Li et al. Etv6 Supplementary Data

a

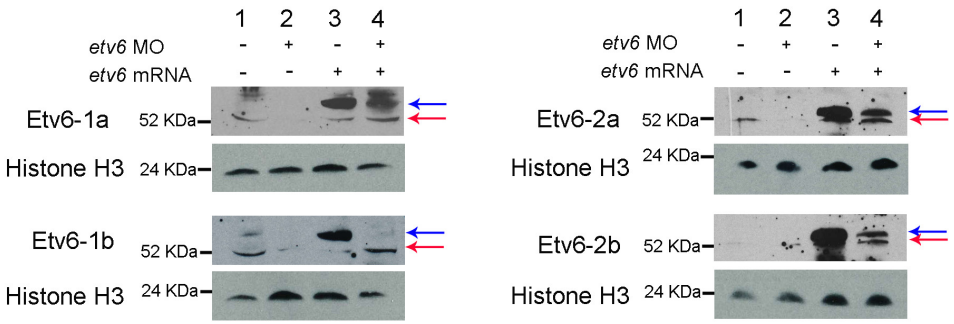
MSSAQCYVKQEQIPYSPPESPAPNCTTSSPSQLNASGPRRYRMEEETVRLPAHLRLPPAHWSREDVSQWL
peptide #1 pointed domain
RLAENEYSLHPIDANTFEMNGKALLLLTKEDFRYRCPHSGDVLYEVLQHTLKERKPRLVHSSLLHTGNSLHG
peptide #2
QSEALISQNHHNDHHSNRPLRTPSHNTPLNTPTIELLHRSRSPFANHHSSPDSESRPLRSPMETPLRRLSPA

ERLHRHASETNHHAQESYPLSVSPAEANHCPSEPINKPNSPRQENPRVIQLMPSPIMHPLLLNTRHSMEFK

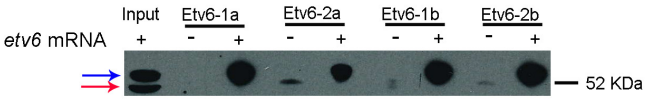
QPRLEDGQPREGKPINLSHREDMAYLNHMMVSVSPPGDHGMPIGRIADCRLLWDYVYQLLSDSRYENFIR
ETS domain
WEDKESMVFRIMDPNGLARLWGNHKNRTNMTYEKMSRALRHYYKLNIIRKEPGQRLLFRFMKTPEEIMSG

RTDRLEHLESQELDEQMYQEDEC
peptide #3

b

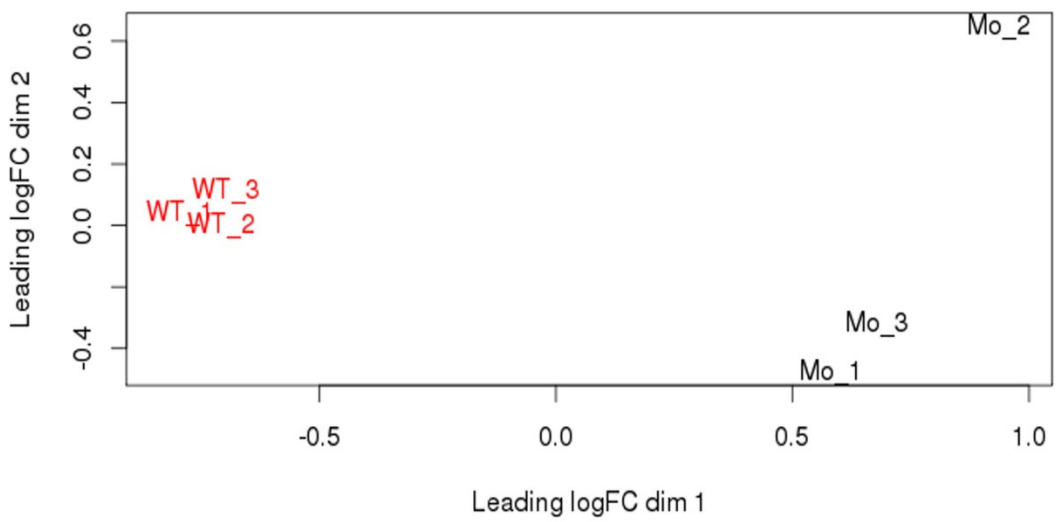


c

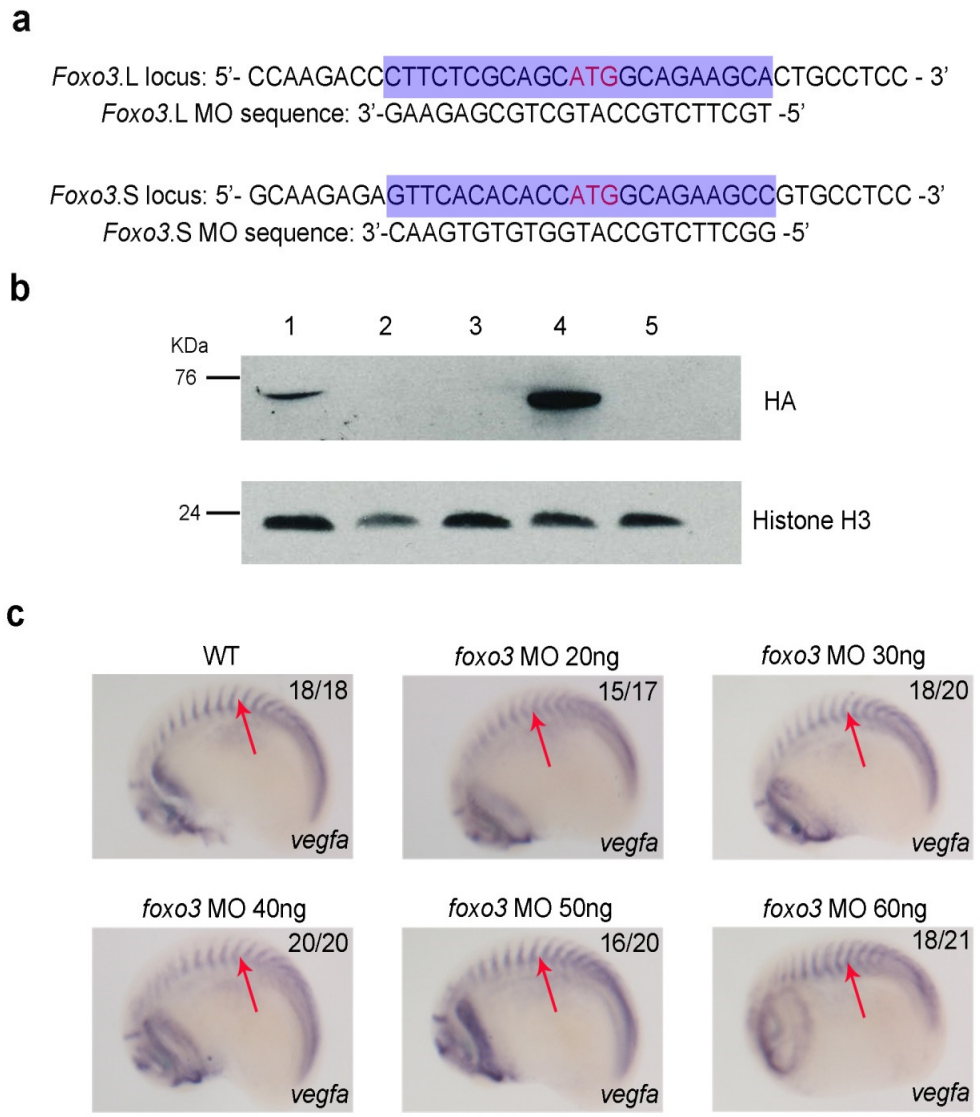


Supplementary Figure 1. Generation and characterization of ChIP grade antibodies against *Xenopus* Etv6. *(**a**) Amino acid sequence for Xenopus Etv6 (Accession Number NP_001124423.1). Pointed domain and ETS domain are indicated in green and blue, respectively. The sequences of the three peptides used for the generation of antibodies are indicated in red. Two rabbit polyclonal antibodies were obtained per peptide: Etv6-1a, -1b, -2a -2b, -3a, -3b. (**b**) Western blot validating the specificity of Etv6 antibodies. Protein extracts from stage 22 wild-type (WT), etv6-deficient (etv6 MO), etv6-overexpressing (HA-etv6 mRNA) or etv6-deficient + etv6-overexpressing (etv6 MO+HA-etv6 mRNA) somites were used for western blot analysis. While polyclonal antibodies generated from peptide 1 (Etv6-1a and Etv6-1b)*

*and peptide 2 (Etv6-2a and Etv6-2b) detect both endogenous and exogenous expression of Etv6, polyclonal antibodies generated from peptide 3 (Etv6-3a and Etv6-3b) did not detect expression of Etv6 and cross-reacted with an unknown protein (data not shown). Lane 1, WT; lane 2, 40 ng etv6 MO; lane 3, 1.0 ng etv6 mRNA; lane 4, 40 ng etv6 MO + 1.0 ng etv6 mRNA. The predicted molecular weight of Etv6 is around 52 KDa. (http://web.expasy.org/compute_pi/). Blue and red arrows point to exogenous and endogenous expression of Etv6, respectively. (***c***) Immunoprecipitation assay validating the affinity of anti Xenopus Etv6 antibodies to endogenous Etv6. Protein extracts from the somites of stage 22 WT embryos were used for immunoprecipitation; somite extracts from embryos injected with exogenous etv6 mRNA were used as positive control. Polyclonal antibodies generated from peptide 1 (Etv6-1a and Etv6-1b) and peptide 2 (Etv6-2a and Etv6-2b) were tested for their capacity to immunoprecipitate Etv6 protein. Western blot was performed using antibody Etv6-1b as primary antibody. Blots show that antibodies Etv6-2a and Etv6-2b could immunoprecipitate endogenous Etv6 protein. Antibody Etv6-2a showed greater affinity to Etv6 and was selected for subsequent experiments. Blue and red arrows point to exogenous and endogenous expression of Etv6, respectively.*

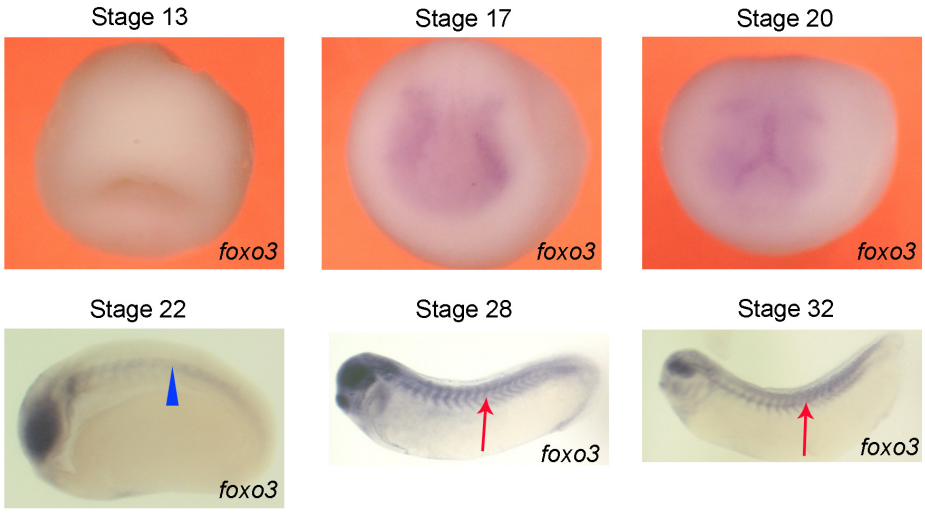


Supplementary Figure 2. PCA plot illustrating the significant differences in the transcriptome of *etv6*-deficient (*etv6* MO) somites when compared to that of wild type (WT) somites. *Three independent biological replicates were used for each condition.*

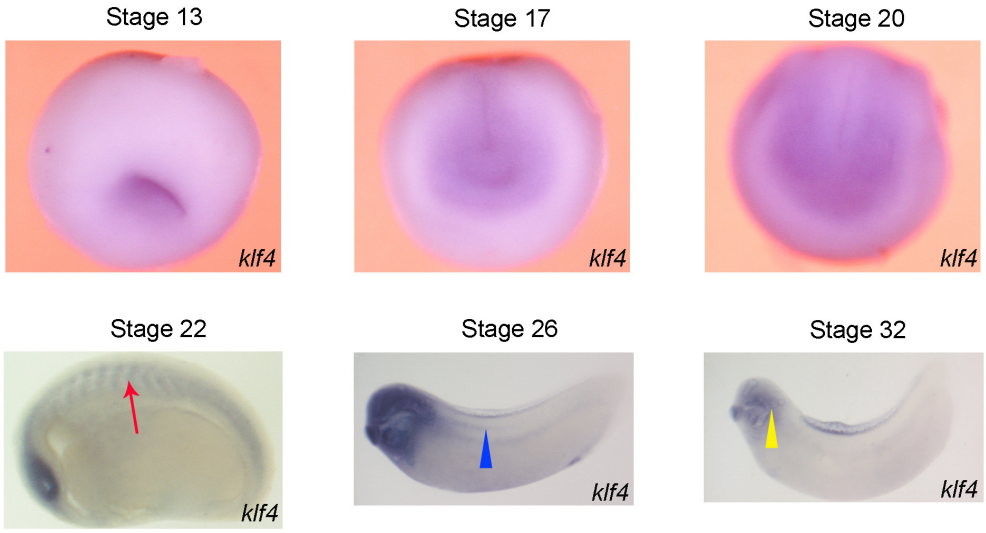


Supplementary Figure 3. *Foxo3* deficiency has no effect on *vegfa* expression in the somites at stage 22 of development. *(**a**) cDNA alignment showing the sequence differences between foxo3.L and foxo3.S. Due to lack of homology in the region of the transcription starting codon, a single MO targeting both genes could not be generated. Therefore, two MOs, each one targeting a specific foxo3 gene, were designed; their target sequence is highlighted in purple. (**b**) Western blot showing that foxo3 MOs block efficiently the translation of their targets. To assess the efficiency of the foxo3 MOs, the cDNAs for foxo3.L and foxo3.S, containing the sequences targeted by the MOs, were HA-tagged, mRNA was synthesised in vitro and injected into 2-cell stage embryos (0.5 ng per embryo). Translation of these cDNAs was blocked when co-injected with their corresponding blocking MO (30 ng). Lane 1, 0.5*

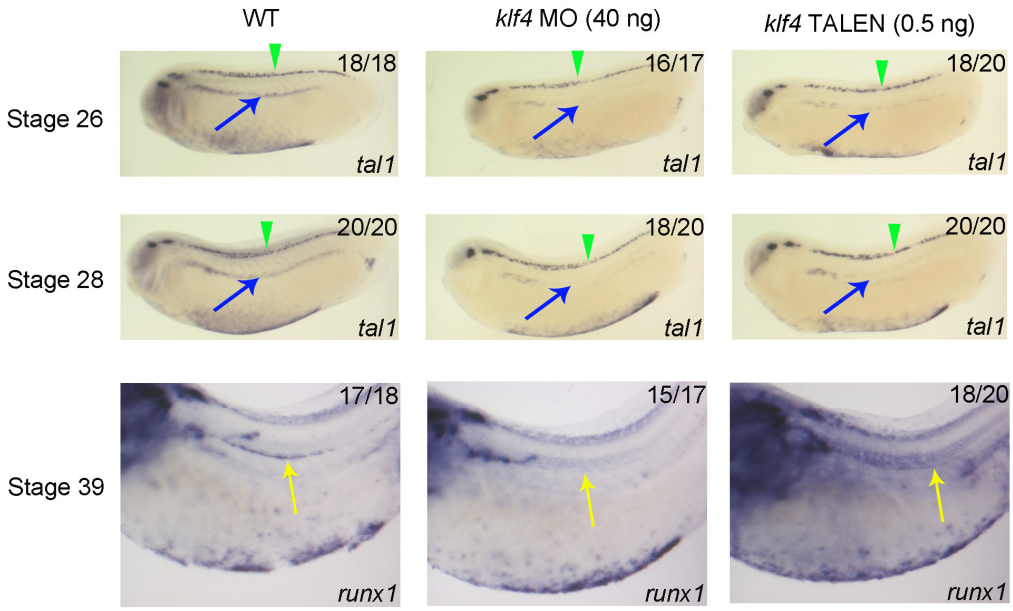
*ng foxo3.L-HA; lane 2, 0.5 ng foxo3.L-HA + 30 ng foxo3.L MO; lane 3, uninjected; lane 4, 0.5 ng foxo3.S-HA; lane 5, 0.5 ng foxo3.S-HA + 30 ng foxo3.S MO. Histone H3 was used as a loading control. (***c***) WISH showing that vegfa expression in the somites (arrows) is not affected in foxo3-deficient embryos. Foxo3 depletion was performed by co-injecting foxo3.L MO and foxo3.S MO in a 1:1 ratio to the total concentrations indicated. Images show stage 22 embryos in lateral view with anterior to the left and dorsal to the top. Numbers in top right corner indicate the number of embryos exhibiting the phenotype pictured.*



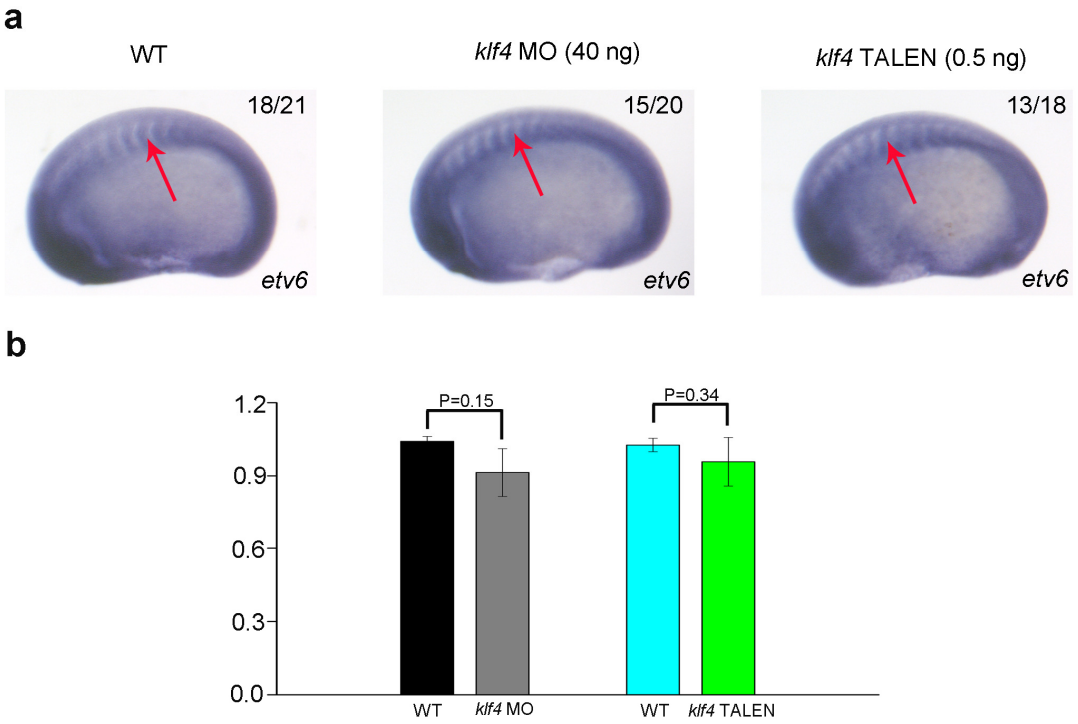
Supplementary Figure 4. WISH showing the expression pattern of *foxo3* during early *Xenopus* development. *Foxo3 mRNA is not detected before stage 22 of development. At stage 22, its expression is detected in the notochord (arrowhead). Expression in the somites is detected at stage 28, i.e. after the establishment of definitive hemangioblast in the lateral plate mesoderm. Arrows in stage 28 and 32 embryos indicate expression in the somites. Stage 13-20 embryos are shown in anterior view with dorsal to the top. Stage 22-32 embryos are shown in lateral view with anterior to the left and dorsal to the top.*



Supplementary Figure 5. WISH showing that *klf4* is transiently expressed in the somites of *Xenopus* embryos. *Klf4 mRNA is not detected before stage 22 of development. At stage 22, its expression is detected in the somites (red arrow) but this expression is extinguished by stage 26 (blue arrowhead indicates expression in the notochord). Yellow arrowhead in stage 32 embryo indicates expression in the head. Stage 13-20 embryos are shown in anterior view with dorsal to the top. Stage 22-32 embryos are shown in lateral view with anterior to the left and dorsal to the top.*



Supplementary Figure 6. *Klf4* is required for the emergence of HSC. *WISH demonstrating that the establishment of definitive hemangioblasts in the lateral plate mesoderm, as indicated by tal1 expression (arrows in stage 26 and 28 embryos), and hemogenic endothelium in the ventral wall of the dorsal aorta, as indicated by runx1 expression (arrows in stage 39 embryos), is impaired in klf4-deficient embryos. Arrowheads in stage 26 and 28 embryos indicate expression of tal1 in neurons, which is unaffected by klf4 depletion. Embryos are shown in lateral view with anterior to the left and dorsal to the top. Numbers in top right corner indicate the number of embryos exhibiting the phenotype pictured.*



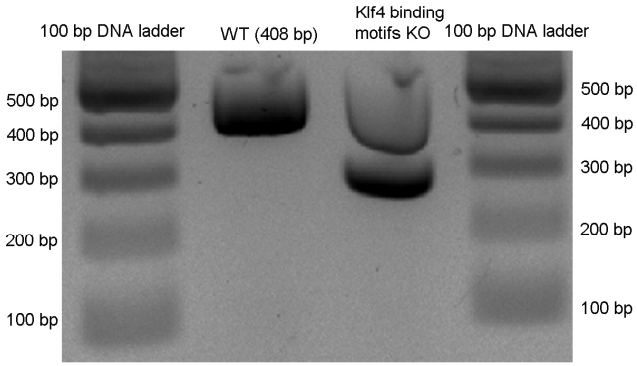
Supplementary Figure 7. *Etv6* expression in the somites is not affected in *klf4*-deficient embryos. *(**a**) WISH showing etv6 expression in the somites (arrows) of stage 22 WT, klf4 MO- and klf4 TALEN-injected embryos. Embryos are shown in lateral view with anterior to the left and dorsal to the top. Numbers in top right corner indicate the number of embryos exhibiting the phenotype pictured. (**b**) RT-qPCR confirming that similar levels of etv6 mRNA are expressed in the somites of WT and klf4-deficient embryos at stage 22. Expression was normalized to odc1. Error bars represent SEM of three biological replicates.*

a

ATTATCCAAAAGTTACACGCACACCGAAGCAATTCTCTTTTAAGATTTATTAGAAAAATAATTAA
TGAAACATCACATCGACGATTCGATTCCTGGTTTAAGCCTTTCTCAAGATGCTTCATCTCTATA
GAGAAGGCAGGCTGCAGCCTCAGTGTCACATAACTAGGGGCGGCGGCGAGGGAGTGGCTTC
AGTGAGGGCGGGGCTTTGCTCAATTGGCTAGCCTGTGAGCAGAACCGGAGCTGACACAGAC
AGAAGAACGCAGTGTAAGAAGAGAGACCCGGGAACAGGGAAGGGCTGAATTGCCGTTGTATA
GGACGGTACAAGGCTACAGGGCACAGCAGGATTATTCACTCACGGTTGGCATATAGAGCAGG
CACTGAGTGCTGGAGATCGGGACCGGGGCATTTACGCGCGATGCATTGAGAGTTGGCGGAA
CTTTCCTGGGATCTTGTTACATTGTATCCGTGTCTGTTACGTTGTATCAACCAGTGGAGCCGTA
GGTTTTACCCAAAAGGATTATGTGGCCGCTGCTTCCTGCTCTGGGGGCTGAGTGAGGGCCCG
TCCAATCGTTGCTCATTTACCTACTGGGGGAGCCGGGACTTCTCCCTAATCTTGGGTTCCCCC
CACTCCCTGAGCCCCTCACCGGAAAATCATG

TSS

b



c

mutation #1: GAGCAGAACCG 11 bp deletion
mutation #2: TGAGCAGAACC 11 bp deletion
mutation #3: AGCCTGTGAGCAGAACCGG 19 bp deletion
mutation #4: AAGCCTTTCTCAAGATGCTTCATCTCTATAGAGAAGGCAGGCTGCAGCCTCAGT
GTCACATAACTAGGGGCGGCGGCGAGGGAGTGGCTTCAGTGAGGGCGGGGC
TTTGCTCAATTGGCTAGCCTGTGAGCAGAAC 136 bp deletion

Supplementary Figure 8. Klf4 binding motifs in the *vegfa* promoter region were deleted using TALENs. *(**a**) DNA sequence for the X. laevis vegfa promoter (-653 bp ~ +3 bp) containing the Etv6 ChIP peak. The Etv6-peak sequence is indicated in red; Klf4 binding motifs under Etv6-peak are indicated in blue; the transcription start site is indicated in green; the TALENs designed for deletion of the Klf4 binding motifs are indicated in yellow and purple, respectively. (**b**) TALEN activity generated a range of deletions in the vegfa promoter. Genomic DNA was obtained from 20 WT and TALEN-injected stage 22 somites and PCR-amplified. The smaller product detected from TALEN-injected somites when compared to WT somites (~270 bp versus ~400 bp) represents the majority of the mutated sequences, as shown in (**c**). (**c**) Sequences showing the range of mutations caused by TALEN injection. The PCR products obtained in (**b**) were cloned and Sanger sequenced. TALENs caused mutations in*

100% of the clones sequenced (10/10). Mutation #1 was detected in two clones, mutation #2 in two clones, mutation #3 in one clone, and mutation #4 in five clones (50%).

Figure 4e top panel

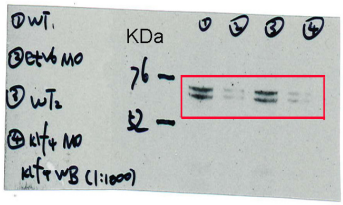
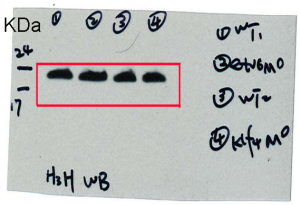
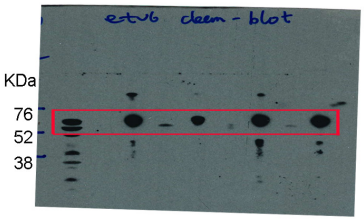


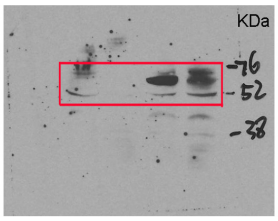
Figure 4e bottom panel



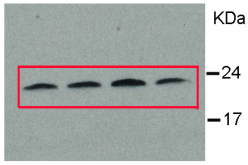
Supplementary Figure 1c



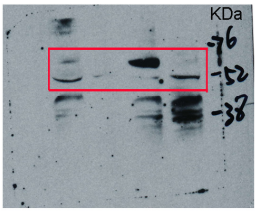
Supplementary Figure 1b, first panel, left side



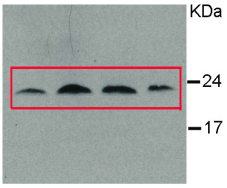
Supplementary Figure 1b, second panel, left side



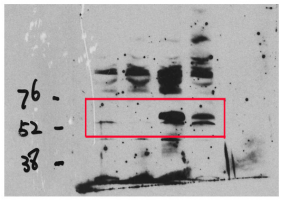
Supplementary Figure 1b, third panel, left side



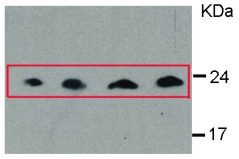
Supplementary Figure 1b, bottom panel, right side



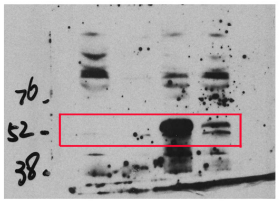
Supplementary Figure 1b, first panel, right side



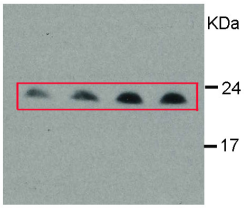
Supplementary Figure 1b, second panel, right side



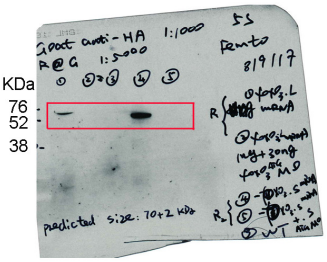
Supplementary Figure 1b, third panel, right side



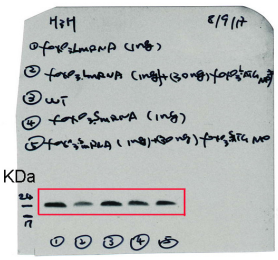
Supplementary Figure 1b, bottom panel, right side



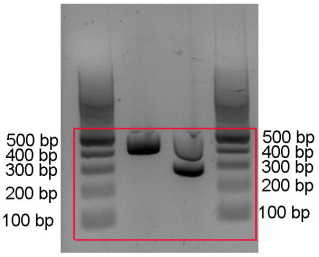
Supplementary Figure 3b top panel



Supplementary Figure 3b bottom panel



Supplementary Figure 8b



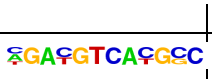
Supplementary Figure 9. Uncropped western blots and agarose gel electrophoresis raw pictures used in the main and Supplementary Figures.

Supplementary Table 2. *De novo* motif analysis on the Etv6 ChIP-seq peaks located in the TSS region.

Rank	Motif	P-value	% of Targets	% of Background	Best Match
1		1e-922	66.01%	10.76%	Klf/Sp/Homer(0.90)
2		1e-334	30.22%	5.28%	NFY(CCAAT)/Promoter/Homer(0.94)
3		1e-106	21.32%	7.31%	MA0443.1_btd/Jaspar(0.690)
4		1e-80	17.26%	6.11%	bZIP_CREB /Jaspar(0.93)
5		1e-74	62.36%	43.87%	MA0498.1_Meis1/Jaspar(0.907)
6		1e-62	15.29%	15.29%	MA0528.1_ZNF263/Jaspar(0.724)
7		1e-47	31.16%	18.79%	MA0319.1_HSF1/Jaspar(0.762)
8		1e-45	15.87%	7.31%	POL010.1_DCE_S_III/Jaspar(0.761)
9		1e-39	17.79%	9.16%	PB0122.1_Foxk1_2/Jaspar(0.818)
10		1e-37	3.53%	0.59%	AtMYB15(MYB)/Arabidopsis thaliana/AthaMap(0.676)
11		1e-32	3.49%	0.68%	GFY(?)/Promoter/Homer(0.928)
12		1e-31	6.93%	2.45%	STB5/STB5_YPD/Yeast(0.749)
13		1e-31	10.74%	4.87%	PB0056.1_Rfxdc2_1/Jaspar(0.911)
14		1e-28	8.94%	3.87%	MA0127.1_PEND/Jaspar(0.816)
15		1e-27	5.78%	1.99%	YY1(Zf)/Promoter/Homer(0.760)
16		1e-26	11.28%	5.57%	MA0374.1_RSC3/Jaspar(0.698)
17		1e-18	2.34%	0.54%	Stat3(Stat) /Homer(0.898)
18		1e-17	3.90%	1.40%	SeqBias: polyA-repeat(0.888)
19		1e-15	1.15%	0.14%	nub /fly(0.692)
20		1e-14	0.62%	0.03%	MA0326.1_MAC1/Jaspar(0.659)
21		1e-9	0.37%	0.02%	MA0537.1_BLMP-1/Jaspar(0.607)
22		1e-5	0.16%	0.01%	MA0362.1_RDS2/Jaspar(0.645)

Supplementary Table 6. *De novo* motif analysis on Etv6 ChIP-seq peaks associated with the TSS region of genes activated or repressed by Etv6.

Motifs overrepresented in the Etv6 peaks associated with the TSS region of genes activated by Etv6

Rank	Motif	P-value	% of Targets	% of Background	Best Match
1		*1e-65*	*68.97%*	*15.33%*	*Klf/Sp/Homer(0.87)*
2		*1e-24*	*21.67%*	*2.97%*	*NFY(CCAAT)/Promoter/Homer(0.93)*
3		*1e-12*	*16.75%*	*3.70%*	*hkb/fly(0.850)*
4		*1e-12*	*2.46%*	*0.01%*	*CREB/Jaspar (0.74)*
5		*1e-12*	*2.46%*	*0.01%*	*Hox/Jaspar(0.68)*
6		*1e-12*	*4.43%*	*0.10%*	*Smad/Homer (0.62)*

Motifs overrepresented in the Etv6 peaks associated with the TSS region of genes repressed by Etv6

Rank	Motif	P-value	% of Targets	% of Background	Best Match
1		*1e-123*	*66.37%*	*11.78%*	*Klf/Sp/Homer(0.89)*
2		*1e-46*	*23.98%*	*3.15%*	*NFY(CCAAT)/Promoter/Homer(0.91)*
3		*1e-26*	*23.68%*	*5.87%*	*MA0260.1_che-1/Jaspar(0.668)*
4		*1e-23*	*19.59%*	*4.49%*	*Klf/Sp/Homer(0.804)*
5		*1e-21*	*12.57%*	*1.9%*	*PB0167.1_Sox13_2/Jaspar (0.67)*
6		*1e-17*	*19.30%*	*5.64%*	*NPAS2(bHLH) /Homer (0.84)*
7		*1e-16*	*8.77%*	*1.17%*	*bZIP_CREB /Jaspar (0.87)*
8		*1e-16*	*20.18%*	*6.55%*	*MA0498.1_Meis/Jaspar (0.76)*
9		*1e-14*	*16.37%*	*4.98%*	*MA0374.1_RSC3/Jaspar (0.72)*
10		*1e-13*	*17.84%*	*5.88%*	*Tcf3(HMG) /Homer (0.69)*
11		*1e-13*	*6.14%*	*0.65%*	*ELF1(ETS) /Homer (0.65)*
12		*1e-12*	*3.80%*	*0.18%*	*ETS1(ETS)/Promoter/Homer (0.58)*

Supplementary Table 8. Probes used for *in situ* hybridization. Probes were designed to target both L and S genes unless otherwise indicated.

Gene Name	Accession Number	Restriction Enzyme	RNA Polymerase	Reference
Meox2	*L20432*	*SacII*	*SP6*	*This Report*
Crim1	*EU882845*	*ApaI*	*SP6*	*This Report*
Sox18	*BC072123*	*SalI or SmaI*	*T7*	*This Report*
Vegfa.L		*BamHI*	*T7*	*Cleaver et al., 1997*[1]
Foxo3	*AJ783964*	*SalI*	*T7*	*This Report*
Klf4	*BC055956*	*SalI or SacI*	*T7*	*This Report*
Runx1		*SalI*	*T7*	*Tracey et al., 1998*[2]
Tal1		*XhoI*	*SP6*	*Ciau-Uitz et al., 2010*[3]
Etv6	*EU760352*	*NotI or SacI*	*T7*	*Ciau-Uitz et al., 2010*[3]

Supplementary Table 9. MO sequences.

Gene Name	MO sequence	References
Etv6	*GTAACACTGGGCTGAAGACATTTCC*	*Ciau-Uitz et al., 2010*[3]
Foxo3.L	*TGCTTCTGCCATGCTGCGAGAAG*	*This report*
Foxo3.S	*GGCTTCTGCCATGGTGTGTGAAC*	*This report*
Klf4	*CTGCCTCATTAATCTGGGAGGGTCA*	*This report*

Supplementary Table 10. Primer sequences for cloning.

Gene		Primer sequences	References
Etv6.S mRNA	*Forward*	*actctagagatatcgagctcgaggtttctgtttcccagagg*	*This report*
	Reverse	*acatcgtatggatatcgggatccgcattcatcctcctgatac*	
Foxo3.L mRNA	*Forward*	*cgactctagacttctcgcagcatggcagaagcactgcc*	*This report*
	Reverse	*agacgtcgacgcctggcacccagctc*	
Foxo3.S mRNA	*Forward*	*cgactctagagttcacacaccatggcagaagccgtgcc*	*This report*
	Reverse	*agacgtcgacgcctggcacccagctc*	
Klf4 talen test	*Forward*	*tagtcactggctttggctgc*	*This report*
	Reverse	*gcaggatactcacattgcct*	
Klf4 binding motif KO talen test	*Forward*	*cgcacaccgaagcaattctc*	*This report*
	Reverse	*ctcaatgcatcgcgcgtaaa*	

Supplementary Table 11. Primer sequences for RT-qPCR. Primers were designed to target both L and S genes.

Gene Name		Primer sequences	References
Foxo3	*Forward*	*tccgctccgtgccctactt*	*This report*
	Reverse	*cgtcttcttggtgcctttcc*	

Klf4	*Forward*	*tgcggaaagacctataccaag*	*This report*
	Reverse	*ttcggtagtggcgggtcag*	
Etv6	*Forward*	*gccgcctactctgggattac*	*This report*
	Reverse	*gcgcagagcccgtgacatc*	
Odc	*Forward*	*ggcgacattgtgaaaaagca*	*This report*
	Reverse	*cgggtttgcatagataatcc*	

Supplementary Table 12. Primer sequences for ChIP-qPCR. Primers were designed to target both L and S genes unless otherwise indicated.

Gene Name		Primer sequences	References
Foxo3	*Forward*	*agcctgtcaccctgcgcgcc*	*This report*
peak	*Reverse*	*ccagggctcctccccttc*	
Foxo3	*Forward*	*tgattctcccctgtcccctatg*	*This report*
exon2	*Reverse*	*tgctgtgaagaagtgagagaaatg*	
Klf4	*Forward*	*gcggggcttgtctccacgtgat*	*This report*
peak	*Reverse*	*gccagggcaccggaac*	
Klf4	*Forward*	*agcatctccagacagccaaagt*	*This report*
intron2	*Reverse*	*gtaggtggtgcaatatctcttaa*	

Vegfa.L peak	*Forward*	*tgcagcctcagtgtcacataac*	*This report*
	Reverse	*cccgggtctctcttcttacact*	
Vegfa.L intron1	*Forward*	*ggccgacagttaagcgattca*	*This report*
	Reverse	*cccccaccccaggaaatgat*	

Supplementary references

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