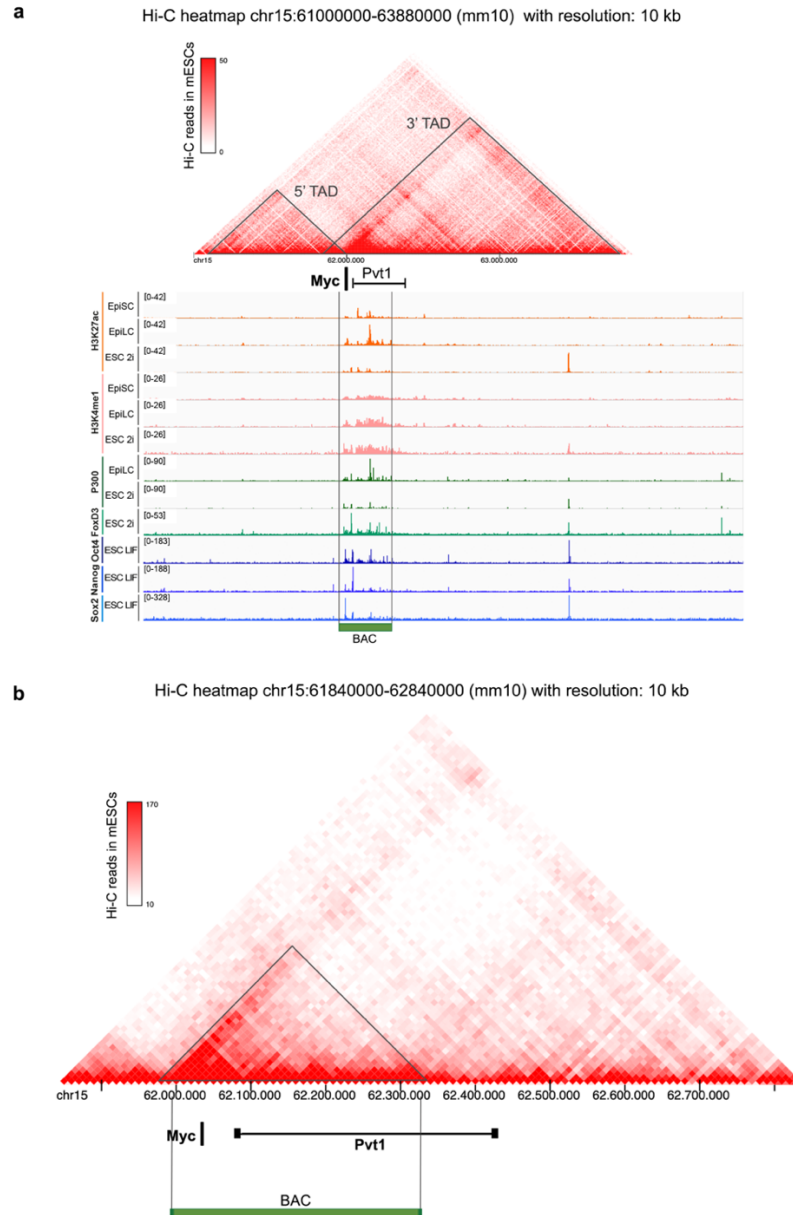
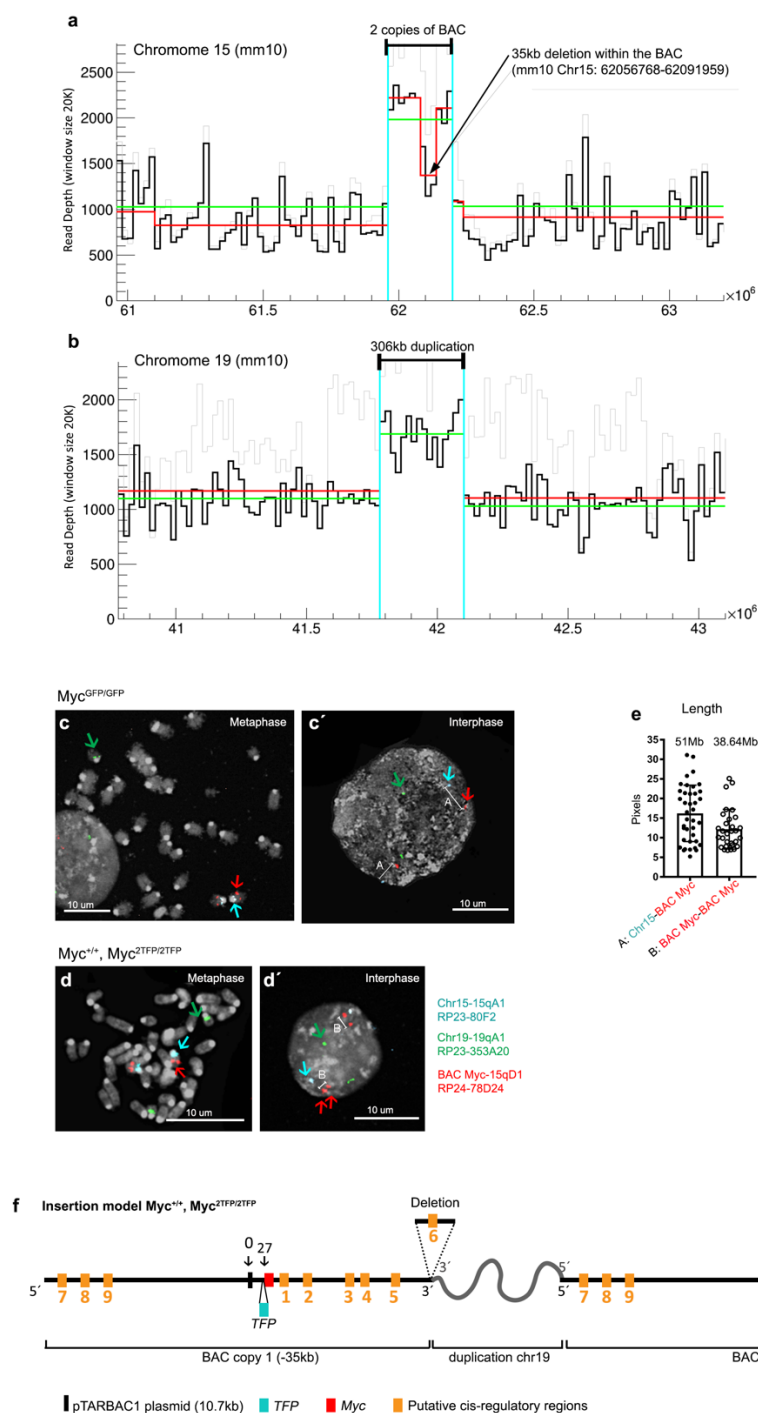


Supplementary Figures and Tables



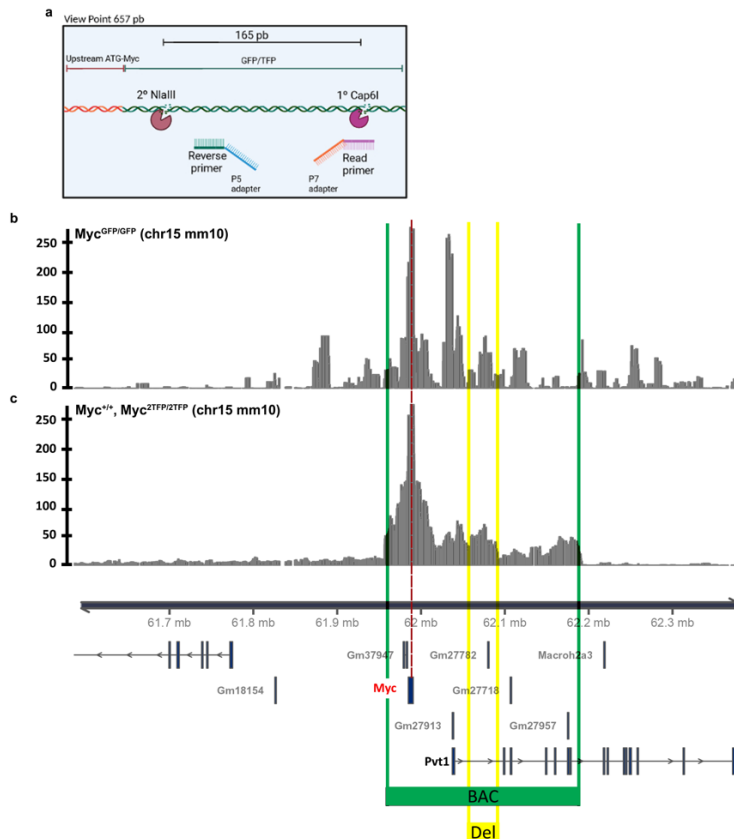
Supplementary Figure 1. Epigenetic modifications and Hi-C heatmap of the genomic region surrounding *Myc* locus in mouse ESCs. **a**, The 3Mb region around *Myc*, showing 2 major TADs upstream and downstream the *Myc* transcription unit. Hi-C reads in mESC from⁷⁶ are represented as a heatmap. The intensity of the red color correlates with the frequency of interactions. Scaled representation of the *Myc* 3Mb gene desert in the genome and in BAC RP24-78D24 (green box). Below, H3K27ac (orange) and H3K4me1 (pink) distribution in EpiSCs (primed pluripotency), EpiLCs (formative pluripotency) and ESCs 2i (pro-naïve pluripotency). P300 (dark green) binding in EpiLCs and naïve ESCs. FOXD3 (light green), OCT4, NANOG and SOX2 (blue) binding in ESCs. Source of the data is the same as in Figure 1. **b**, 1Mb region around *Myc* of the downstream region showing a sub-TAD.



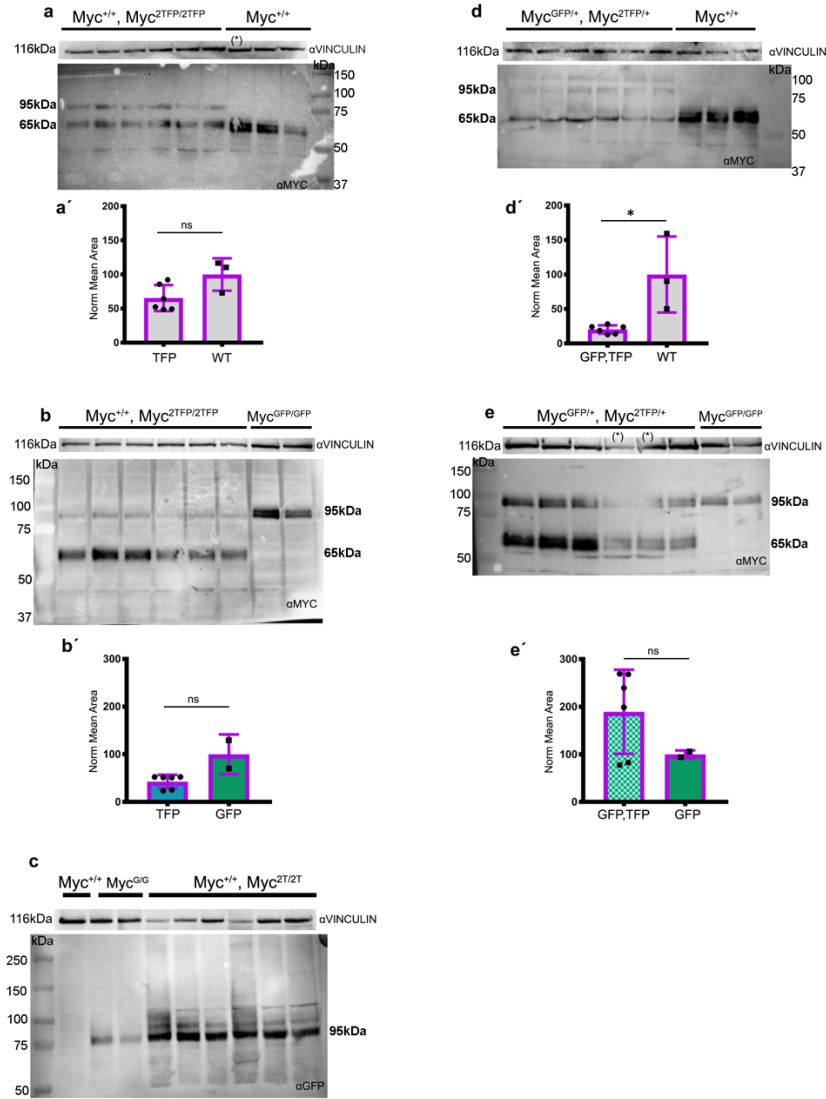
Supplementary Figure 2. Characterization of the BAC RP24-78D24 transgenic insertion.

a-b, Graph presenting a 20Kb sliding window showing copy number detection from genomic DNA deep sequencing of mice carrying the transgene BAC transgene insertion in heterozygosity. The red line represents the read depth compared to the average in the surrounding genome. The green line represents the read depth compared to the average of the entire genome. **a**, Chromosome 15: the BAC sequences (delimited by blue lines) are detected in double amount than the surrounding sequences, indicating the detection of the 2 endogenous alleles, and 2 extra-copies from the single

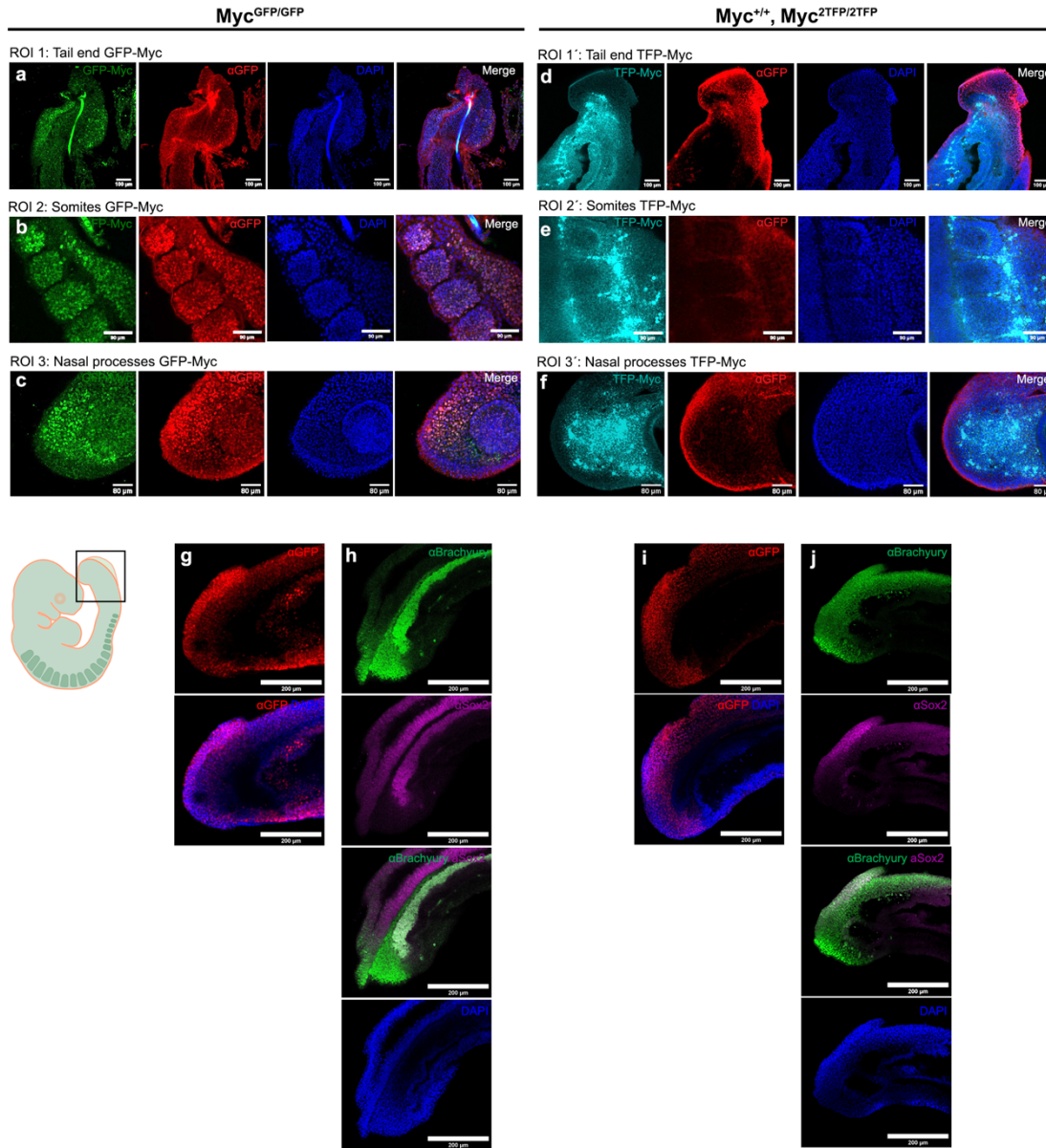
insertion of the BAC. A 1-copy deletion of ~35kb within the BAC can be observed. **b**, Chromosome 19: hybrid reads between the BAC DNA and chromosome 19 DNA were detected. The region between the two breakpoints identified by the hybrid sequences was detected in 3 copies (delimited by blue lines) which suggest that the insertion site of the BAC involved the duplication of this region. **c-d**, FISH hybridization in *Myc*^{GFP/GFP} (**c,c'**) and *Myc*^{+/+}, *Myc*^{2TFP/2TFP} (**d,d'**) cells. 19qA1 region of the chr19 is tagged by a green probe (green arrows). A control 15qA1 region of chr15 is tagged by a blue probe (blue arrows). The BAC, in the 15qD1 region is labelled by a red probe (red arrows). Scale bars=10 microns. In the metaphasic nuclei (**c, d**), the red and the blue probes co-localize in chromosome 15. In interphasic nuclei (**c', d'**), there are two signals of the red probe in *Myc*^{GFP/GFP} cells, and four red signals in *Myc*^{+/+}, *Myc*^{2TFP/2TFP} cells. **e**, Estimation of the distance between the *Myc* locus and the BAC insertion by comparing quantification of the distance *Myc*-Control Probe (A distance) versus *Myc*-BAC (B distance). From the known distance between 15qA1 and *Myc* (51Mb), the distance between *Myc* and the BAC insertion is estimated at 38.64 Mb. **f**, Insertion model of the TFP-*Myc* BAC into ectopic site in chromosome 15, carrying a fragment from chromosome 19. Two copies of BAC were inserted, one copy with a 35Kb deletion. Source data for all graphs are available from the Source Data file and raw data from Figshare (see Data Availability section).



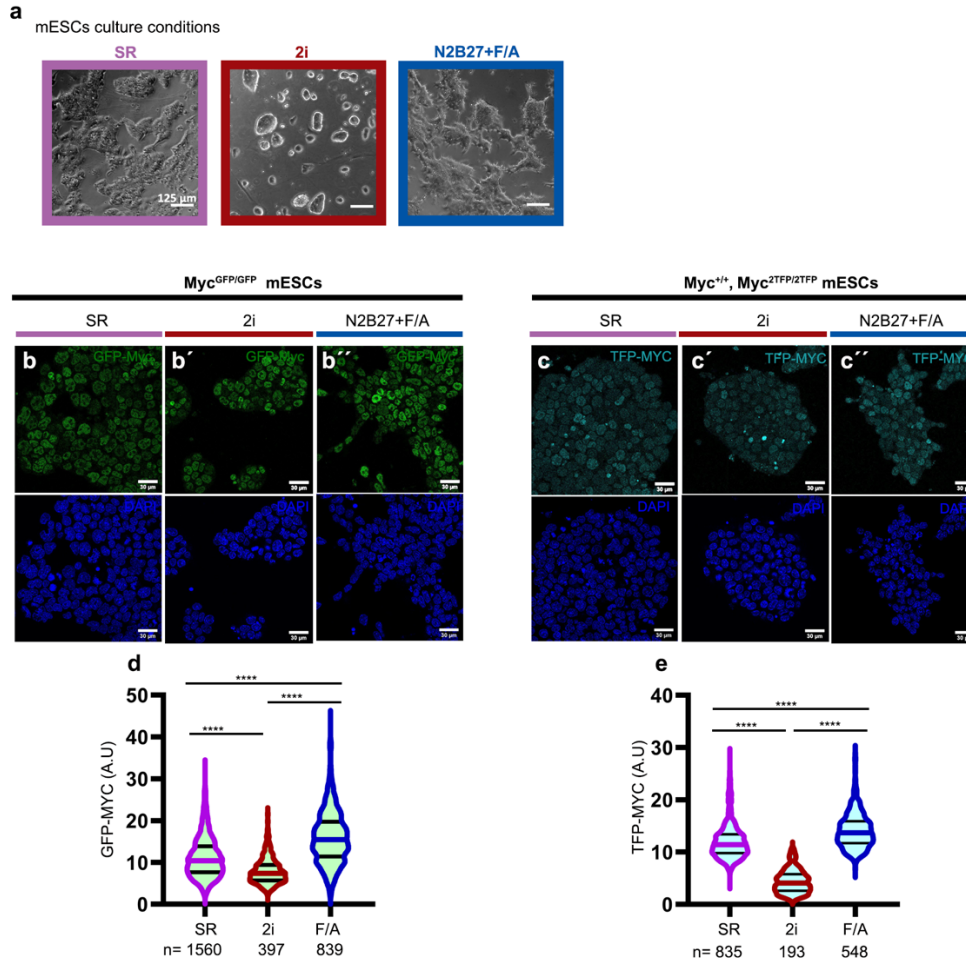
Supplementary Figure 3.4C analysis in *Myc*^{GFP/GFP} and *Myc*^{+/+}, *Myc*^{2TFP/2TFP} ESCs. **a**, Viewpoint design. TFP or GFP was used as viewpoint, which is upstream and in frame with *Myc* coding sequence. The two enzymes and primers used for generating the libraries are represented. Primers are listed in Supplementary Table 2. **b-c**, Reference genes such as *Myc* (red dashed line) and *Pvt1* are represented below. The BAC region is delimited by green lines and the 35Kb deletion in the BAC transgene (Del) is delimited by yellow lines. **b**, *GFP*-*Myc* interactions. **c**, *TFP*-*Myc* interactions.



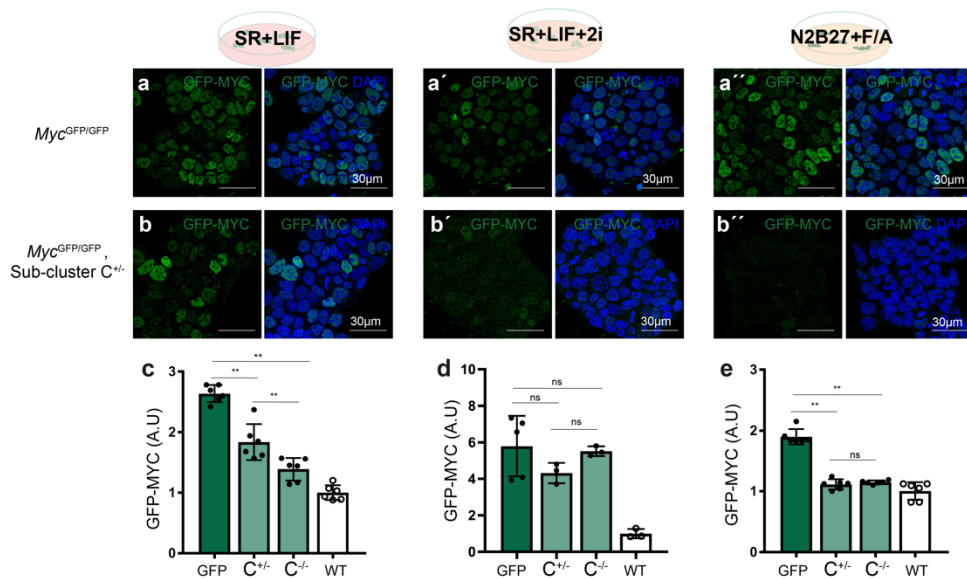
Supplementary Figure 4. Characterization of the TFP-Myc fusion protein expression. **a-c**, Western blot of *Myc*^{+/+}, *Myc*^{2TFP/2TFP} (TFP) ESCs lysates. Anti-vinculin is shown as loading control. **a'**, Graphs show the abundance of WT MYC protein (65kDa) produced by TFP cells and *Myc*^{+/+} cells (WT). **b'**, Graph shows the abundance of FP-MYC fusion protein (95kDa) produced by TFP cells and by *Myc*^{GFP/GFP} cells (GFP). **c**, anti-GFP antibody in WT, GFP, and TFP cells, FP-MYC fusion protein is absent in WT cells, and GFP and TFP-fusion proteins had the same size (95kDa). **d-e**, Western blot of *Myc*^{GFP/+}, *Myc*^{+/2TFP} (GFP,TFP) ESCs lysates. **d'**, Graph shows the abundance of the MYC protein produced by GFP;TFP and WT cells. **e'**, Graph shows the abundance of FP-MYC fusion protein produced by GFP;TFP cells and GFP cells. **a'-e'**, Mann Whitney test; ns: P-value >0,05; *: P-value=0.0238; All quantifications were normalized to vinculin and to the control quantity of WT or FP-fusion proteins. Each dot represents an independent Western-Blot lane analysis. bars indicate the Mean and error bars, the Standard Deviation. n= 6 and 3 in **a'** and **d'** and 6 and 2 in **b'** and **e'**. The images shown here are representative examples of the different experiments quantified. (*) aberrant bands were not used for quantifications. Source data for all graphs are available from the Source Data file and raw data from Figshare (see Data Availability section).



Supplementary Figure 5. Comparative *GFP-Myc* and *TFP-Myc* expression in E9.5 embryos. **a-c**, Confocal images show the posterior embryonic bud (**a**), the newly formed somites (**b**) and the nasal processes (**c**) of E9.5 *Myc^{GFP/GFP}* embryos with detection of native GFP-MYC signal, immunostaining against GFP and DAPI. N = 6 GFP-MYC embryos. **d-f**, similar images as in **a-c** for a *Myc^{+/+}, Myc^{2TFP/2TFP}* embryo. **a,d**, Scale bar= 100 microns. N = 10 TFP-MYC embryos. **b,e**, Scale bar= 60 microns. **c,f**, Scale bar= 80 microns. **g-j**, Confocal images show optical transversal sections of the posterior embryonic bud in *Myc^{GFP/GFP}* (**g,h**) and *Myc^{+/+}, Myc^{2TFP/2TFP}* (**i, j**) E9.5 embryos. Scale bar= 200 microns. FP-MYC is detected by antiGFP immunostaining (**g, i**) and NMPs are detected by double staining with anti-Brachyury and anti-SOX2 antibodies (**h, j**).

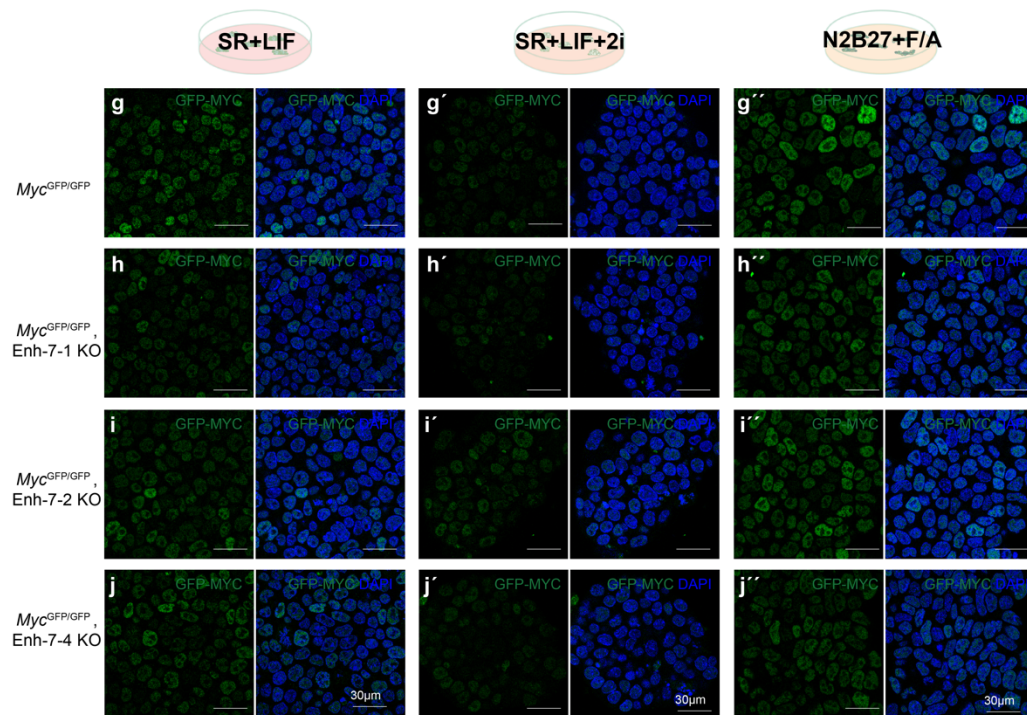


Supplementary Figure 6. *TFP-Myc* expression analysis by mESC cultured in SR+LIF, 2i and 2 days-F/A conditions. **a**, Brightfield images of mESCs in the three culture conditions used in this work: SR+LIF (purple), in 2i (red), and in 2 days-F/A (blue). Scale bar= 125 microns. **b-c**, Confocal images show GFP-MYC or TFP-MYC endogenous signals and DAPI in *Myc*^{GFP/GFP} (**b-b''**) (N=2 clones) and *Myc*^{+/+}, *Myc*^{2TFP/2TFP} (**c-c''**) (N=2 clones) in the three culture conditions. Scale bar= 30 microns. **d**, **e**, Violin plots show median and quartiles of GFP-MYC or TFP-MYC endogenous signals of *Myc*^{GFP/GFP} (**d**) and *Myc*^{+/+}, *Myc*^{2TFP/2TFP} (**e**) mESCs in the 3 culture conditions indicated. The number of cells quantified (n) is shown. Mann Whitney test; ns: P-value >0,05, ****: P-value<0,0001. Source data for all graphs are available from the Source Data file and raw data from Figshare (see Data Availability section).

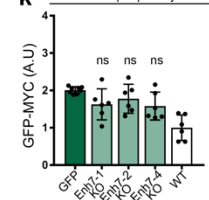


f

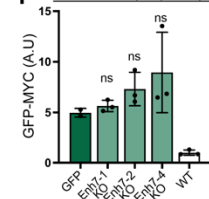
| Sub-cluster | Putative region | Putative enhancer | position | size (bp) | cCRE ENCODE | Position | Score | Size (bp) | H3K27ac max-Z | Cell type (High H3K27ac) |
|-------------|-----------------|-------------------|-----------------------------|-----------|--------------|-------------------------|-------|-----------|---------------|--------------------------------------|
| c | 7 | 7_1 | chr15:62,085,072-62,088,281 | 3211 | EM10E0600677 | chr15:62085273-62085583 | 182 | 311 | 2.39 | 129 ES-E14 B10.H-2aH-4bp/Wts CH12.LX |
| | | | | | EM10E0600679 | chr15:62087830-62088156 | 242 | 327 | 2.68 | |
| | | 7_2 | chr15:62,089,322-62,092,601 | 3281 | EM10E0600682 | chr15:62091244-62091460 | 219 | 217 | 3.15 | |
| | | | | | EM10E0600683 | chr15:62091806-62092032 | 225 | 227 | 3.69 | |
| | | 7_3 | chr15:62,092,596-62,095,870 | 3276 | EM10E0600685 | chr15:62092738-62093087 | 451 | 350 | 3.29 | |
| | | | | | EM10E0600689 | chr15:62096850-62097138 | 186 | 289 | 2.58 | |
| | | 7_4 | chr15:62,096,720-62,099,694 | 2976 | EM10E0600690 | chr15:62098817-62099164 | 289 | 348 | 2.63 | |
| | | | | | EM10E0600691 | chr15:62099282-62099619 | 338 | 338 | 2.87 | |



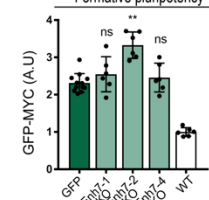
k Mixed pluripotency



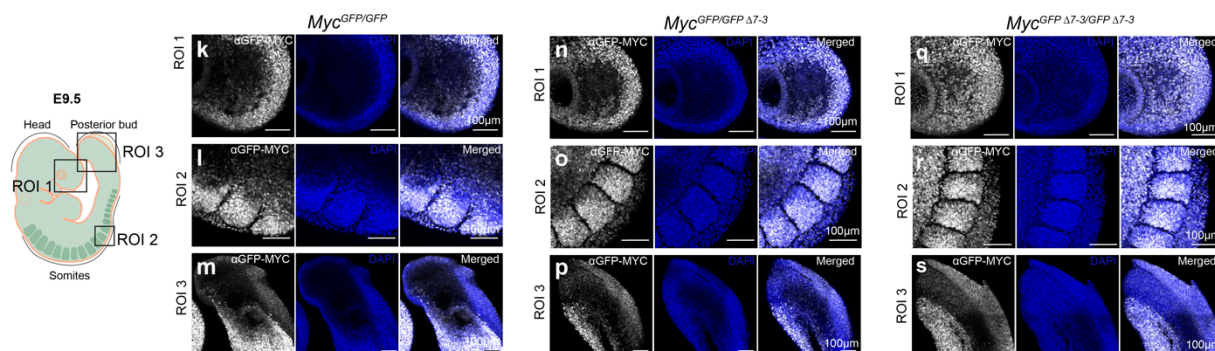
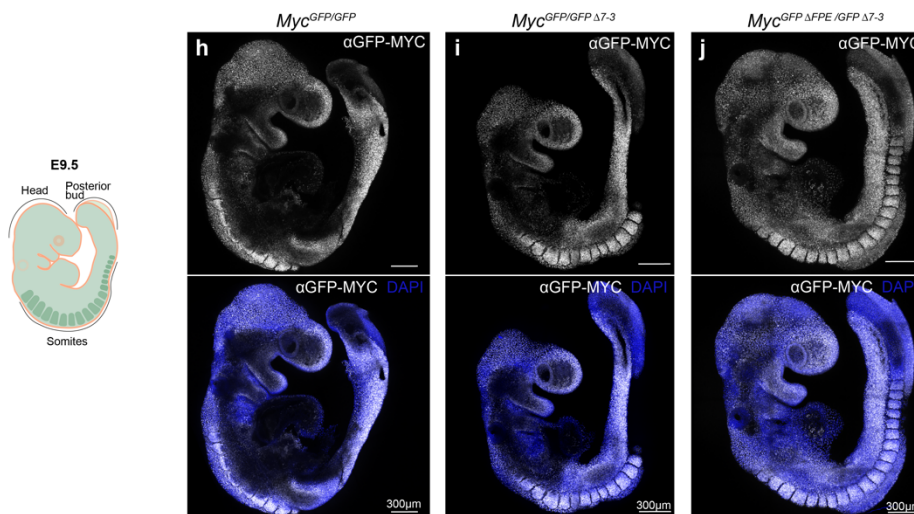
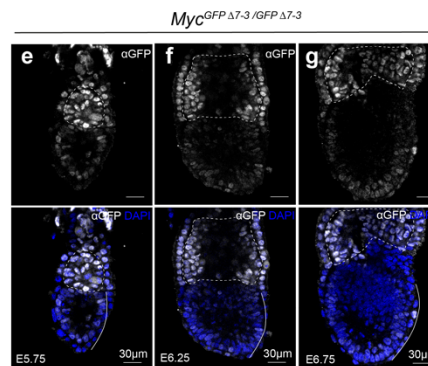
l Pro-Naïve pluripotency



m Formative pluripotency



Supplementary Figure 7. GFP-Myc expression analysis in ESC with heterozygous deletion of Sub-cluster C and homozygous deletion of putative enhancers 7-1, 7-2 and 7-4. **a-b**, Confocal images show GFP-MYC endogenous fluorescence and DAPI in control *Myc^{GFP/GFP}* cells (**a-a''**) and cells with heterozygous deletion of sub-cluster C (**b-b''**) in SR+LIF, SR+2i and 2 days-N2B27+F/A culture conditions. Scale bar= 30 microns. **c-e**, Graph shows normalized median intensity of sub-cluster C deletion in heterozygosity or homozygosity and comparison to GFP-Myc and Control ES cells. 3 independent clones with two biological replicates each were measured in **c** and **e**. 5 GFP-Myc independent clones and 3 independent clones for the rest of genotypes were measured in **d**. GFP-MYC endogenous levels were analyzed by FACs in SR+LIF (**c**) and 2 days-F/A (**e**) conditions. In SR+2i condition (**d**) GFP-MYC levels were analyzed in confocal images. The number of cells quantified per clone/biological replicate is available from the source data in Figshare. The bars show the Median and the error bars show the Standard Deviation. Mann Whitney test; ns: P-value >0,05; **: P-value=0.0022 except for C^{+/-} vs C^{-/-} comparison in which it was 0.0043. **f**, Table shows the genomic coordinates of putative enhancers and cCRE included in sub-cluster C. Name, position, size score, max-z and cell type are indicated. **g-j**, Confocal images show GFP-MYC endogenous fluorescence and DAPI in control *Myc^{GFP/GFP}* cells (**g-g''**) and cells with a homozygous deletion of enhancer 7-1 (**h-h''**), enhancer-7-2 (**i-i''**) and enhancer-7-4 (**j-j''**) cultured in 3 conditions. Scale bar= 30 microns. **k-m**, Bars show normalized median intensity and error bars show Standard Deviation. 3 independent clones with two biological replicates each were measured in **k** and **m**, except for the GFP-MYC condition, in which 5 clones were used. 2 GFP-Myc independent clones and 3 independent clones for the rest of genotypes were measured in **d**. GFP-MYC endogenous levels were analyzed by FACs in SR+LIF (**k**) and 2 days-F/A (**l**) conditions. In SR+2i condition (**m**) GFP-MYC levels were analyzed by in confocal images. The number of cells quantified per clone/biological replicate is available from the source data in Figshare. One-way ANOVA with Dunnett's correction; ns: P-value >0,05; **: P-value=0,0011. Source data for all graphs are available from the Source Data file and raw data from Figshare (see Data Availability section).

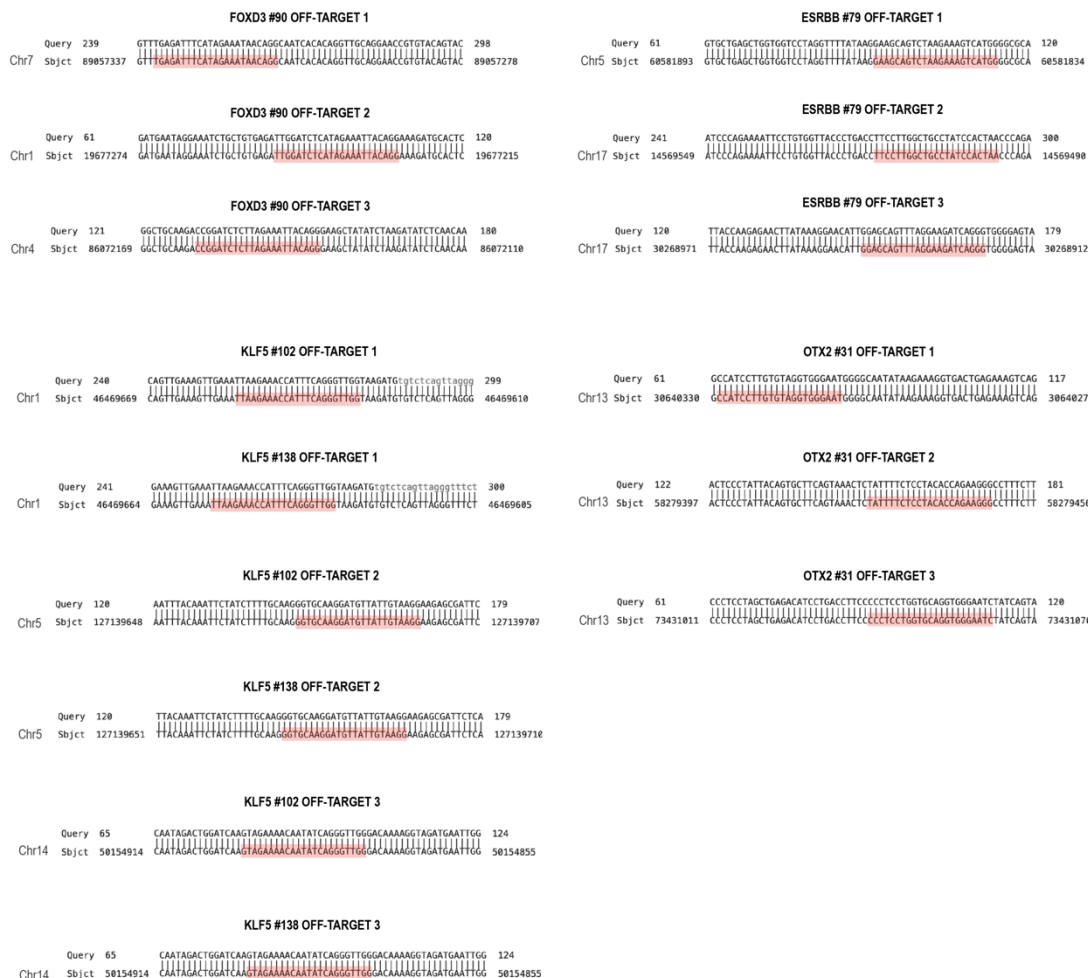


Supplementary Figure 8. Analysis of the requirement for Enhancers 2 and 7-3 activity from E3.5 to E9.5. **a**, Dot plot showing GFP-MYC expression levels in trophectoderm (TE) and epiblast cells (EPI) of *Myc*^{GFP/GFPΔ2} and *Myc*^{GFPΔ2/GFPΔ2} blastocysts. **b**, Dot plot showing GFP-MYC expression levels in trophectoderm (TE) and epiblast cells (EPI) of wild type, *Myc*^{GFP/GFP}, *Myc*^{GFP/GFPΔ7-3} and *Myc*^{GFPΔ7-3/GFPΔ7-3} blastocysts. In **a**, **b**, each column represents an individual embryo and each dot represents the intensity of single cells, with the number of cells scored indicated above. **c**, Violin plot showing GFP-MYC expression levels in extra-embryonic ectoderm (EE) and epiblast cells (EPI) of *Myc*^{GFP/GFPΔ2} and *Myc*^{GFPΔ2/GFPΔ2} E6.5 embryos. **d**, Violin plot showing GFP-MYC expression levels in extra-embryonic ectoderm (EE) and epiblast cells (EPI) of wild type, *Myc*^{GFP/GFP}, *Myc*^{GFP/GFPΔ7-3} and *Myc*^{GFPΔ7-3/GFPΔ7-3} E6.5 embryos. In **c**, **d**, each column represents an individual embryo, and the plots represent the intensity values of single cells, with the number of cells scored indicated above each column. Violin plots show median and quartiles. **e-g**, Confocal images show anti-GFP immunofluorescence and DAPI in E5.75, E6.25 and E6.75 *Myc*^{GFPΔ7-3/GFPΔ7-3} embryos. Scale bar= 30 microns. **h-j**, Confocal images with Maximum Intensity Z-projection show immunostaining against GFP and DAPI in *Myc*^{GFP/GFP} (h, N=4), *Myc*^{GFP/GFPΔ7-3} (i, N=5) and *Myc*^{GFPΔ7-3/GFPΔ7-3} (j, N=6) E9.5 embryos. Scale bar= 300 microns. **k-i**, Confocal images show antiGFP immunofluorescence and DAPI in E9.5 embryos. Scale bar= 100 microns. **k-m**, Nasal process (**k**), somites (**l**) and posterior embryonic bud (**m**) regions of a *Myc*^{GFP/GFP} embryo. **n-p**, Nasal process (**n**), somites (**o**) and posterior embryonic bud (**p**) regions of a *Myc*^{GFP/GFP} embryo. **q-s**, Nasal process (**q**), somites (**r**) and posterior embryonic bud (**s**) regions of a *Myc*^{GFPΔ7-3/GFPΔ7-3} embryo. Source data for all graphs are available from the Source Data file and raw data from Figshare (see Data Availability section).

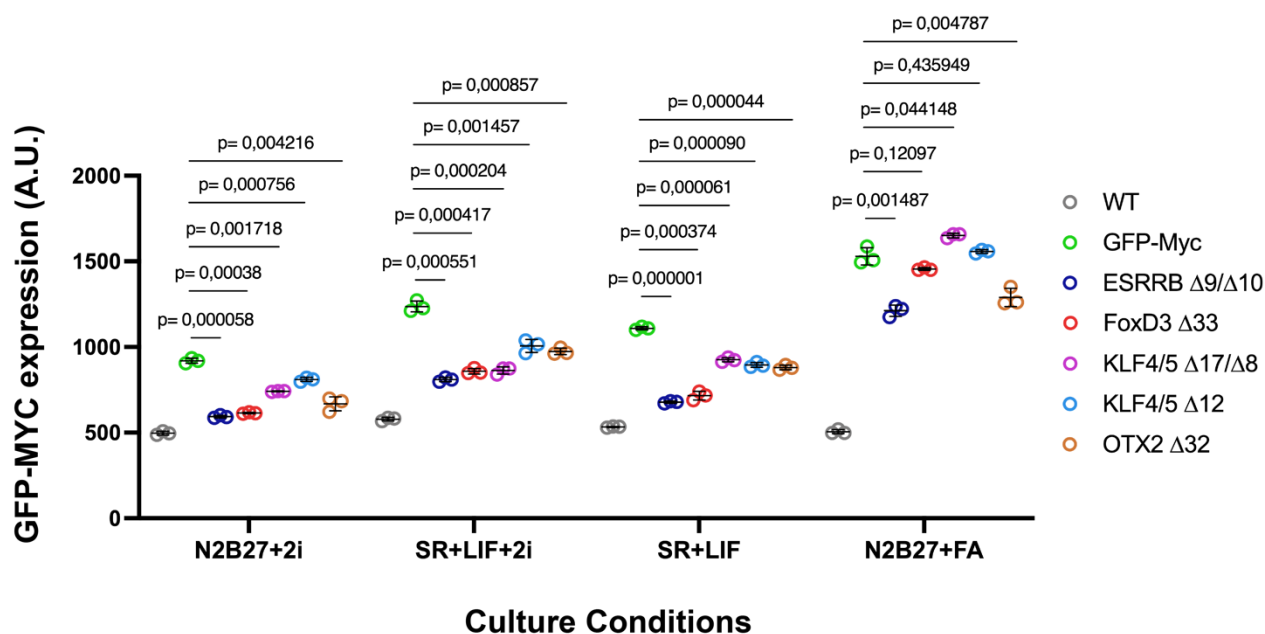
a



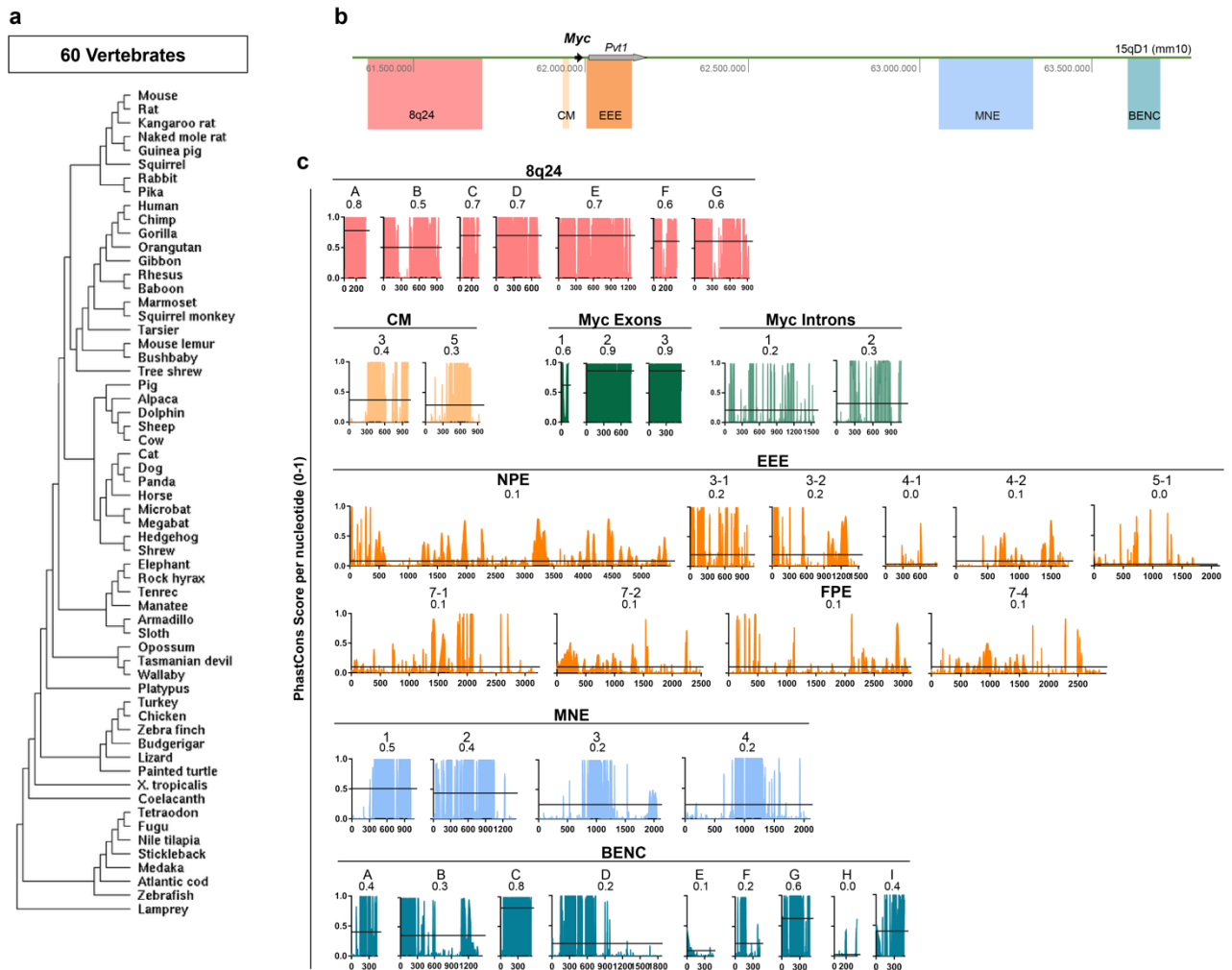
b



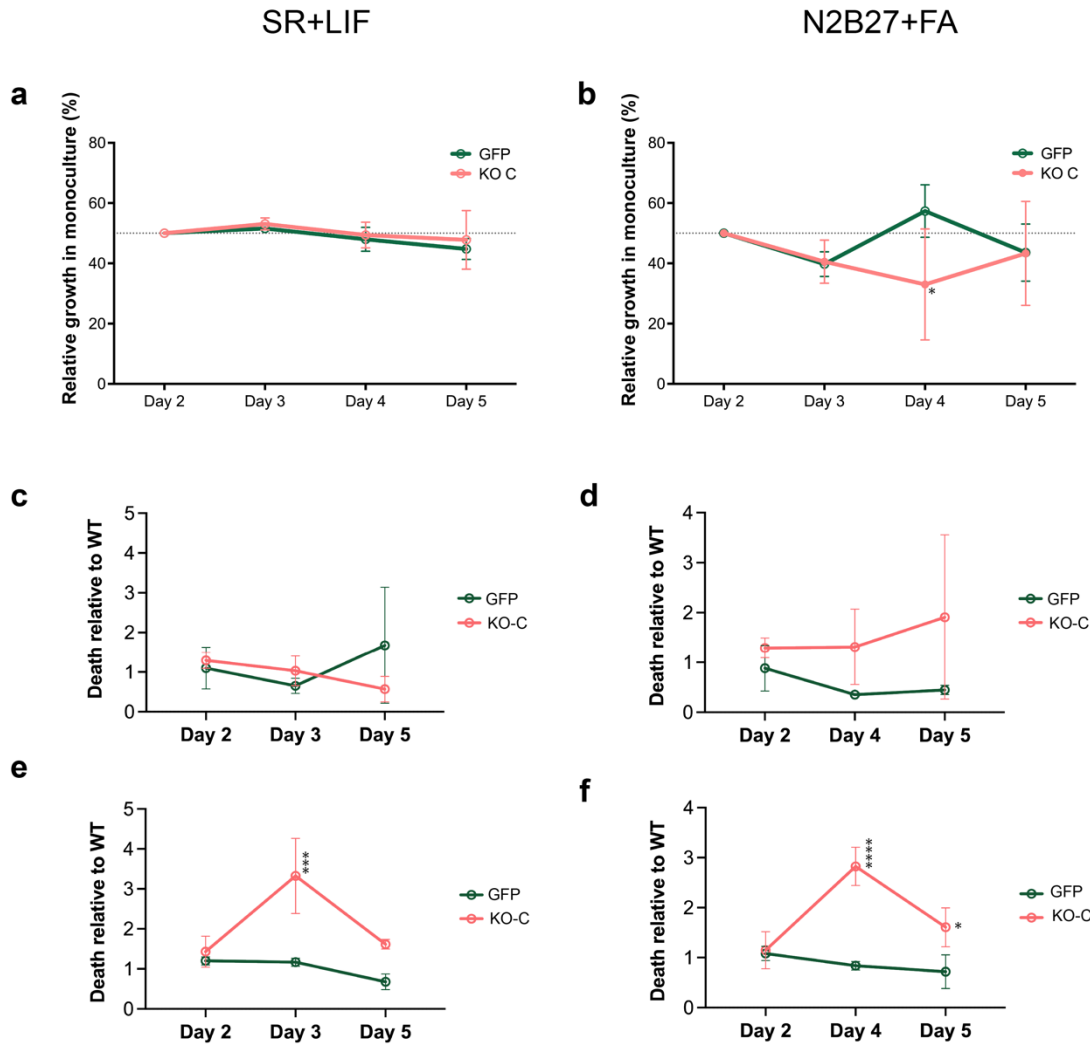
Supplementary Figure 9. OFF-Target analysis in ESC clones with CRISPR-Cas9 deletion of Transcription Factor Binding sites. **a**, shows the bands containing the OFF-target sequences amplified from each clone's Genomic DNA. See Supplementary Table 4 for primers used. Below the gels, the expected band size is indicated. H lanes are negative controls containing water instead of Genomic DNA solution. **b** shows the alignment of the sequence of the amplified bands with the GRCm39 C57Bl/6J genome sequence. The OFF-target sequences appear highlighted in red.



Supplementary Figure 10. GFP-MYC expression levels in mESCs deleted for ESRRB, FoxD3, KLF4/5 and OTX2 candidate binding sites. Dot plot shows flow cytometry GFP-MYC mean intensity of 3 biological replicates of WT, *Myc^{GFP/GFP}* and *Myc^{GFP/GFP}* deleted in homozygosity or trans-heterozygosity for the indicated mutations in four culture conditions shown on the X axis. The number of cells quantified per clone/biological replicate is available from the source data in Figshare. Ordinary one-way ANOVA with Šidák's multiple comparisons test. Exact P-values are shown above each comparison. Source data for all graphs are available from the Source Data file and raw data from Figshare (see Data Availability section).

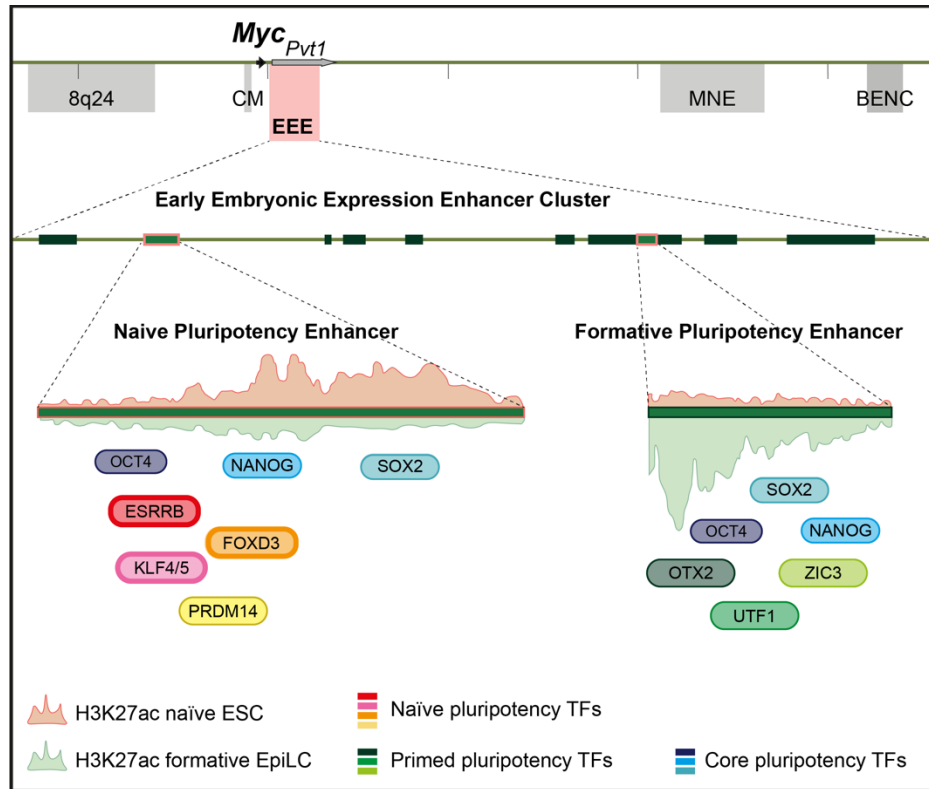


Supplementary Figure 11. Evolutionary conservation of *Myc* enhancers. **a**, The 60 vertebrate species included in this comparative study. **b**, scaled representation of previously described enhancers belonging to different enhancer cluster regions and the Early Embryonic Expression region described here. Including 8q24 enhancers A-G, CM3, CM5, EEE enhancers 2 to 7-4 (including NPE and FPE), MNE enhancers 1-4 and BENC enhancers A to I. *Myc* (black arrow) and *Pvt1* (grey arrow) transcription units are illustrated. The location, size and reference for all the elements shown are listed in Supplementary Table 5. **c**, Graphs show conservations as PhastCons scores (ranged from 0 to 1) per nucleotide. The average of the conservation scores of each element is represented by a black horizontal line and the numerical value appears above the graph.



Supplementary Figure 12. Proliferation and cell death in ESCs with Cluster-C deletion.

a, shows the relative proportion between the populations of GFP-Myc ESCs and GFP-Myc ESCs carrying a sub-cluster-C deletion with Control Tomato+ cells cultured independently in SR+LIF. **b** shows similar results when ESCs are cultured in N2B27+F/A conditions. All populations were cultured independently and mixed 1:1 in volume for characterization of the cell populations proportions by Flow Cytometry. **c** and **d** show the proportion of cell death measured in the parental GFP ESC line and in ESCs carrying a sub-cluster-C deletion when cultured independently in SR+LIF (**c**) or N2B27+F/A (**d**). **e** and **f** show similar results, respectively, when cells were co-cultured with the Tomato+ control cells. Cell death was determined by DAPI incorporation in Cytometry. 3 independent clones were measured for each genotype in **a-f**. Dots indicate the Mean and error bars show the standard deviation. The number of cells quantified per clone/biological replicate is available from the source data in Figshare. Two-way ANOVA with Šídák's multiple comparisons test; ns: P-value >0,05 (not shown); *: P-value=0,0448 in **b** and 0.0127 in **f**; ***: P-value=0,0001; ****: P-value<0,0001. Source data for all graphs are available from the Source Data file and raw data from Figshare (see Data Availability section).



Supplementary Figure 13. Model for the modular regulation of *Myc* transcription in early mammalian embryos and during different pluripotency states

Supplementary tables

| Name | Chr | Localization (mm10) | Type |
|------------|--------------|--------------------------------|-------------------|
| MycDel3Mb | chr15 | chr15:60301761-63718995 | Deletion |
| InvMyc1 | chr15 | chr15:60301761-62336788 | Inversion |
| Delcent3 | chr15 | chr15:61518771-61982613 | Deletion |
| Del40 | chr15 | chr15:62336788-63353788 | Deletion |
| Del11 | chr15 | chr15:62836993-63353788 | Deletion |
| Del21 | chr15 | chr15:63364914-63718995 | Deletion |
| BAC | chr15 | chr15:61958143-62188886 | RP24-78D24 |

Supplementary Table 1. Chromosome rearrangements and BAC localization.
mm10 genome coordinates

| 4C | | | |
|------|-------------------|---|---|
| | | Forward | Reverse |
| PCR1 | adapter | AATGATACGGCGACCACCGAACTCTTTCCCTAC ACGACGCTCTTCCGATCTggggcacaagctggagtac- | CAAGCAGAAGACGGCATACGA GTGTCGCCCTCGAACT |
| PCR2 | Index- Library | AATGATACGGCGACCACCGAGATCTACACTCTTTC CCTACACGACGCTCTTCCGATCT | (GFP 2I) Rv P7 index1: 5'CAAGCAGAAGACGGCATACGAGATCGTGAT GTGACTGGAGTTCAGACGTGTGCT-3' (GFP 2I) Rv P7 index2: 5'- CAAGCAGAAGACGGCATACGAGATACATCGG TGACTGGAGTTCAGACGTGTGCT-3' (GFP EPI) Rv P7 index3: 5'- CAAGCAGAAGACGGCATACGAGATGCGTAAG TGACTGGAGTTCAGACGTGTGCT-3' (GFP EPI) Rv P7 index4: 5'- CAAGCAGAAGACGGCATACGAGATTGGTCAG TGACTGGAGTTCAGACGTGTGCT-3' (TFP 2I) Rv P7 index5: 5'- CAAGCAGAAGACGGCATACGAGATCACTGTG TGACTGGAGTTCAGACGTGTGCT-3' (TFP 2I) Rv P7 index6: 5'- CAAGCAGAAGACGGCATACGAGATATTGGCG TGACTGGAGTTCAGACGTGTGCT-3' (TFP EPI) Rv P7 index7: 5'- CAAGCAGAAGACGGCATACGAGATGATCTGG TGACTGGAGTTCAGACGTGTGCT-3' (TFP EPI) Rv P7 index8: 5'- CAAGCAGAAGACGGCATACGAGATCAAGTG TGACTGGAGTTCAGACGTGTGCT-3' |

Supplementary Table 2. 4C primers. mm10 genome coordinates

| crRNAs used for CRISPR deletions | | | | |
|----------------------------------|-----------------------------|--------------|----------------------|----------------------|
| | Localization (mm10) | Size (bp) | 5′ | 3′ |
| Putative enhancers cluster | | | | |
| Cluster A | chr15:61,990,505-62,014,068 | 23.764 | GAATGGCTTCGGTTAACTGT | TGTACATATCCGCACCTTCC |
| | | | GGGTGTCCTTCCGCTGGTTA | GTTGAACTGAAAGCGACCAA |
| Cluster B | chr15:62,039,218-62,055,983 | 16.664 | GACTGGGAAAAACCTCGTGG | ACCTCTGACAAAGCCTACGA |
| | | | GTTGAGTTCAAATAACCGTA | GAAGGAGCTACGTCAACCTA |
| Cluster C | chr15:62,078,497-62,099,928 | 22.185 | CCAGCCTTAAGTGAATGACC | TGCCATTGATGTCGCTGACT |
| | | | TCCAGCAGGGGCACGTGAT | TAAACTGAGCCGGTAACCCG |
| Cluster D | chr15:62,103,836-62,133,033 | 28.395 | GTGGGCTGCGTTCTGTATTC | GTGTACAATGAGCGTAGTCA |
| | | | ACTCCCTTGAACACGGGTCC | CTAGGAAGCCTTGTTGATG |
| Putative enhancers | | | | |
| Cluster A- enhancer 2 | chr15:62,008,447-62,014,334 | 5.888 | AGCTGCAGTGATCCAGTACG | TGTACATATCCGCACCTTCC |
| | | | CAATGTCCTGTTACGGATTA | GTTGAACTGAAAGCGACCAA |
| Cluster C- enhancer 7-1 | chr15:62,085,072-62,088,281 | 3.211 | CTATGTTTGCCTGCGTACCC | TGAGATTGAGATCCGTTCTC |
| | | | AGTTCCCTTAACAAGGCCAC | TATCGTAGTTAAGTTTCCCA |
| Cluster C- enhancer 7-2 | chr15:62,089,322-62,092,601 | 3.281 | GAGTTGTGACATGCCATACT | CGCATGTGCTATTGCCTTTC |
| | | | GTTTAAGGGTGCATCTCGGC | GAGGATTGGATTCCCCTCCT |
| Cluster C- enhancer 7-3 | chr15:62,092,596-62,095,870 | 3.276 | GGCCCTGTGTGTCGATAAC | TTCATTCTAGCATGTGCAGG |
| | | | CCAAGGTCAGGTTGTCGGGG | AAGGTATGTCATATCCAAGT |
| Cluster C- enhancer 7-4 | chr15:62,096,720-62,099,694 | 2.976 | GACAGTACCTGCTCAGAAT | AAGCAGTATCTAGAGGACGT |
| | | | AAAGAACACATGTAAAGTCG | AAGTGAAGCTTTCTATGACG |
| TF binding sites (BS) | | | | |
| ESRRB BS | chr15:62009970-62009979 | 9 | GAAGCAGTCAAGGAAGGTCA | |
| | chr15:62009963-62009971 | 10 | | |
| FoxE1 BS | chr15:62010890-62010923 | 33 | TTGGATCTCTAGAAATAAC | |
| KLF5 a BS | chr15:62010999-62011015 | 17 | GTAGGTAACCATTTCAGGGT | |
| | chr15:62,011,006-62,011,013 | 8 | | |
| KLF5 b BS | chr15:62011000-62011011 | 12 | | |
| OTX2 BS | chr15:62092929-62092960 | 32/33 | CATTTCTCTACACCAGGA | |

Supplementary Table 3. crRNA used for sub-cluster and putative enhancer deletions

| Primers for genotyping | | | | | | | |
|-----------------------------------|--------|-----------|--------------------------|-------------------------|--|--------------------------------|-------------------------|
| | allele | size (pb) | Forward | Reverse | | | |
| Putative enhancers cluster | | | | | | | |
| Cluster A | KO | 466/1663 | TATGGGAAGGGGTATCATTG | AGTGGTCTTCTGCCTCTG | | | |
| | WT | 680 | TCCTTTGTACATCAAGCC | GTTGAAGAATCAAAAGTCTG | | | |
| Cluster B | KO | 568/645 | TGTGTGTCTGTGTGTTGG | GCAAGTAGAGCATGCCTG | | | |
| | WT | 538 | CAGTGTGTGTAGCATGTTG | GCAATAGTGGCATGCATG | | | |
| Cluster C | KO | 444/1136 | GAGACAGGGTCTCTAAGTAG | AGATCTAGTTCCAACAATG | | | |
| | WT | 530 | TAGTCTGCCTGTCAAAAC | AAGGACAGCAGTAAACTAAG | | | |
| Cluster D | KO | 451/751 | TCTGGCTTATTGGGTCCTG | CCAGGAACAGTTTTTGAAAC | | | |
| | WT | 398 | GCTCTGCCTCTCAGTCTAC | CACACCCACACATCTCTG | | | |
| Putative enhancers | | | | | | | |
| Cluster A- enhancer 2 | KO | 952 | GTAAGGAGGTTGAAATGGCTCAAG | GGGAGTAATGGATGCTGTGGT | | | |
| | WT | 1496 | CTTCTCCTGGTTGACACGCT | ATTGTGGTTTTCTGCCGTGC | | | |
| Cluster C- enhancer 7-1 | KO | 585/855 | TGGTATGGACAGCATTGTAG | CTATACCTAGTACTCGGAG | | | |
| | WT | 524 | GCTGTTGCTTGTCTGGC | AGCTGGGCATTAACACCAC | | | |
| Cluster C- enhancer 7-2 | KO | 506/700 | TCCTGGGCTGAAATAAC | GCCTAGGCAGCATCTGGC | | | |
| | WT | 500 | TCTCGAATCTTGAGATGTTTC | TACTCTGGGAGGAGATATTC | | | |
| Cluster C- enhancer 7-3 | KO | 388/401 | CACCTTTATGAGAGGCCTTAGG | CCCTGTCTTGAAAAACAG | | | |
| | WT | 505 | AGCGTTGCTGTATATCAGATAC | GTCCCAGCACTGAAGAGG | | | |
| Cluster C- enhancer 7-4 | KO | 600/775 | CAGATTATCACACCCAAG | CTGTGGCCAGCAGAGATG | | | |
| | WT | 541 | CTTCTACCATATGTAAGGGATC | TCTCTCTGGTGGCAAAAC | | | |
| TF binding sites (BS) | | | | | PCR products restriction | | |
| ESRRB BS | WT/KO | 314 | CAGGCTGTTTGGAGAGCTGA | TAAAGGGCCACACAGCTGAC | ESRBB_HinfI (wt): 185+130 bp ESRBB_HinfI (del): 300 bp | | |
| FoxE1 BS | WT/KO | 577 | TCTCATCCCTCGCCAAAGA | CAACCCTTCCCATGTCCAG | Foxd3-72_BstYI (wt): 359 +218 bp Foxd3-72_BstYI (del): 577bp | | |
| KLF5 a BS | WT/KO | 640 | TCAGAGACTCTACTGCCCAT | TCATGTGCTATGTCTCAGCC | Klf5_BsII (wt): 391 +125 + 63 +61 bp | | |
| KLF5 b BS | | | | | Klf5_BsII (del): 454 +125 +61 bp | | |
| OTX2 BS | WT/KO | 335 | TAAGGAGGGGAGGAAGAGGC | CTGGAAGGAAAGGCAGGGAG | Otx2_BstNI (wt): 118 + 76 + 60+ 57+ 24pb Otx2_BstNI (del): 133 + 118+ 60+ 24 pb | | |
| OFF-Target Sequence amplification | | | | | OFF-target position | MIT OFF- Target Score | OFF-target Sequence |
| ESRBB crRNA | 1 | 428 | CTTCTGAAAGGAGGAGTCATCA | TTGGAACCTATTGCTGGGATAA | chr5:60424497-60424519:- | 0.20 | GAAGCAGTCTAAGAAAGTCATGG |
| | 2 | 427 | TGTCCTGCTGTCTTCTCTG | GGCCATTCCAGGTAAGCCTTC | chr17:14349242-14349264:+ | 2.51 | TAGGCAGCCAAGGAAGGTCAGGG |
| | 3 | 476 | TGTGCATAATAGTCCCTTACCG | ATACGTTTGAGTGCTTGGACTTC | chr19:30291521-30291543:- | 0.34 | GGAGCAGTTTAGGAAGATCAGGG |
| KLF5 crRNA | 1 | 600 | TGATTTGCCTTTAGTGGATAGCT | TCTCTGAGGACTGGTCTGGAAG | chr1:53702359-53702381:+ | 1.47 | TTAAGAAACATTTCAGGGTTGG |
| | 2 | 440 | TAAGCTAAGCAATCTCACCAGC | GAGTTTCAAAGAGTCTCCAGG | chr5:127062610-127062632:+ | 0.37 | GGTGCAAGGATGTTATTGTAAGG |
| | 3 | 465 | CCCGAACTTACTAATCTCCAGT | CACAATAGAGGAGTGGTTGGGT | chr14:49917419-49917441:- | 0.29 | GTAGAAAAAATATCAGGGTTGG |
| FoxE1 crRNA | 1 | 535 | CCTGCATCATCTCCTTCA | TTGTGCTATGTTGGGAACCA | chr7:89408104-89408126:- | 0.85 | TGAGATTTCATAGAAATAACAGG |
| | 2 | 492 | GAAAAACCAAAACCCATCAA | AATTCGCCCTTCTCTTCT | chr1:19607003-19607025:- | 1.36 | TTGGATCTCATAGAAATTACAGG |
| | 3 | 473 | GTTCTTTACCCCTCAAAACAG | GGCTCATCTCTGGGAAAGACTA | chr4:86153900-86153922:- | 0.66 | CCGGATCTCTAGAAATTACAGG |
| OTX2 crRNA | 1 | 510 | ACATAATTGAGGGAAGCCAGGG | AAGGGACCAAGCATGACTGAAT | chr13:30456324-30456346:+ | 0.48 | CATTCCCACCTACACAAGGATGG |
| | 2 | 532 | TGAGGGTAGGAACCCGAGAG | CTCTCACTACCCCACTCCA | chr13:58131612-58131634:+ | 0.68 | TATTTTCTCCTACACCAAGAGGG |
| | 3 | 485 | TGAGAGGCAGGGTGCTATCT | ATCCACCAAGAGAGGCCATT | chr13:73282921-73282943:- | 0.70 | CCCTCTGGTGCAAGGTGGGAATC |

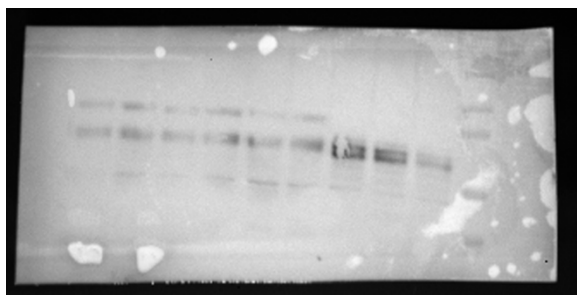
Supplementary Table 4. Primers for genotyping CRISPR-Cas9 deletions and OFF-target sites. mm10 genome coordinates

| name | ref | coordinates (mm10) | size bp |
|------------------------|-----------------------|-----------------------------|---------|
| 8q24 enhancer A | Sotelo et al., 2010 | chr15:61356836-61357193 | 358 |
| 8q24 enhancer B | Sotelo et al., 2010 | chr15:61365625-61366591 | 967 |
| 8q24 enhancer C | Sotelo et al., 2010 | chr15:61395374-61395696 | 323 |
| 8q24 enhancer D | Sotelo et al., 2010 | chr15:61608209-61608962 | 754 |
| 8q24 enhancer E | Sotelo et al., 2010 | chr15:61618832-61620086 | 1.255 |
| 8q24 enhancer F | Sotelo et al., 2010 | chr15:61653313-61653698 | 386 |
| 8q24 enhancer G | Sotelo et al., 2010 | chr15:61689984-61690912 | 929 |
| CM3 | Hallikas et al., 2006 | chr15:61949429-61950423 | 995 |
| CM5 | Hallikas et al., 2006 | chr15:61959148-61960079 | 932 |
| Myc | exon (UCSC) | chr15:61,985,922-61,989,908 | 1369 |
| | exon 1 | chr15:61,985,922-61,985,951 | 30 |
| | exon 2 | chr15:61,987,504-61,988,279 | 776 |
| | exon 3 | chr15:61,989,346-61,989,908 | 563 |
| Myc | intron (UCSC) | chr15:61,985,922-61,989,908 | 2.622 |
| | intron 1 | chr15:61,985,951-61,987,504 | 1.554 |
| | intron 2 | chr15:61,988,279-61,989,346 | 1.068 |
| Enhancer 2 | ROI | chr15:62,008,447-62,014,334 | 5.888 |
| Cluster B-enhancer 3-1 | ROI | chr15:62,038,009-62,039,109 | 1.101 |
| Cluster B-enhancer 3-2 | ROI | chr15:62,039,143-62,040,653 | 1.511 |
| Cluster B-enhancer 4-1 | ROI | chr15:62,043,253-62,044,145 | 893 |
| Cluster B-enhancer 4-2 | ROI | chr15:62,044,235-62,046,157 | 1.923 |
| Cluster B-enhancer 5-1 | ROI | chr15:62,052,822-62,054,955 | 2.134 |
| Cluster C-enhancer 7-1 | ROI | chr15:62,085,072-62,088,281 | 3.210 |
| Cluster C-enhancer 7-2 | ROI | chr15:62,089,322-62,092,601 | 3.280 |
| Cluster C-enhancer 7-3 | ROI | chr15:62,092,596-62,095,870 | 3.275 |
| Cluster C-enhancer 7-4 | ROI | chr15:62,096,720-62,099,694 | 2.975 |
| MNE Enhancer 1 | Uslu et al., 2014 | chr15:63052149-63053208 | 1.060 |
| MNE Enhancer 2 | Uslu et al., 2014 | chr15:63163294-63164714 | 1.421 |
| MNE Enhancer 3 | Uslu et al., 2014 | chr15:63189194-63191298 | 2.105 |
| MNE Enhancer 4 | Uslu et al., 2014 | chr15:63339415-63341511 | 2.097 |
| BENC-A | Bahr et al., 2018 | chr15:63611116-63611556 | 441 |
| BENC-B | Bahr et al., 2018 | chr15:63616791-63618232 | 1.442 |
| BENC-C | Bahr et al., 2018 | chr15:63648126-63648667 | 542 |
| BENC-D | Bahr et al., 2018 | chr15:63657837-63659699 | 1.863 |
| BENC-E | Bahr et al., 2018 | chr15:63665359-63665959 | 601 |
| BENC-F | Bahr et al., 2018 | chr15:63678942-63679359 | 418 |
| BENC-G | Bahr et al., 2018 | chr15:63704706-63705184 | 479 |
| BENC-H | Bahr et al., 2018 | chr15:63709542-63709926 | 385 |
| BENC-I | Bahr et al., 2018 | chr15:63736276-63736746 | 471 |

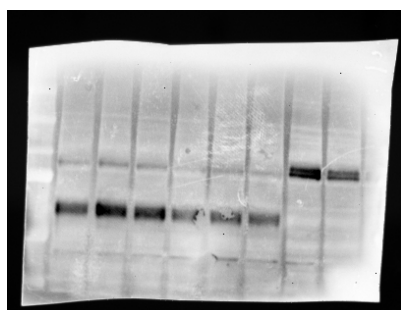
Supplementary Table 5. Location, reference and size of the enhancers used for the conservation study. mm10 genome coordinates

Uncropped gels from Supplementary Figure 4

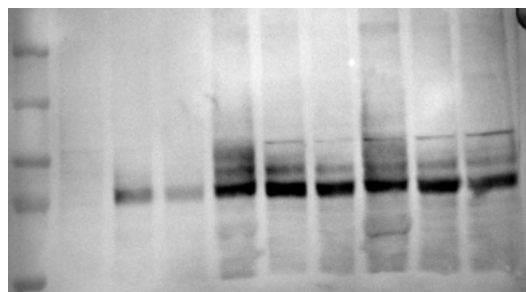
a



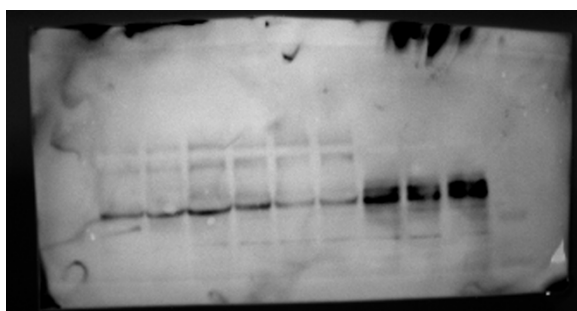
b



c



d



e

