Developmental Cell, Volume *20*

Supplemental Information

Genome-Wide Analysis of Simultaneous GATA1/2, RUNX1, FLI1, and SCL Binding in Megakaryocytes Identifies Hematopoietic Regulators

Marloes R. Tijssen, Ana Cvejic, Anagha Joshi, Rebecca L. Hannah, Rita Ferreira, Ariel Forrai, Dana C. Bellissimo, S. Helen Oram, Peter A. Smethurst, Nicola K. Wilson, Xiaonan Wang, Katrin Ottersbach, Derek L. Stemple, Anthony R. Green, Willem H. Ouwehand, and Berthold Göttgens

Supplemental Experimental Procedures

Histone modification ChIP-Sequencing

Megakaryocytes were cultured as described for TF ChIPs. ChIP assays were performed using 33.4 million cells using anti-trimethyl-Histone H3 (Lys4; clone MC315, Millipore), Anti-acetyl-Histone H3 (Millipore), and Anti-trimethyl-Histone H3 (Lys27, Millipore). Patterns for H3K27me3, H3K4me3 and H3K9ac around the HOXA loci show active genes on the right hand side with repressive marks on the left. The homo sapiens promoter database (Cold Spring Harbor Laboratory) was used to depict the histone marks on all promoters.

Validation of the ChIP material by Real-time PCR

Enrichment for the GATA1 ChIP was determined with the following primers: SCL –16kb forward; 5'-TGTAAGTGGCAGTGGTGATGTCT-3', reverse; 5'-AACAGAACACACTTGAAGTGGCA-3'. SCL +40kb forward; 5'-TGGGAATGAGCGATAAGGAT-3', reverse; 5'-TTACAGCCCTTCACCCTCAC-3'. Enrichment for all other ChIPs was quantified with the following primers: RUNX1 +23kb forward; 5'-CCTGTGGTTTTCTCGCTCTC-3', reverse; 5'-ATGCTGACAGCCTCAGATGG-3'. RUNX1 +31kb forward; 5'-AAAGCCACAAAAAGAGATCTGG-3', reverse; 5'-AGCACCTGCCAGAAGACATC-3'.

Peak calling

Peak calling programs and parameters used for the different TFs were as follows. GATA1: overlap of all three programs with Findpeaks – FDR <0.01 called, MACS – mfold=16 --tsize=35 --bw=100 --pvalue=1e-9 --gsize=2200000000 –nolambda, Peakseq – p=0.01. GATA2: MACS – mfold=16 --tsize=35 --bw=100 --pvalue=1e-9 --gsize=2200000000 –nolambda. RUNX1: overlap of all three programs with Findpeaks – FDR 0 called, MACS – mfold=16 --tsize=35 --bw=100 --pvalue=1e-15 --gsize=2200000000 –nolambda, Peakseq – p=1e-7. FLI1: Findpeaks – default setting for ChIPseq analysis, peaks with FDR < 0.05 called. SCL: Peakseq – standard settings (p=0.05). When the overlap of three algorithms was used, MACS peak coordinates were used as reference for further analysis.

Generation of *Pdzk1ip1* targeted ES cell lines

To inactivate *Pdzk1ip1* a targeting vector was generated by bacterial recombineering which was designed to delete 131bp of exon 1 of *Pdzk1ip1* (containing the ATG site) and insert a LacZ reporter gene and a LoxP-PGK-Neo-LoxP cassette (Liu et al., 2003). The targeting vector was used for homologous recombination in AB2.2 ES cells. Targeted cells were transiently transfected with PGK-Cre plasmid for deletion of the Neo cassette.

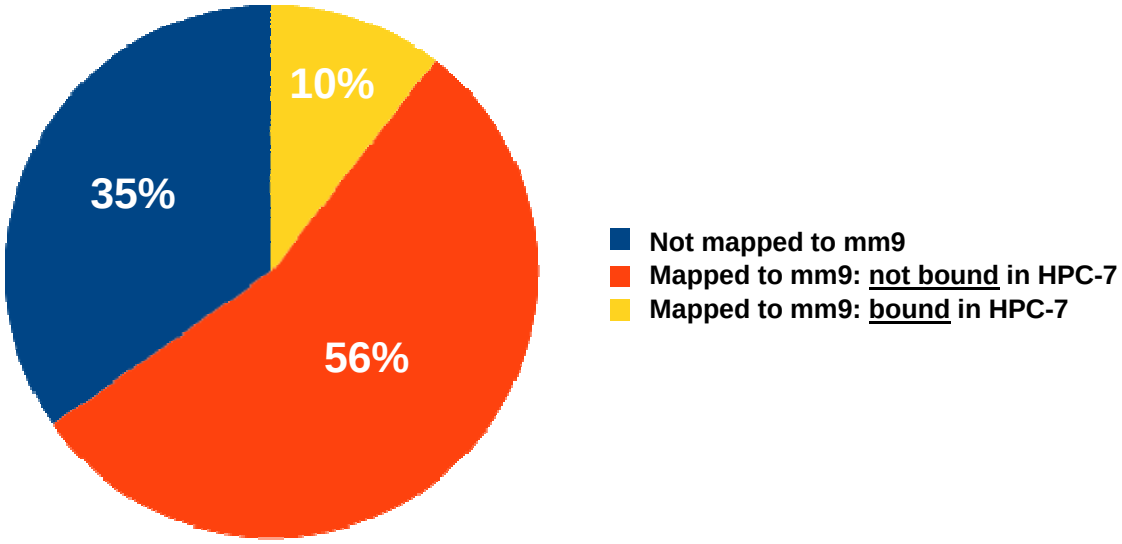
Supplemental References

Liu,P., Jenkins,N.A., and Copeland,N.G. (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res. 13, 476-484.

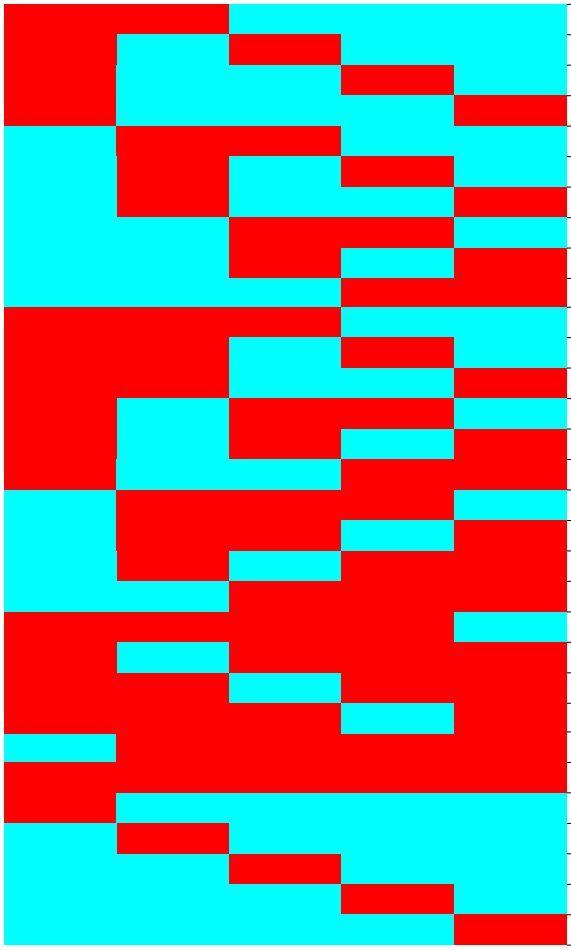
Matsumura,G. and Sasaki,K. (1989). Megakaryocytes in the yolk sac, liver and bone marrow of the mouse: a cytometrical analysis by semithin light microscopy. J. Anat. 167, 181-187.

Ye,T., Krebs,A.R., Choukrallah,M.A., Keime,C., Plewniak,F., Davidson,I., and Tora,L. (2010). seqMINER: an integrated ChIP-seq data interpretation platform. Nucleic Acids Res.

A



B



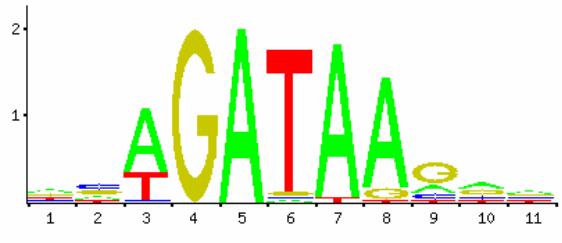
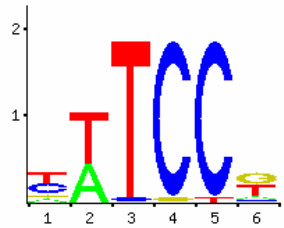
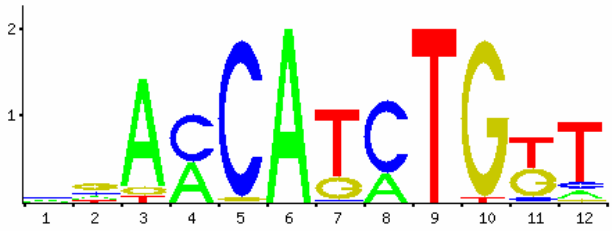
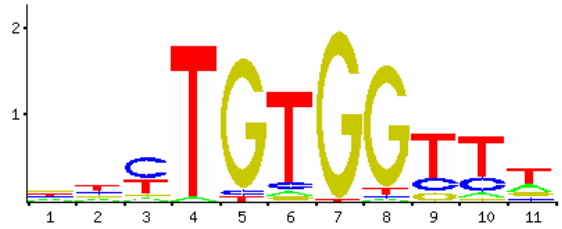
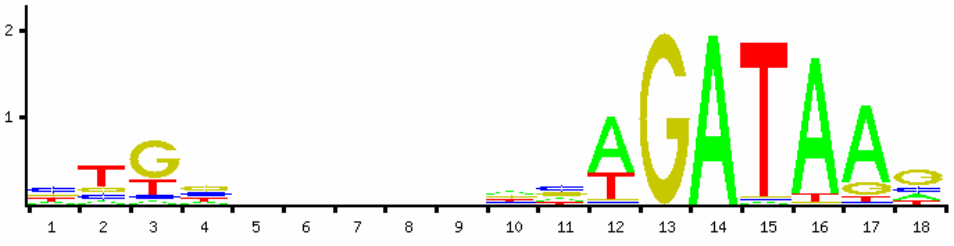
Peaks	Mapped to mm9 (%)	Mapped to mm9: not bound in HPC-7	Mapped to mm9: bound in HPC-7 (%)
224	17 (8)	17	0 (0)
419	216 (52)	208	8 (4)
241	157 (65)	147	10 (6)
371	249 (67)	222	27 (11)
44	33 (75)	33	0 (0)
324	60 (19)	60	0 (0)
18	7 (39)	7	0 (0)
1586	1336 (84)	1253	83 (6)
83	54 (65)	49	5 (9)
335	234 (70)	206	28 (12)
66	55 (83)	46	9 (16)
72	28 (39)	27	1 (4)
64	54 (84)	41	13 (24)
221	155 (70)	145	10 (6)
140	100 (71)	86	14 (14)
264	188 (71)	156	32 (17)
34	19 (56)	18	1 (5)
2	2 (100)	2	0 (0)
26	8 (31)	8	0 (0)
133	105 (79)	92	13 (12)
104	32 (31)	26	6 (19)
235	174 (74)	174	0 (0)
54	38 (70)	30	8 (21)
46	33 (72)	25	8 (24)
2	1 (50)	1	0 (0)
144	103 (72)	80	23 (22)
1821	1069 (59)	997	72 (7)
858	487 (57)	462	25 (5)
3644	2716 (75)	2594	122 (4)
4539	3095 (68)	2340	755 (24)
1149	707 (62)	665	42 (6)

Tijssen et al Supplemental Figure 1

Figure S1. Comparison of the Megakaryocyte Data with Hematopoietic Progenitor Data, related to Figure 2

(A) All peaks for the 5 TFs were converted to the mouse genome (mm9). For the mapped peaks it was next determined whether these regions were also bound by the same transcription factor in mouse HPC-7 cells. Depicted are the percentage of the total number of peaks in our human megakaryocyte data that could be mapped to the mouse genome and what percentage of these are bound or not bound by the same TFs in HPC-7. Human megakaryocyte GATA1 and GATA2 peaks were compared with HPC-7 GATA2 data as no GATA1 data is available.

(B) The same analysis as for (A) was done for the 31 binding patterns of the 5 TFs.

Gata1	
ETS1	
TAL1::TCF3	
Runx1	
Tal1::Gata1	

Tijssen et al Supplemental Figure 2

Figure S2. Consensus Binding Sites from the JASPAR Database for the TFs Examined, related to Figure 3

(A) GATA1

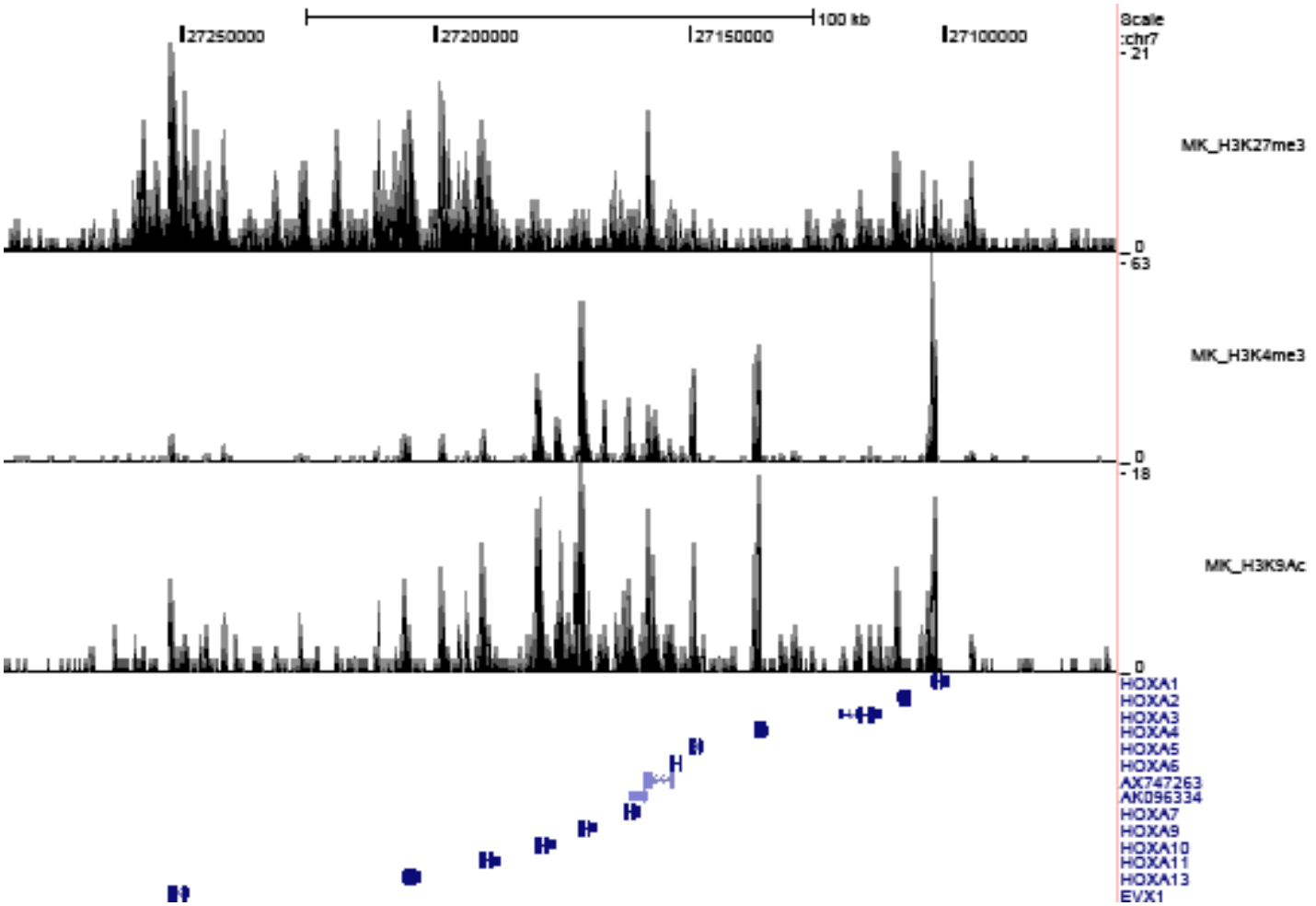
(B) RUNX1

(C) ETS1 (FLI1)

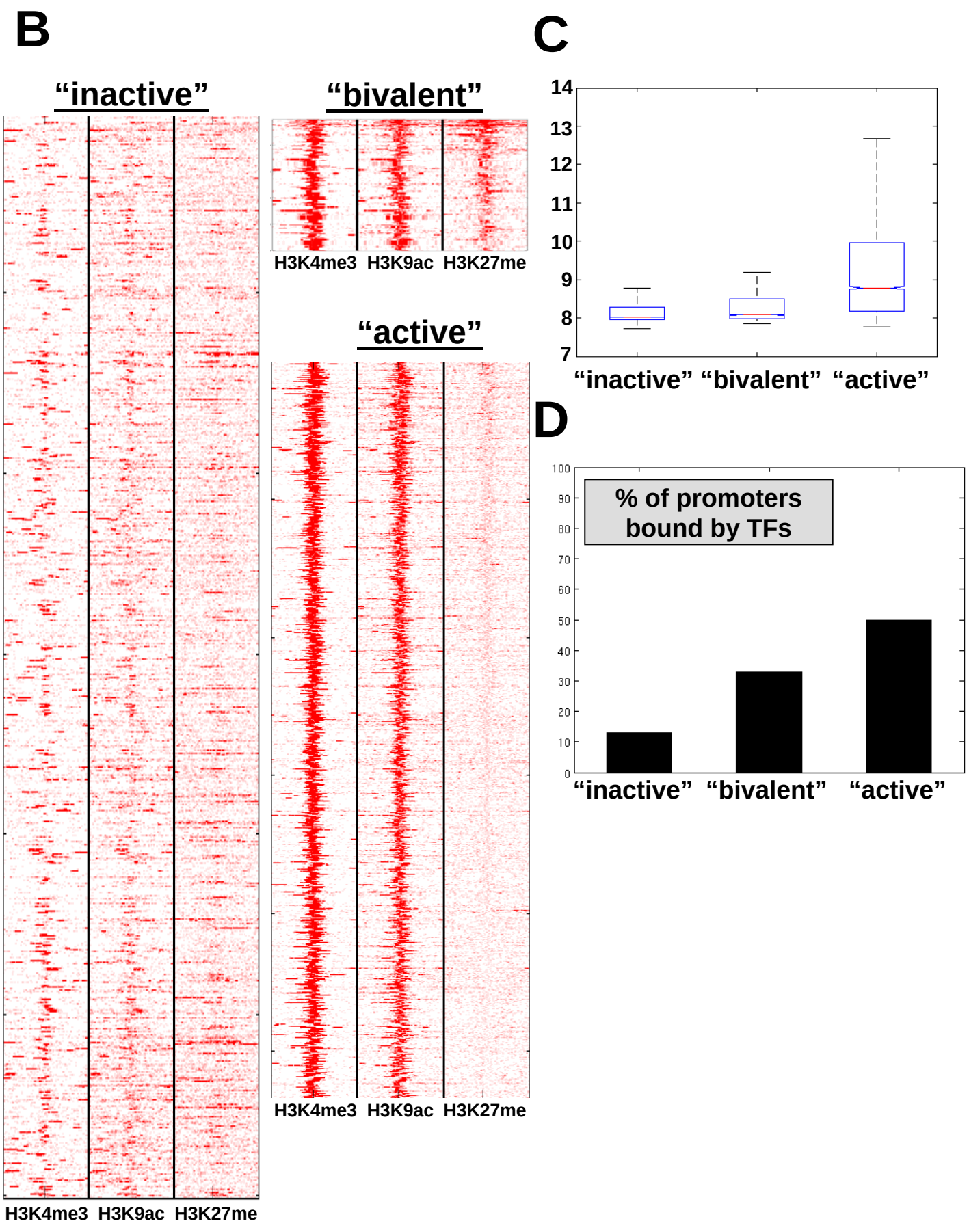
(D) E-box (SCL)

(E) SCL::GATA1

A



Tijssen et al Supplemental Figure 3



Tijssen et al Supplemental Figure 3

Figure S3. Histone Modifications in the Human Megakaryocyte Genome, related to Figure 4

Megakaryocytes were cultured as before (data not shown).

(A) Patterns for H3K27me3, H3K4me3 and H3K9ac around the HOXA loci show active genes on the right hand side with repressive marks on the left.

(B) A list of all human promoters was obtained from the mammalian promoter database. ChIP-Seq enrichment across 10 kb regions centered on all promoter sequences was determined, partitioned using Seqminer (Ye et al., 2010) and displayed as “heatmaps”, which allowed us to categorize promoters into “active”, “bivalent” and “inactive” in human megakaryocytes.

(C) Analysis of gene expression levels in megakaryocytes (Haematlas (Watkins et al., 2009)) shows the highest level of expression for genes with “active” promoters (log2 transformed gene expression scores are displayed as Boxplots).

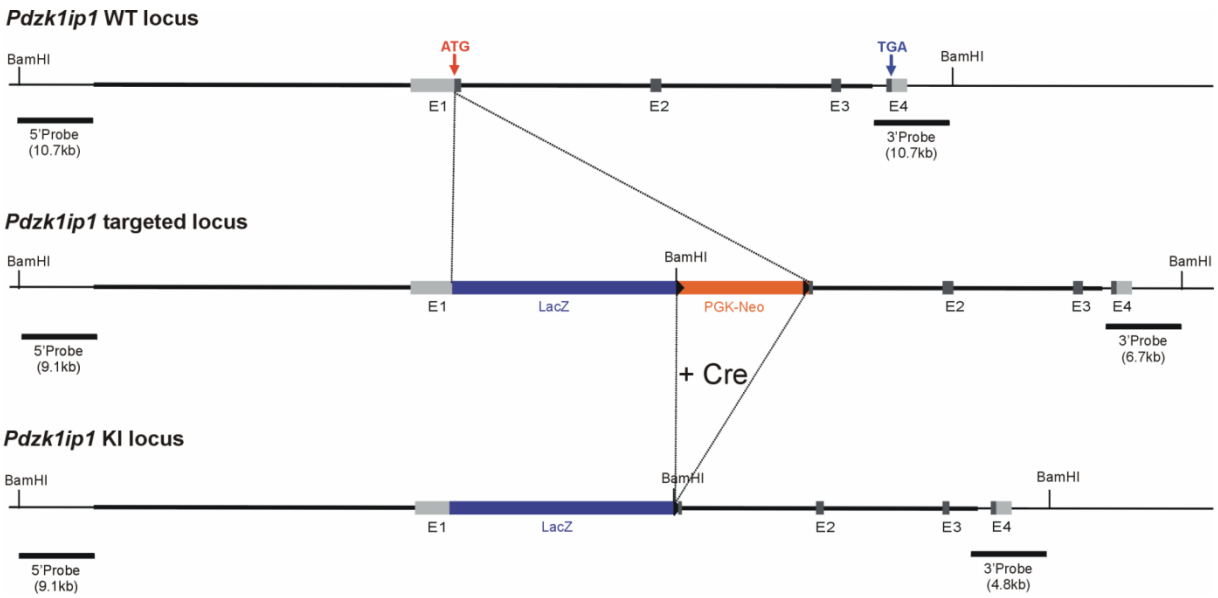
(D) Binding of GATA1, GATA2, RUNX1, FLI1 and SCL is most prevalent for promoters “active” in MKs, with intermediate levels seen for “bivalent” promoters.

Tijssen et al Supplemental Figure 4

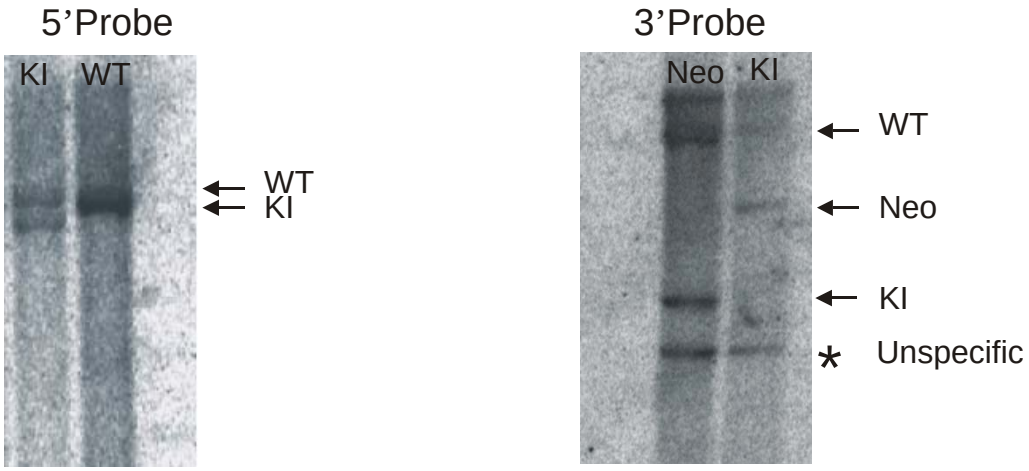
Figure S4. Morpholino Screen in *cd41* Transgenic Zebrafish Embryos, related to Figure 5

Representative pictures at 72 hpf of 5 control transgenic *Tg(cd41:EGFP)* zebrafish embryos or injected with MOs. The GFP positive cells represent the number of presumed hematopoietic stem cells (*cd41low*) and thrombocytes (*cd41high*). For *march2* (n=44), *max* (n=63), *smox* (n=60), *pttg1lp* (n=50), *emilin1* (n=65) and *sufu* (n=65), a severe decrease of *cd41* positive cells was observed. *Ncor2* (n=53) depletion resulted in a mild phenotype, and *pdzk1ip1l* (n=49) and *nfatc1* (n=60) MO injected embryos showed no phenotype.

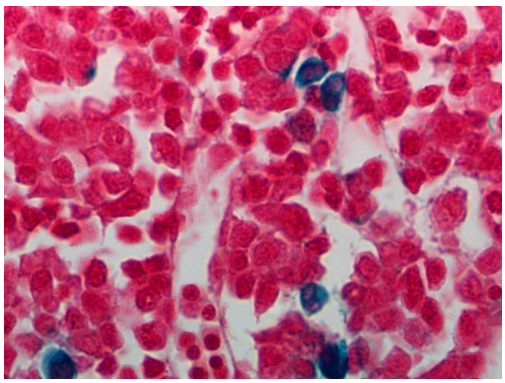
A



B



C



Tijssen et al Supplemental Figure 5

Figure S5. Disruption of the *Pdzk1ip1* locus by homologous recombination, related to Figure 6

(A) The structure of the wild-type allele of the murine *Pdzk1ip1* locus is illustrated with exons as grey boxes (untranslated regions in light grey and translated regions in dark grey). In the targeted allele, 131bp of *Pdzk1ip1* exon 1 (containing the ATG site) was deleted and a cassette containing the LacZ gene and a LoxP-PGK-Neo-LoxP cassete were introduced by recombineering. The Knock-in allele was generated by deletion of the LoxP-PGK-Neo-LoxP cassette using Cre recombinase. BamHI sites and probes used for Southern blot analysis are shown. The predicted band size for each probe is indicated.

(B) Representative Southern blots obtained with 5'probe (left panel) and 3' probe (right panel). WT- AB2.2 ES cells; Neo-*Pdzk1ip1* targeted ES cells; KI- Knock-in ES cells (Neo deleted); * unspecific band.

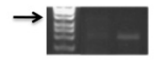
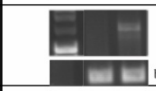
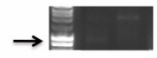
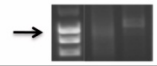
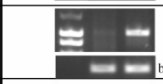
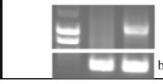
(C) Fetal liver cells of day 12.5 transgenic mouse embryos with the +19 enhancer fused to the *Pdzk1ip1* promoter. The lacZ positive cells in this picture have double lobed nuclei characteristic for megakaryocytes at this stage of embryogenesis (Matsumura and Sasaki, 1989).

Transcriptional Control	Signal Transduction	Other		No GO Annotation	
CBFA2T3	BMP2K	ABCC1	OAT	ANKRD33B	HTT
CDK5RAP2	CD84	ACSF3	PDE3A	ASB8	KIAA0125
CDK9	CDKL1	AP1M1	PLA2G6	BCL8	LAPTM5
ENO1	CHN2	ASS1	RAB7A	C11orf24	LOC100272217
G3BP2	CXCL2	BCL2L1	RCN1	C12orf68	LOC284805
HFM1	DIAPH1	C6orf103	RDH16	C14orf70	MDM1
ILK	DUSP28	CACNA1C	RHBDL3	C1orf150	MIR557
KLF2	FERMT3	CCND3	RNPEPL1	C1orf212	MIR663
LYL1	GNA12	CDH23	RPN1	C1orf55	MIR760
MAX	GNB5	COPZ1	RSPO2	C21orf121	NCRNA00164
MEX3B	GP1BB	CTNND2	SEPT5	C5orf15	PDZK1IP1
NCOR2	GPR182	DAB2IP	SEPT9	C5orf56	PRR5
NEUROG1	GRIK4	DLK1	SLC39A3	C8orf75	PTTG1IP
NFATC1	IRS2	DNAJB2	SLC39A4	CCDC19	RSPH1
NFE2	ITGA2B	EFHC2	SLCO5A1	CEP68	SAMD14
PMF1	LRP5	EMILIN1	SMOX	CUX1	SLC43A3
PPBPL2	MACC1	EPB49	ST8SIA6	CXADRP2	TIFAB
PPIE	P2RX1	EVL	STOM	DLG2	TMEM129
RERE	PLXNA4	FNTB	SYNRG	FAM73B	TMEM64
RUNX1	PTGIR	GCNT1	TAGLN2	GJA4	TMEM88
RXRA	RAMP1	GGTA1	TNFAIP8	GNG8	TSPAN33
SLBP	RGS9	IMPDH2	TPM4	HAUS2	UBASH3A
SUFU	SEMA4D	LIMS1	UBE2H	HCCA2	
TAL1	SH2D3C	MARCH2	UCP3		
	TNIK	MICAL3	UMODL1		
	TREML2	MYL4	ZC3HC1		
	USP20	MYRIP	ZMYND8		
	XCL2				

Tijssen et al Supplemental Table 4

Table S4. Candidate Target Genes of GATA1, GATA2, RUNX1, FLI1 and SCL in Primary Human Megakaryocytes, related to Figure 5

151 candidate target genes of the 144 regions bound by all 5 TFs separated in GO-annotation groups.

Gene	Ensembl ID	Morpholino sequence 5'-3'	Primer Name	Sequence	Tm (C)	Product Size (bp)	gel
NCOR2	ENSDART00000001092	GTTATTCTGCGAGCACAGAAAATCA	NCOR2F1-2000	AACTCTGAGAAGCAGATGCGT	59.25	2000	
			NCOR2R1-2000	GGGGGTTCTGGAATATGGA	59.55		
MARCH2	ENSDART00000088419	GAGCCACTGTTGAAAAATGACAAGT	MARCH2F162-1595	TCTTTGTGGCTTGAGGTGTG	59.87	1434	
			MARCH2R162-1595	TGGCCCTGTAATACCCATGT	60.07		
SMOX	ENSDARG00000036967	TTTTACCTGTGGAGATAGGAAGGAA	SMOXF19-1981	CGGTTTACGCTGAAGACGAT	60.27	1970	
			SMOXR19-1981	CAGAAGGCACTTACAAAAATGG	58.81		
NFATC1	ENSDART00000052521	CGCATCTGTAAGGTACAATCACATT	NFATC1F105-2238	CGTTGTCATTTCTTGCGTTTT	60.16	2134	
			NFATC1R105-2238	TTTGTGGGCAGGTATGTGAA	59.96		
PTTG1IP	ENSDART00000058565	GCAGTCTGTGAAAAGGACACATTTA	PTTG1IPF9-1063	CCCAACTTCCAGCAGAAAAC	59.71	1037	
			PTTG1IPR9-1063	GGTGCACAAAACAAATCACAA	59.48		
EMILIN1	ENSDART00000035612	AGTCCGATACCTGTGGTGAGATATT	EMILIN1F323-1346	CCACTCTATTGGCGCTGATT	60.24	1024	
			EMILIN1R323-1346	CGACCTCCATTGCCATTACT	59.96		
PDZK1IP1I	ENSDART00000012604	CCGCAGTCTCTGAAAGAAACACAAA	PDZK1IP1IF50-1111	TGGGAAAACCAGTCTCTGTG	58.69	1062	
			PDZK1IP1IR50-1111	CCATTGAGACAATCCCCTTT	58.84		

Gene	Ensembl ID	Morpholino sequence 5'-3'
MAX	ENSDART00000044462	ATATCATCGTTGTCGCTCATTCTTC
SUFU	ENSDART00000079356	CGCTGCTAGGCCGCATCTCATCCAT

Tijssen et al Supplemental Table 5

Table S5. Morpholino Sequences for Targeted Genes and Primers Used to Verify Their Efficiency, related to Figure 5

mRNA levels of *ncor2, march2, smox*, *nfatc1*, *pttg1lp*, *emilin1* and *pdzk1ip1l* in MO-injected embryos (next to ladder) and control were measured by RT-PCR, from equal amounts of total RNA. Splice modifications are seen as a band shift, as indicated with the black arrows. Amplification of β-actin, was used as a control where MO injection resulted in mRNA degradation.

Gene ID	Gene name	Function	Homology	MO knock-down Thrombocytes	MO knock-down Erythrocytes
NCOR2	nuclear receptor co-repressor 2	Promotes chromatin condensation to mediate the transcriptional repression activity of certain nuclear receptors	45%	✓	✓
MARCH2	membrane-associated ring finger (C3HC4) 2	E3 ubiquitin-protein ligase involved in regulating endosomal trafficking	77%	✓	✓
SMOX	spermine oxidase	Polyamine oxidase involved in the regulation of the intracellular polyamine concentration	70%	✓	✓
NFATC1	nuclear factor of activated T-cells	Plays a role in the inducible expression of cytokine genes in T-cells. Also controls gene expression in embryonic cardiac cells.	52%	✗	✗
PTTG1IP	pituitary tumor-transforming 1 interacting protein	Binds pituitary tumor-transforming gene 1 protein (PTTG1) facilitating PTTG1's nuclear translocation and potentiating PTTG1's transcriptional activation of fibroblast growth	45%	✓	✓
EMILIN1	elastin microfibril interfacer 1	Extracellular matrix glycoprotein that associates with elastin fibres at the interface between elastin and microfibrils	33%	✓	✓
SUFU	suppressor of fused homolog	Negative regulator in the hedgehog signaling pathway	80%	✓	✓
MAX	MYC associated factor X	Transcriptional regulator that forms homodimers and heterodimers. The MYC-MAX complex is a transcriptional activator while the MAD-MAX complex is a transcriptional repressor	82%	✓	✓
PDZK1IP1	PDZ-domain containing protein 1 interacting protein 1	May play an important role in tumor biology	37%	✗	✓

Tijssen et al Supplemental Table 6

Table S6. Candidate Target Genes of GATA1, GATA2, RUNX1, FLI1, and SCL Selected for Knockdown in Zebrafish, related to Figure 5

A short description of the function of the genes and the percentage of homology between human and zebrafish is given. A summary of phenotypes of MO knock down in zebrafish is depicted in the last two columns.

control	*ncor2*	*march2*	*smox*	*nfatc1*	*pttg1ip*	*emilin1*	*sufu*	*max*	*pdzk1ip1l*
63	38	1	9	65	1	3	3	1	53
49	37	3	10	50	3	3	4	1	41
83	24	8	11	74	2	5	7	0	71
70	26	3	5	48	6	6	4	0	58
59	45	3	11	50	4	10	2	2	63
67	24	0	12	47	7	3	2	0	67
51	32	3	5	38	1	7	3	2	61
77	31	8	8	68	5	6	4	1	48
69	48	4	9	50	5	8	3	0	37
67	32	4	10	57	4	6	5	1	78
88	25	1	5	68	3	8	5	0	50
68	48	4	11	64	0	5	2	1	58
53	45	1	6	61	4	5	4	0	64
63	32	5	9	72	0	8	0	0	58
64	20	2	11	68	1	0	6	1	40
50	30	2	10	60	3	4	3	4	51
86	33	9	12	49	1	2	2	0	56
54	36	0	12	50	4	4	4	1	74
59	31	7	8	70	4	7	1	1	40
56	43	4	10	39	6	8	4	0	76
average/embryo	**64.8**	**34**	**3.6**	**9.2**	**57.4**	**3.2**	**5.4**	**3.4**	**0.8**	**57.2**

Tijssen et al Supplemental Table 7

Table S7. Number of *cd41*-Positive Cells in 20 Control Embryos and 20 Embryos Treated with Morpholinos for Each of the Candidate Target Genes, related to Figure 5