	71.8
55.55	68.45
	BRA
no DOX	100ng/ml DOX
12.74	4.57
3.2	15.8
16.25	31.5
0.05	0.58
0.13	0.56
0.07	0.09

Table 8: Raw data from the flow cytometry experiment to test the impact of OCT4 overexpression before differentiation. The values represent the percentage of the marker in the population. Figure 3g

1	BRA+	DPOS	DNEG	SOX1+
SHOH	25.5	0.9	61.5	12.1
SHOL	25.8	0.325	67.3	6.59
SLOH	20.5	0.49	67.2	11.8
SLOL	7.63	0.17	88.7	3.51
2	BRA+	DPOS	DNEG	SOX1+
SHOH	2.905	0.07	90.3	6.69
SHOL	0.65	0.081	97.2	2.1
SLOH	0.53	0.076	95.3	4.09
SLOL	0	0	96.25	3.74
3	BRA+	DPOS	DNEG	SOX1+
SHOH	9.675	0.375	70.8	19.15
SHOL	0.895	0.0535	95.95	3.08
SLOH	12.4	0.365	73.3	13.95
SLOL	0.42	0	98.5	1.05
4	BRA+	DPOS	DNEG	SOX1+
SHOH	11.115	0.635	71.75	16.45
SHOL	3.52	0.26	91.7	4.49
SLOH	17.34	0.695	72.75	9.24
SLOL	2.95	0.23	93.9	2.935
5	BRA+	DPOS	DNEG	SOX1+
SHOH	0.5375	0.1325	73.8	25.55
SHOL	0.084	0.084	87.1	12.7
SLOH	1.11	0.1665	80.45	18.25
SLOL	0.165	0.0725	94.6	5.145

Table 9: Raw data from the flow cytometry experiment to test the differentiation outcome after sorting for different SOX2 and OCT4 level combinations in G1 phase. The values represent the percentage of the marker in the population. Figure 3i

1	BRA+	DPOS	DNEG	SOX1+
SHOH	4.17	0.48	58.2	37.2
SHOL	5.15	0.34	72.7	21.8
SLOH	7.75	0.61	64.1	27.5
SLOL	7.79	0.32	72.2	19.7
2	BRA+	DPOS	DNEG	SOX1+
SHOH	0.62	0.15	80.2	19
SHOL	0.61	0.21	79.7	19.4
SLOH	1.56	0.41	71.1	26.9
SLOL	1.46	0.35	73.2	24.9
3	BRA+	DPOS	DNEG	SOX1+
SHOH	0.43	0.135	73.3	26.1
SHOL	0.455	0.0995	81.8	17.65
SLOH	2.705	0.67	67.45	29.15
SLOL	1.34	0.28	84.55	13.8
4	BRA+	DPOS	DNEG	SOX1+
SHOH	16.9	1.56	66.2	15.4
SHOL	3.75	0.27	90.1	5.84
SLOH	3.34	0.41	86.6	9.67
SLOL	9.84	1.4	73.5	15.3
5	BRA+	DPOS	DNEG	SOX1+
SHOH	13.45	0.775	76.05	9.745
SHOL	13.8	0.765	77.8	7.57
SLOH	22.3	1.205	64.95	11.5
SLOL	20.4	0.93	69.35	9.29

Table 10: Raw data from the flow cytometry experiment to test the differentiation outcome after sorting for different SOX2 and OCT4 level combinations in S phase. The values represent the percentage of the marker in the population. Figure 3j

	Nanog	Esrrb	Otx2	Fgf5	Fgf4	Gata4	Gata6	Eomes	Ascl1
SHOH	1.18	0.81	1.35	1.53	1.23	0.43	0.95	0.75	1.21
SHOH	0.76	0.96	0.99	0.96	0.86	0.55	0.39	0.87	1.96
SHOH	0.62	0.85	1.53	1.06	1.29	0.66	0.45	1.10	3.61
SHOL	1.10	0.93	1.11	0.93	0.90	0.96	1.06	0.81	0.64
SHOL	0.90	0.76	0.85	0.82	0.82	0.46	0.80	0.69	0.93
SHOL	1.16	1.25	0.92	0.78	0.93	1.55	1.06	0.93	1.30
SLOH	0.97	0.84	1.66	1.49	1.13	0.46	1.18	1.00	1.95
SLOH	0.79	0.67	1.09	1.08	0.93	0.35	0.47	0.73	1.55
SLOH	0.54	0.98	1.26	1.13	1.16	1.13	0.55	1.10	1.18
SLOL	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SLOL	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SLOL	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table 11: qPCR data after 24 hours of differentiation normalised to SOX2-low, OCT4 low cells. Figure 3k

1	BRA+	DPOS	DNEG	SOX1+	DPOS + BRA+
OCT4 low	0.51	0.27	94.40	4.85	0.78
OCT4 high	0.14	0.23	71.15	28.45	0.37

2	BRA+	DPOS	DNEG	SOX1+	SOX1+
OCT4 low	0.00	0.00	91.85	8.14	0.00
OCT4 high	0.23	1.10	69.00	29.65	1.33

3	BRA+	DPOS	DNEG	SOX1+	SOX1+
OCT4 low	0.06	0.19	78.30	21.40	0.25
OCT4 high	0.31	0.20	62.40	37.10	0.51

4	BRA+	DPOS	DNEG	SOX1+	SOX1+
OCT4 low	0.43	0.43	88.40	10.70	0.86
OCT4 high	0.78	0.78	72.50	25.90	1.56

Table 12: Raw data from the flow cytometry experiment to test the differentiation outcome after sorting for different OCT4 levels in EOMES KO A cells in G1 phase. The values represent the percentage of the marker in the population. Figure 4g

SOX1	
no DOX	100ng/ml DOX
4.0	0.8
6.0	0.3
24.2	1.7
5.0	0.3
20.0	0.8
23.6	2.2
BRA	
no DOX	100ng/ml DOX
0.4	1.9
26.3	43.1
21.6	44.0
72.4	78.7
25.6	47.6
29.7	49.0

Table 13: Raw data from the flow cytometry experiment to test the impact of OCT4 overexpression during differentiation. The values represent the percentage of the marker in the population. Figure S4d

G1	S	marker
7.43	26.55	SOX1
3.25	22.55	SOX1
9.26	21.68	SOX1
7.98	11.55	SOX1
15.90	9.53	SOX1
19.55	6.22	BRA
0.79	1.06	BRA
5.31	1.23	BRA
8.41	8.46	BRA
1.76	17.49	BRA

Table 14: Raw data showing the % of SOX1+ or BRA+ cells in G1 or S phase for differentiating SBROS cells. The values represent the percentage of the marker in the population. Figure S4h

OL	OH	
0.75	2.55	SBROS
0.90	10.20	SBROS
0.00	14.85	SBROS
1.90	3.30	SBROS
2.30	2.64	SBROS
0.78	0.37	EOMES KO A
0.00	1.33	EOMES KO A
0.25	0.51	EOMES KO A
0.86	1.56	EOMES KO A
0.31	0.37	EOMES KO B
0.71	0.63	EOMES KO B
0.57	0.26	EOMES KO B
0.98	1.23	EOMES KO B
0.44	0.84	EOMES KO B
0.00	1.50	EOMES KO B

Table 15: Raw data showing the amount of BRA+ plus DPOS cells (% of total mCherry+ cells) in G1 sorted cells. The values represent the sum of the markers in the population. Figure S5i

SBROS	EOMES KO A	marker
41.70	50.90	SOX1
26.50	50.00	SOX1
23.48	46.30	SOX1
14.30	0.00	BRA
13.70	0.03	BRA
7.91	0.01	BRA

Table 16: Raw data showing the percentage of the given marker in unsorted SBROS or EOMES KO A cells. Figure S5j

SBROS	EOMES KO A	EOMES KO B
15.84	0.04	0.03
14.70	0.06	0.02
8.95	0.05	0.13

Table 17: Raw data showing the amount of BRA+ plus DPOS cells (% of total mCherry+ cells) in unsorted cells. The values represent the sum of the % of the markers in the population. Figure S5k