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Supplemental Information

Otx2 and Oct4 Drive Early Enhancer Activation during Embryonic Stem Cell Transition from Naive Pluripotency

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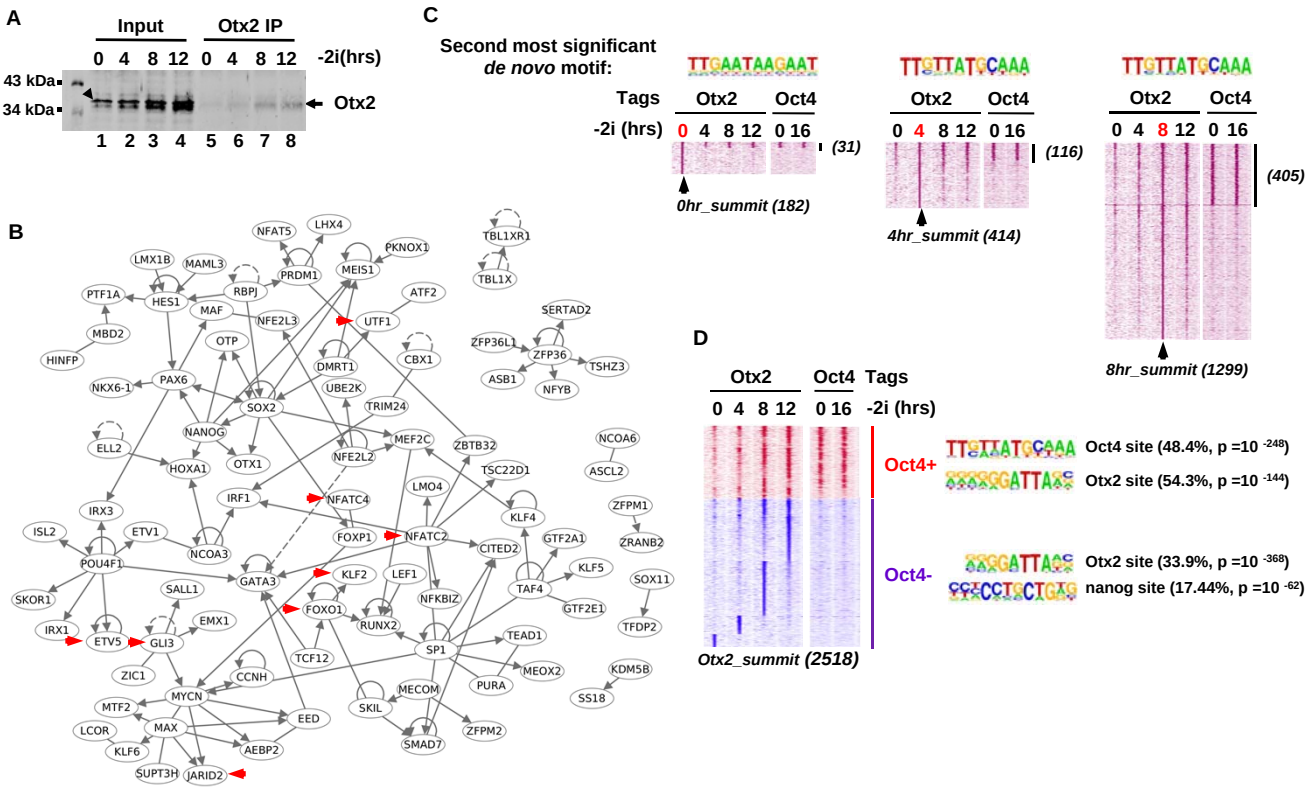

Figure S1 (related to Figure 1). Otx2 and Oct4 bind similar regulatory regions. (A) Western blot of Otx2 immunoprecipitation used in ChIP-seq experiments over a 12 hours time course following release from pluripotency (-2i) in *Rex1GFP*d2 mESC cells. 1% input sample (lanes 1-4) and 10% of immunoprecipitated material (lanes 5-8) are shown. The arrowhead marks the position of a non-specific cross-reacting band in the input samples. (B) Ingenuity pathway analysis of genes associated with all of the Otx2 binding regions (see Fig. S1D) reveals a network of interacting transcriptional regulators. Arrows indicate genes that were identified in a genome-wide siRNA screen for factors involved in promoting escape from pluripotency (Yang et al., 2012). (C) Heat map of normalised Otx2 and Oct4 tag densities at the indicated times following release from pluripotency (-2i), plotted onto 4 kb regions centred on the Otx2 peak summits that time point (indicated in red). The numbers of regions bound by Otx2 at each time point are given in brackets. Data are partitioned based on whether there is co-occurrence of Otx2 and Oct4 binding (Otx2+/Oct4+; top) or Otx2 binding alone (Otx2+/Oct4-; bottom). The second most significant *de novo* motif discovered in the Otx2 binding regions at each of the time points are shown above the heat maps and in each case resembles the Oct4 consensus site. (D) Heat maps of normalised Otx2 and Oct4 tag densities at the indicated times following release from pluripotency (-2i) and is plotted for 1 kb either side on the union set of all Otx2 peak summits across all timepoints. The total number of regions bound by Otx2 is given in brackets. Data are partitioned based on whether there is co-occurrence of Otx2 and Oct4 binding (Otx2+/Oct4+) or Otx2 binding alone (Otx2+/Oct4-). The two most significant *de novo* motifs discovered in the Otx2+/Oct4+ (top) or Otx2+/Oct4- (bottom) binding regions at each of the time points are shown to the right.

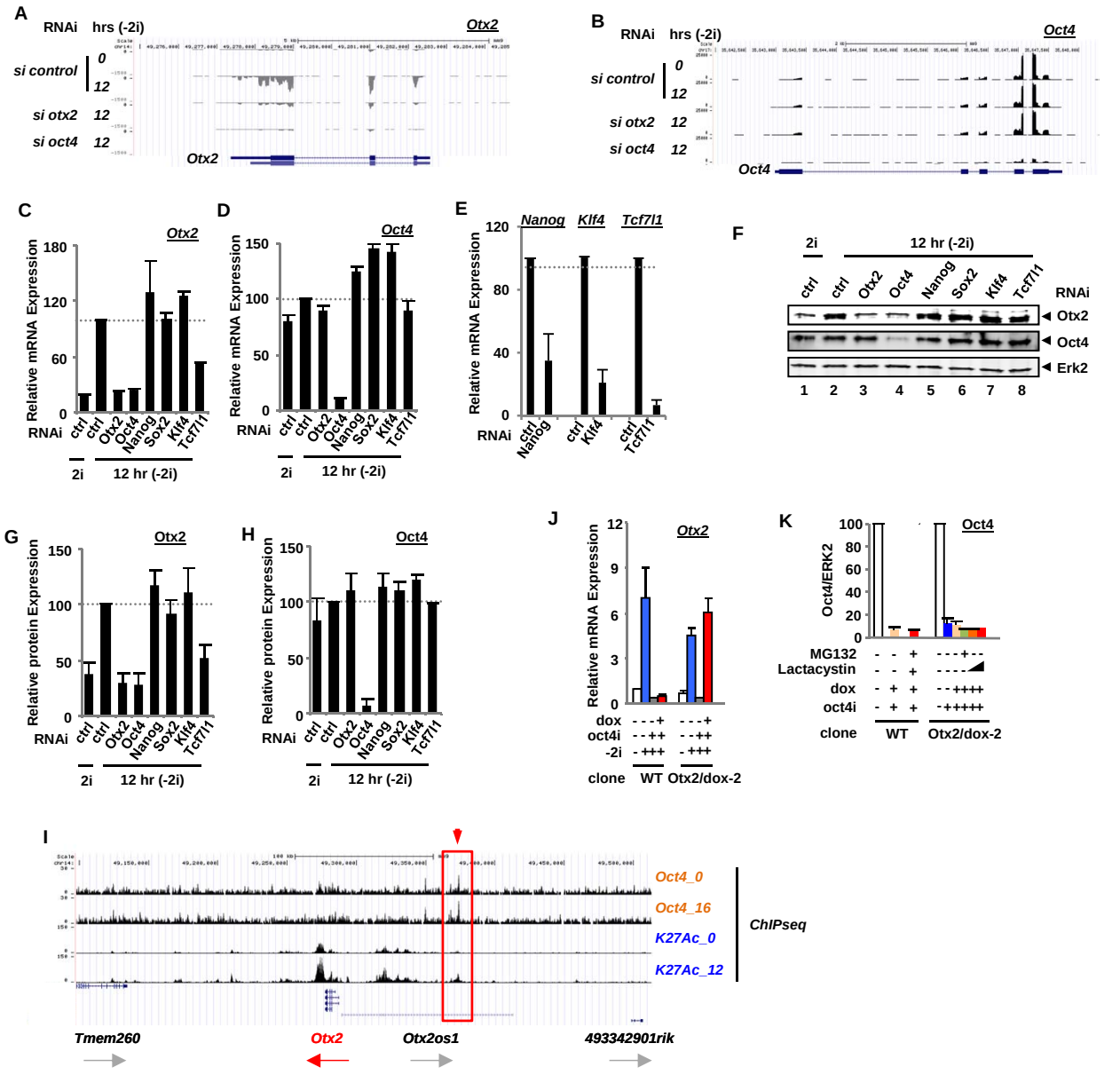

Figure S2 (related to Figure 2). Interplay between Oct4 and Otx2 expression. (A & B) UCSC genome browser view of RNAseq tags associated with the *Otx2* (A) or *Oct4* (B) locus. Data are shown for the indicated times following release from 2i and treatment with control (ctrl) or the indicated RNAi duplexes. (C-E) RT-qPCR analysis of mRNA expression of the indicated genes (above each graph) after knockdown of the pluripotency associated genes, as indicated, or in the presence of a non-targeting control siRNA (ctrl) at 12 hours following "2i" withdrawal in mouse ES cells (*Rex1GFP*d2). Data are normalised by the geometric mean of two reference genes (*Ppia* and *Gapdh*) and are presented as means ± SEM and are the average of at least three biological replicates (n≥3). (F) Representative immunoblots showing Otx2 and Oct4 levels at the indicated time of "2i" withdrawal following the depletion of indicated pluripotency associated genes. Quantification of the data from (F) is shown in (G; Otx2) and (H; Oct4) and are normalised for Erk2 levels and presented as means ± SEM (n=3). (I) UCSC genome browser views of Oct4 and H3K27Ac binding profiles associated with the region surrounding the *Otx2* locus. An Oct4 binding region exhibiting enhanced H3K27Ac levels upon release from 2i is boxed. (J) RT-qPCR analysis of mRNA expression of *Otx2* levels in (*Rex1GFP*d2) mESCs and a clonally derived cell line containing a doxycyclin-inducible Otx2 transgene, Otx2/dox-2, from "2i" for 12 hrs. Data are normalised by the geometric mean of two reference genes (*Ppia* and *Gapdh*) and are presented as means ± SEM and are the average of three biological replicates (n=3). (K) Quantification of Western blot (see Fig. 2G) of Oct4 levels upon release of WT (*Rex1GFP*d2) mESCs and a clonally derived cell line containing a doxycyclin-inducible Otx2 transgene, Otx2/dox-2, from "2i" for 12 hrs. *Oct4* depletion (oct4i), the presence (+) or absence (-) of doxycycline (dox) and the proteasome inhibitors, lactacystin and MG132, are indicated. Data are normalised for Erk2 levels and presented as means ± SEM (n=2).

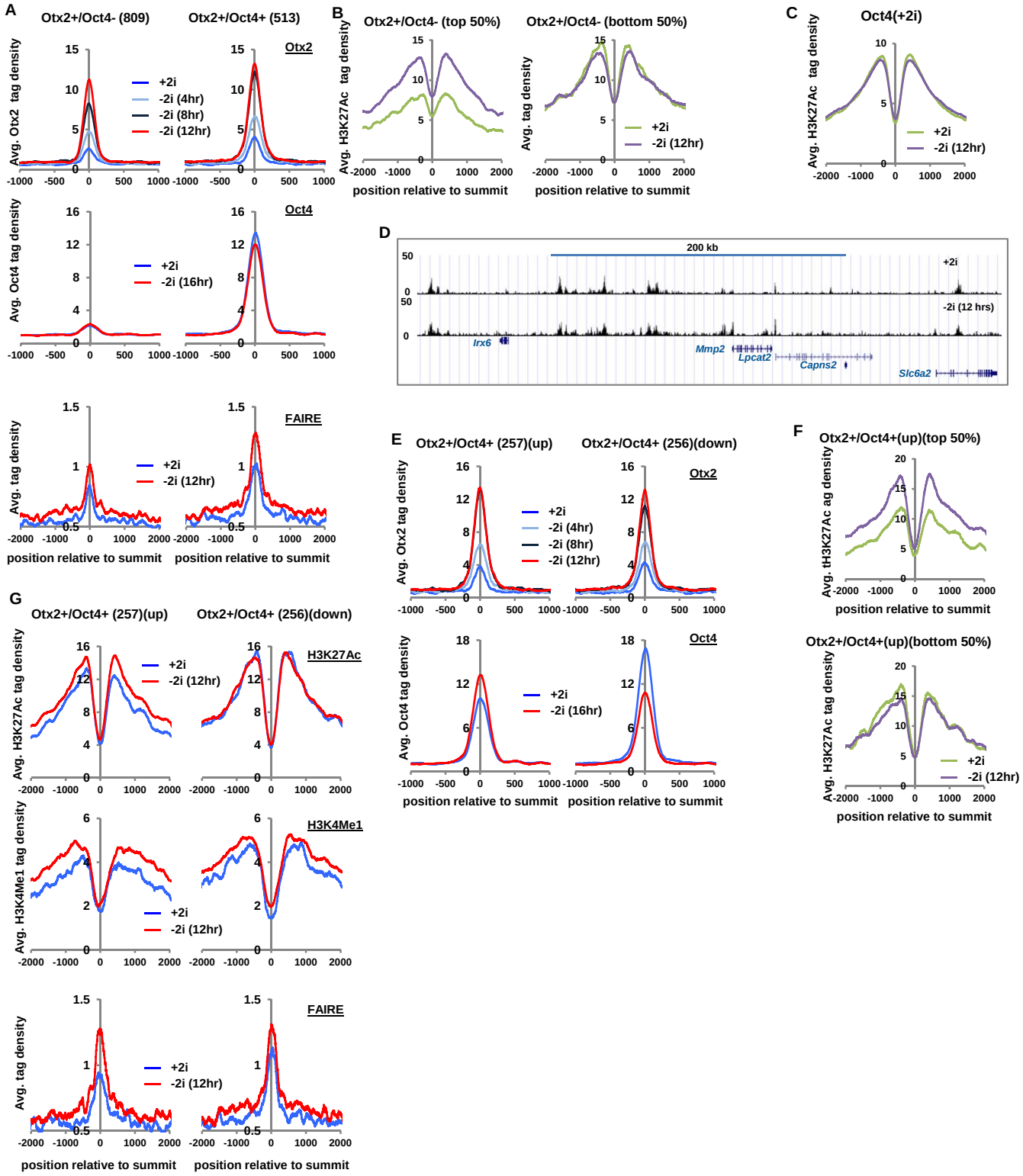

Figure S3 (related to Figure 3). Differential properties of Otx2+/Oct4- and Otx2+/Oct4+ binding regions. (A,B,E-G) Average tag density profiles showing the indicated ChIP-seq or FAIRE-seq data plotted onto the 2 or 4 kb regions respectively, surrounding the summits of Otx2 binding regions from; (A and B) the Otx2+/Oct4- and Otx2+/Oct4+ subgroups; (E, F and G) the Otx2+/Oct4+ subgroup showing increased (up) or no change/decreased (down) Oct4 binding 16 hrs after release from 2i. (C) Average tag density profiles showing the H3K27Ac ChIP-seq plotted onto the 4 kb regions surrounding the summits of all Oct4 binding regions found in "+2i" conditions. Average tag profiles are shown in the presence of 2i (+2i) or for the indicated times following 2i withdrawal. Numbers in brackets are the number of binding regions in each subgroup. In (B) and (F), the data is partitioned into the top and bottom 50% based on the fold change in H3K27Ac observed upon 2i withdrawal from the Otx2+/Oct4- and Otx2+/Oct4+ (up) subgroups respectively. (D) UCSC genome browser views of H3K27Ac binding profiles across a 400 kb region of chromosome 8 in the presence of 2i or upon release from 2i for 12 hrs.

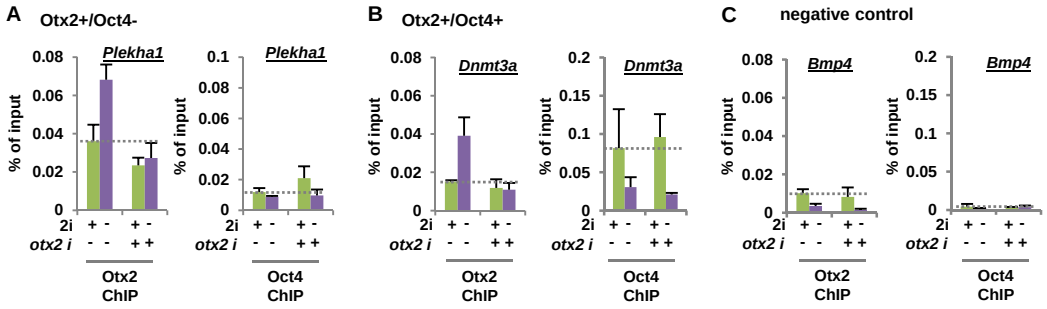

Figure S4 (related to Figure 4). Otx2 and Oct4 binding to chromatin following Otx2 depletion. ChIP-qPCR analysis of Otx2 and Oct4 binding to genomic regions associated with the indicated genes in mouse ES cells. Cells were maintained in 2i (+) or cultured in the absence of 2i (-) for 12 hrs in the presence of siRNAs against Otx2 (+) or control duplexes (-). Examples are given for the Otx2+/Oct4- dataset (A), Otx2+/Oct4+ dataset where increased Oct4 binding does not occur upon release from 2i (B) and a negative control region that lacks Oct4 and Otx2 binding peaks (C). Data are presented as means ± SEM (n=3 (A & B) or n=2 (C)).

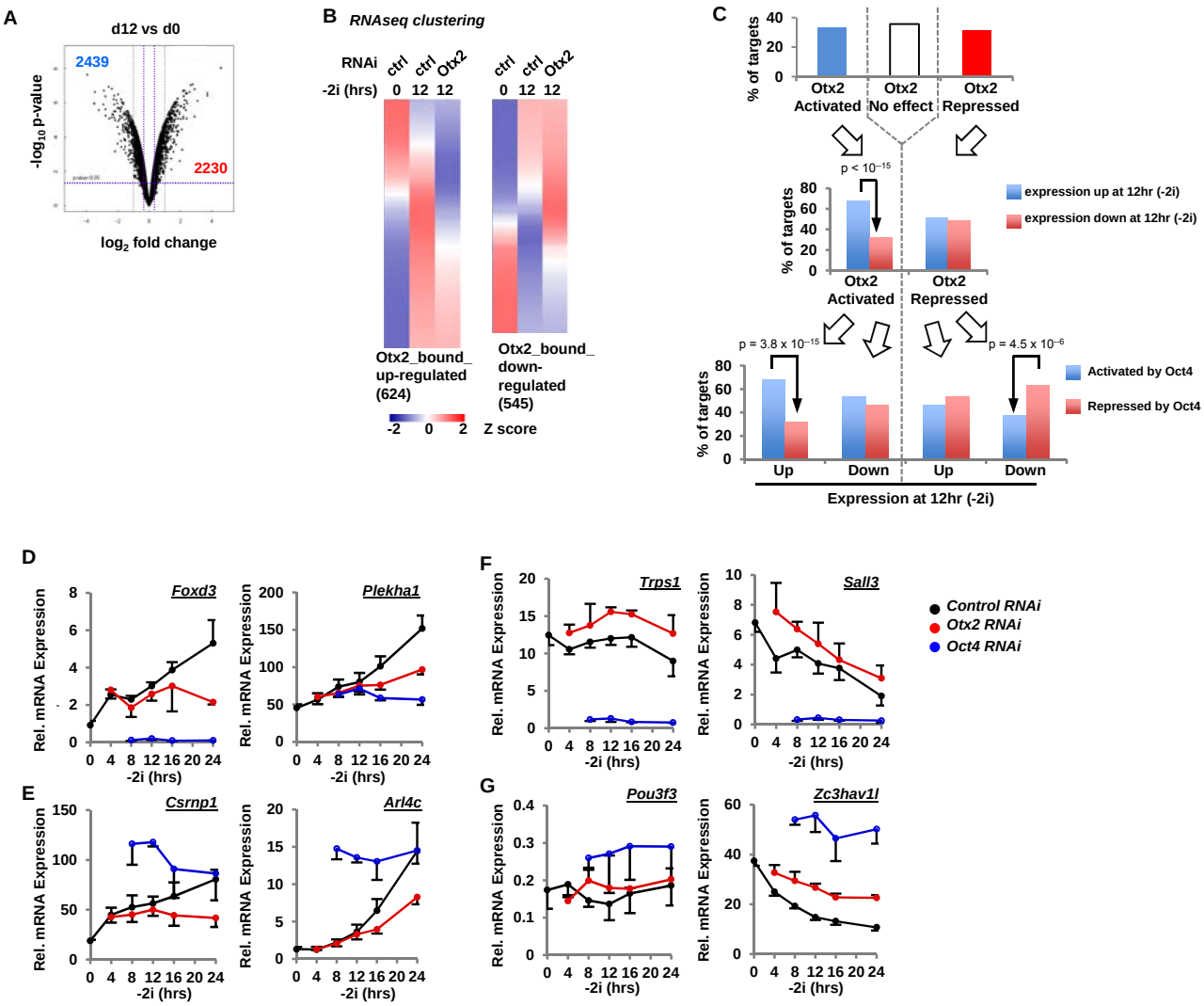

Figure S5 (related to Fig. 5). Otx2 and Oct4 co-regulate target gene expression. (A) Volcano plot of microarray data showing gene expression changes in mouse ES cells (*Rex1GFP*d2) cells in the presence (d12) or absence (d0) of "2i" withdrawal for 12 hrs. (B) Heatmap view of RNA-seq data showing the proportion of genes up or down regulated upon withdrawal of 2i from ES cells for 12 hrs, for subcategories of genes which are either activated (left) or repressed (right) by Otx2. (C) Summary of gene expression changes affected by Otx2 or Oct4 depletion 12 hr after 2i release from RNAseq experiments. Otx2 binding regions were linked to nearby genes and the top graph summarises the proportions of Otx2 targets which are activated, repressed on unchanged Otx2 (as determined by the effect of Otx2 depletion; >1.5 fold change). The expression of genes these different subgroups were compared with their expression changes following release from 2i for 12 hrs (middle graph). Genes within these subgroups were then further partitioned according to their activation or repression by Oct4 (determined by expression changes following Oct4 depletion; >1.5 fold change). (D-G) of RT-qPCR analysis using the Fluidigm system of mRNA expression of the indicated genes over a 24 hour time course following 2i withdrawal. Cells were treated with control, *Otx2* or *Oct4* siRNAs where indicated. Data are presented as means ± SD (n=3).

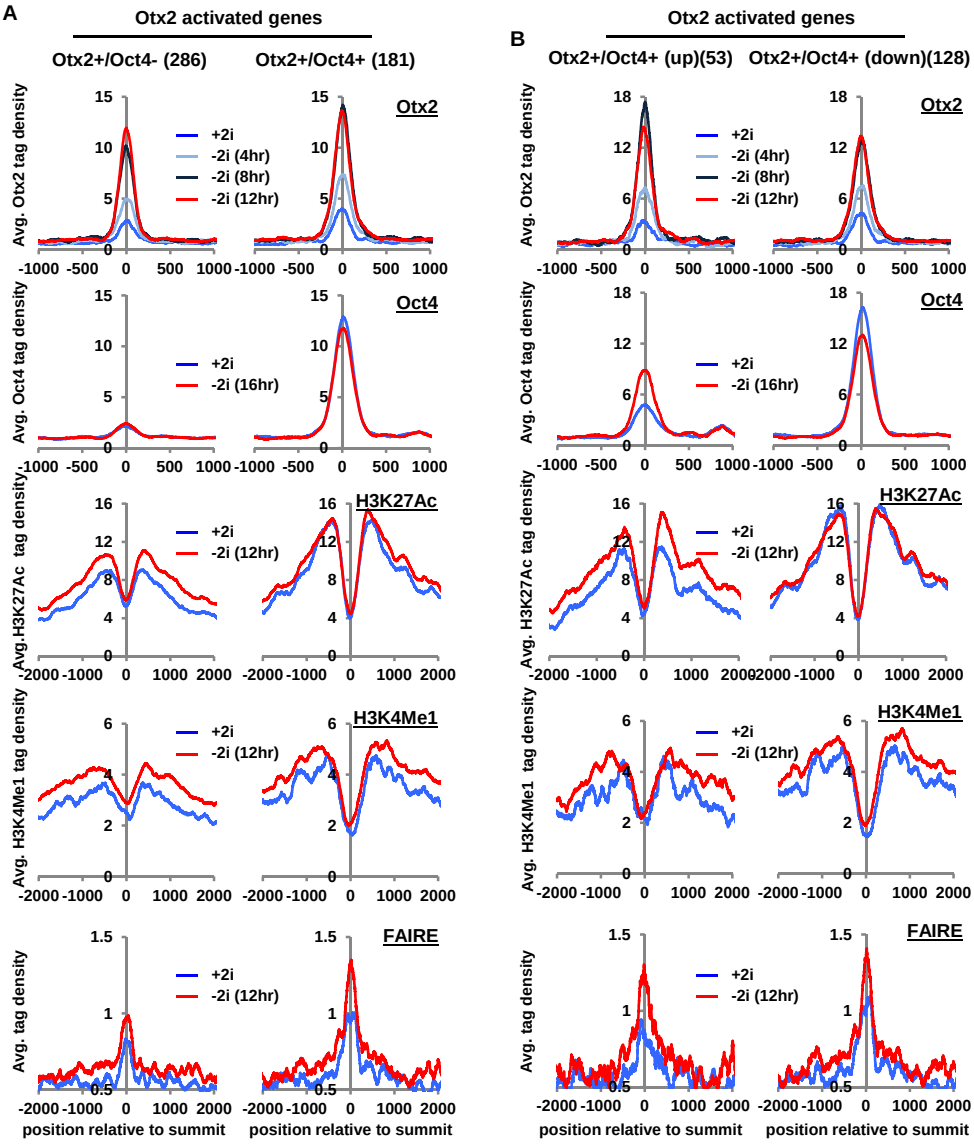

Figure S6 (related to Fig. 6). Otx2 target gene activation correlations with changes in enhancer properties. Average tag density profiles showing the indicated ChIP-seq or FAIRE-seq data plotted onto the 2 or 4 kb regions surrounding the summits (0 position on x-axis) of Otx2 binding regions associated with Otx2 activated genes and from the Oct4- and Oct4+ subgroups (A) or associated with Otx2 binding regions showing increased (up) or decreased/no change (down) in Oct4 binding (B). Data are shown in the presence of 2i (+2i) or for the indicated times following 2i withdrawal (-2i). Numbers in brackets represent the number of binding regions in each subgroup. Note that the Otx2 and H3K27Ac graphs are the same as in Fig.6 and are included here for comparative purposes.

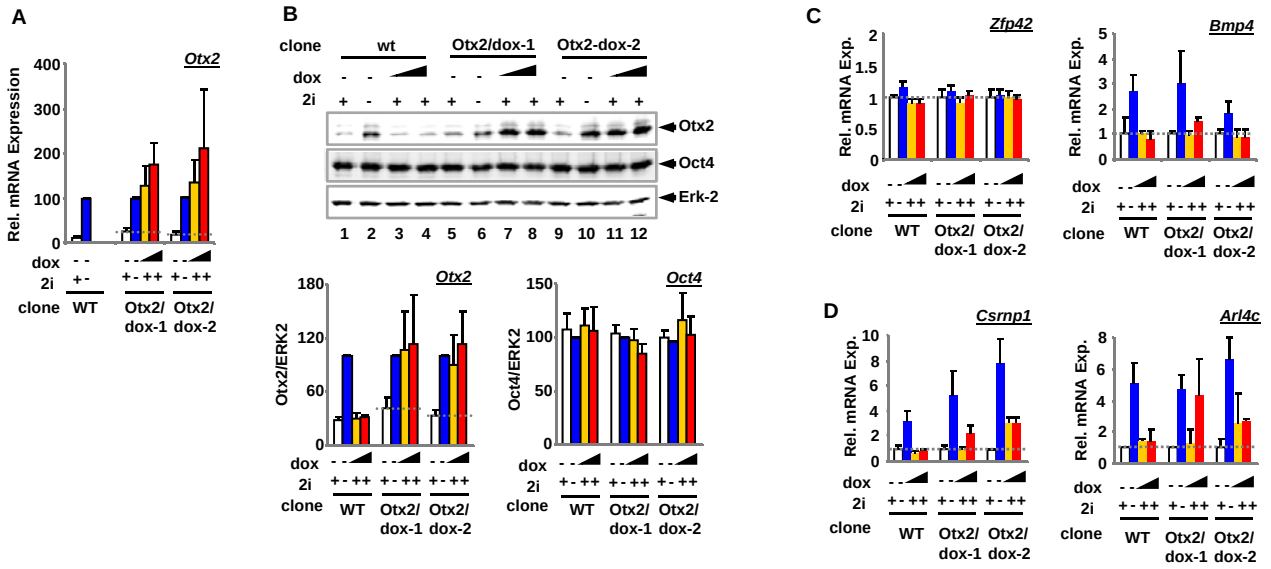

Figure S7 (related to Fig. 7). Target gene expression in the presence of ectopic Otx2 expression. (A- C & D) RT-qPCR analysis of *Otx2* mRNA (A), negative control (C) or selected examples (D) using the Fluidigm system of mRNA expression of the indicated genes in WT (*Rex1GFP*d2), mESCs and two clonally derived cell lines, Otx2/dox-1 and Otx2/dox-2, containing a doxycyclin-inducible *Otx2* transgene. Cells were maintained in 2i media and either left untreated or treated with increasing concentrations (10 and 100 ng/ml) of doxycycline (dox) for 24 hrs. Data are presented as means ± SEM and are the average of at least three biological replicates (n ≥ 3). Gene expression following release from 2i for 12 hrs is also shown. (B) Immunoblots showing Otx2 and Oct4 levels in WT (*Rex1GFP*d2), mESCs and two clonally derived cell lines, Otx2/dox-1 and Otx2/dox-2, containing a doxycyclin-inducible Otx2 transgene. Cells were maintained in 2i media and either left untreated or treated with increasing concentrations (10 and 100 ng/ml) of doxycycline (dox) for 24 hrs. Quantification of the data from (B) is shown below and are normalised for Erk2 levels and presented as means ± SEM (n=3).

Supplementary Table 1 : Oligonucleotide primers

Primers for ChIP analysis

ADS_ID	primer_name	primer_F	ADS_ID	primer_name	primer_R	amplicon
4137	hells_F	CACTTCCTCCCCCAGCTACT	4138	hells_R	GGAAGCCTACCTCCAGGAAT	130
4139	plekha1_F	GGGGATTTGAGCAGGTGATA	4140	plekha1_R	GAGAAAGGTGGGACAGGACA	127
4141	gm98_F	GCAGACAGGATCGGAATCTC	4142	gm98_R	GAGCCCTGGGTTTACCATTT	164
4143	csrnp1_F	AGACCAGGAGCTTCACCAGA	4144	csrnp1_R	CAGCAGGGAATAGGGACAAG	127
4145	slc16a3_F	TTCCCACTGCGTTTTATTCC	4146	slc16a3_R	TGGGGTTGCATCCTATTCTT	155
4147	dnmt3a_2_F	GAAGCCTGGAAAGCTCACAG	4148	dnmt3a_2_R	ATGCAACGATGGGTGGTTAT	176
4149	foxd3_F	AGATTGCTGCCGTGTTTTCT	4150	Roxd3_R	CCTGGTGTCTGAAGGCATTT	167
4151	yes1_F	CAAAAGTTTCCGTCCAGAGC	4152	yes1_R	TCAAGACATAAAAGGAAGCCAAA	123
4153	sgk1_F	CCTCCCACTTCACTTTCCAC	4154	sgk1_R	CCACCTACAAGGTTCCCAAA	120
4155	pim2_F	TTGACCATTTGCCTCCTTCT	4156	pim2_R	TAATGAGTCTGCCCCAGCTT	157
4157	bmp4_F	ACAGCTCCTGGAGGCAGTTA	4158	bmp4_R	CCACGGCTTTGGTTGATAGT	171

Primers for expression analysis

ADS_ID	primer_name	primer_F	ADS_ID	primer_name	primer_R	amplicon
4159	otx2_F	GGAGAGGACGACATTTACTAGG	4160	otx2_R	TTCTGACCTCCATTCTGCTG	197
4161	tal2_F	CCTAGCAAGAAGGAACCTGGA	4162	tal2_R	GATATACCTCATTGCCAAGCGA	174
4163	hesx1_F	ATGTCTTTAGAGTGAACTGCTACC	4164	hesx1_R	GAAACTGTGATTCTCTACGGGAC	143
4167	pou3f1_F	TTTGTATGCCCGACTAAACC	4168	pou3f1_R	ACAGATGAAGAAATGGTGAAGG	117
4169	foxd3_F	TCTACCCAATCCTGGACTCTG	4170	foxd3_R	GAAAGTTATTCCCAGCGGAC	125
4171	dnmt3a_F	GCGATTTCTTGAGTCTAACCC	4172	dnmt3a_R	TTGCTGAACTTGGCTATTCTG	176
4175	gm98_F	GAGCAGTCACAGTCAGACAG	4176	gm98_R	CACCACCAGCATGAAATACCT	176
4177	hells_F	GTTTAGTTGATGGGAATGGAGAG	4178	hells_R	CTTCTACACAAAGACTCGTAGTGG	170
4179	csrnp1_F	CCTTAGCTGAGTTTACACAGGA	4180	csrnp1_R	TGCCAAGTCCTCCTCTACAG	184
4181	fgf15_F	GAAGACGATTGCCATCAAGGA	4182	fgf15_R	CTAAACAGTCCATTTCCTCCCT	127
4183	plekha1_F	AACAGAGTGACATAATGATGAGGG	4184	plekha1_R	AGAGACTGCTTTAATCCAACTGTG	115
4185	slc16a3_F	GCTATGCTCTATGGCACAGG	4186	slc16a3_R	AGGGAAGGCTGGAAGTTGAG	196
4187	lima1_F	AAACACAGAAACTTCAGGCA	4188	lima1_R	CACCTATTTCCCAGTCATCCA	175
4189	arl4c_F	GCAACATCTCCTCCAACATCTC	4190	arl4c_R	GCTCAGTTTGATCTTCTCGGT	160
4191	irgm1_F	CACTCGGATCTTATCATGAAACCA	4192	irgm1_R	CCTTCTCTTAAAGCTCTTTCGG	181
4195	capn3_F	CTTCCATCTCAACAGCCAACTC	4196	capn3_R	GATACCATCGCCATCCTTGTC	153
4197	skor1_F	GGGCTCACTTTGGATGTCAC	4198	skor1_R	GTTGCACCATTTCTTCCCGA	195
4199	fgf5_F	GGGATTGTAGGAATACGAGGA	4200	fgf5_R	CCTGAACTTACAGTCATCCGT	104
4203	zfp521_F	CACTGAGCGACATCACAGAG	4204	zfp521_R	TTCCTCTCCGAAATCACACC	157
4205	spic_F	ACGCCATTGACATATTGATCCA	4206	spic_R	GCAGAACCATTTGTTACACCT	177
4211	car12_F	ACTACACCTCCTTGCTACCC	4212	car12_R	CAGCCTCTCATCGAACTTCTG	176
4215	zc3hav1l_F	AGATGCTTGAAGAGAAAGACC	4216	zc3hav1l_R	GCTCGTTCAGGTTTATTGGG	145
4217	pou3f3_F	CCCTCTTTCTTCCACTTCTTCC	4218	pou3f3_R	CTCTGTTTCTCCCTTCTTCCC	200
4219	nr3c1_F	GCAGAGAATGACTCTACCCT	4220	nr3c1_R	CTTGGCTCTTCAGACCTTCC	157
4221	sall3_F	AAGACCTTCTCCTCAGCCAG	4222	sall3_R	ATGTGTACCTTGAGATTGCCCT	121
4223	trps1_F	AGTTCAGCCATTCCAAAGAC	4224	trps1_R	GTTCCTCCTGAAGTGACCTG	129
4227	tbp_F	TACTGAAGAAAGGGAGAATCATGG	4228	tbp_R	GAGACTGTTGGTGTTCTGAATAGG	166
4229	gapdh_F	ACATCAAGAAGGTGGTGAAGCAG	4230	gapdh_R	GAAGGTGGAAGAGTGGGAGTTG	116
4237	ywhaz_F	TATTGAGCAGAAGACGGAAGG	4238	ywhaz_R	GCCAAGTAACGGTAGTAGTCAC	197
4239	tubb5_F	CTTCAGACCAGACAACTTCGT	4240	tubb5_R	GCACCACATCCAAGACAGAG	109
4241	pgk1_F	GAAGATTACCTTGCCTGTTGAC	4242	pgk1_R	GCTCTCAGTACCACAGTCCA	126
4243	ppia_F	ACTTCATCCTAAAGCATACAGGTC	4244	ppia_R	CTTCCACAATGTTCATGCCT	161
4245	bmp4_F	AACACTGTGAGGAGTTTCCA	4246	bmp4_F	GTTTATACGGTGGAAGCCCT	194
4247	Oct4_F	GTGAAGTTGGAGAAGGTGGA	4248	Oct4_R	GCTGAACACCTTTCCAAAGAG	170
4249	nanog_F	TTACAAGGGTCTGCTACTGAG	4250	nanog_R	TTCTGCTTCTGAAACCTGTC	244
4251	klf4_F	AGTTCTCATCTCAAGGCACAC	4252	KLF4_R	CACACTTCTGGCACTGAAAGG	157
4253	tcf7l1_F	TCTTCTTCCCATAGTTGTCCC	4254	tcf7l1_R	GCAGCCATTAACCAAATCCT	148
4255	rex-1_F	GAAATGGACTAAGAGCTGGGA	4256	rex-1_R	TGAACAATGCCTATGACTCAC	212

Primer for Plasmid constructs

ADS_ID	primer_name	primer_F	ADS_ID	primer_name	primer_R	
4257	Inf_OTX2_F	CCCAGGACCGACCGGTATGATGTCTTATCTAAAG	4258	Inf_OTX2_R	CAGGTCCGGAACTAGTTCACAAAACCTGGAATTTC	870
4259	Inf_TRE3G_F	TGTTTCCTGAGGTTACTTCAAGAATTCCTCGAG	4260	Inf_TRE3G_R	CGCCTAGGCAGTCGACTTTACGAGGGTAGGAAG	380
4261	ROSA26_F	CACCGTGTGTGGGCGTTGTCCTGC	4262	ROSA26_R	AAACGCAGGACAACGCCCACACAC	N/A
4163	ROSA26.2_F	CACCGTGGCAGGAAGCGCGCGCTG	4164	ROSA26.2_R	AAACCAGCGCGCGCTTCCTGCCAC	N/A
4165	ROSA26.3_F	CACCCGGCCAGACTCTGCGGCGCG	4166	ROSA26.3_R	AAACCGCGCCGCAGAGTCTGGCCG	N/A

Supplemental Experimental procedures

Generation of " knock-in-inducible-Otx2" ES cell lines

pAS4428 [pEntL1L3-CAG-Tet3G (Tet3G expression under the control of CAG promoter)] was constructed by ligating an EcoRV/MfeI fragment of pAS4427 [pCAG-Tet3G] into PacI (blunted by fill in)/MfeI sites of pAS4426 [pEntL1L3-EF1α-rtTA2Sm2] vector. pAS4426 [pEntL1L3-EF1α-rtTA2Sm2] was constructed by ligating an AflII/SbfI fragment from pAS4425 [pEntL1L3-EF1α-tTA-2 (Addgene 26803)] into the same sites of pAS4424 [pEntL1L3-rtTA-3 (Addgene 27106)]. pAS4431 [pEntR3L2-TRE3G-mCherry-otx2] was generated by mixing a PCR product encoding Otx2 with AgeI/SpeI cut vector pAS4430 [(pEntR3L2-TRE3G-mCherry-RS1-2)] using in fusion technology. pAS4430 [pEntR3L2-TRE3G-mCherry_Rs1-2] was generated by mixing a TRE3G PCR product with the PacI/SalI digested vector pAS4429 [(pEntR3L2-tetO (sh)-mCherry-Rs1-2, Addgene 24417)]. pAS4433 [R26(CAG-Tet3G-TRE3G-mCherry-otx2] was constructed by mixing pAS4428 [pEntL1L3-CAG-Tet3G], pAS4431 [pEntR3L2-TRE3G-mCherry-otx2] and pAS4432 [pR26 R1R2 RexNeo PI-SceI (Addgene 24418)] plasmids using Gateway system (LR clonase II plus). pAS4435-4437 [pX335-Rosa26, pX335-Rosa26.3 and pX335-Rosa26.3] were constructed by cloning the oligo duplexes encoding the guide RNAs (ADS4261/2, ADS4163/4 and ADS4165/6 respectively) for the Rosa26 locus into a BbsI-digested pAS4434 [pX335 vector (Addgene 42335)]. All plasmids were validated by sequencing.

8 μg of R26(CAG-Tet3G-TRE3G-mCherry-otx2 with either 2 μg of pX335-Rosa26 plasmid (to create Otx2/dox-1/-2 lines) or 4 μg of pX335-Rosa26.2 and pX335-Rosa26.3 plasmids (to create Otx2/dox-3 line) were transfected using Amaxa (A-013 setting) into $2x10^6$ *Rex1GFP*d2 mES cells. Cells were selected with 600 μg/ml of geneticin (G418) for 12 days. Individual cells were cloned by serial dilution and propagated in G418/"+2i" media.

Integration of the expression cassette was verified by inverted PCR followed by sequencing (Otx2/dox-1/-2) or Southern blotting Otx2/dox-3).