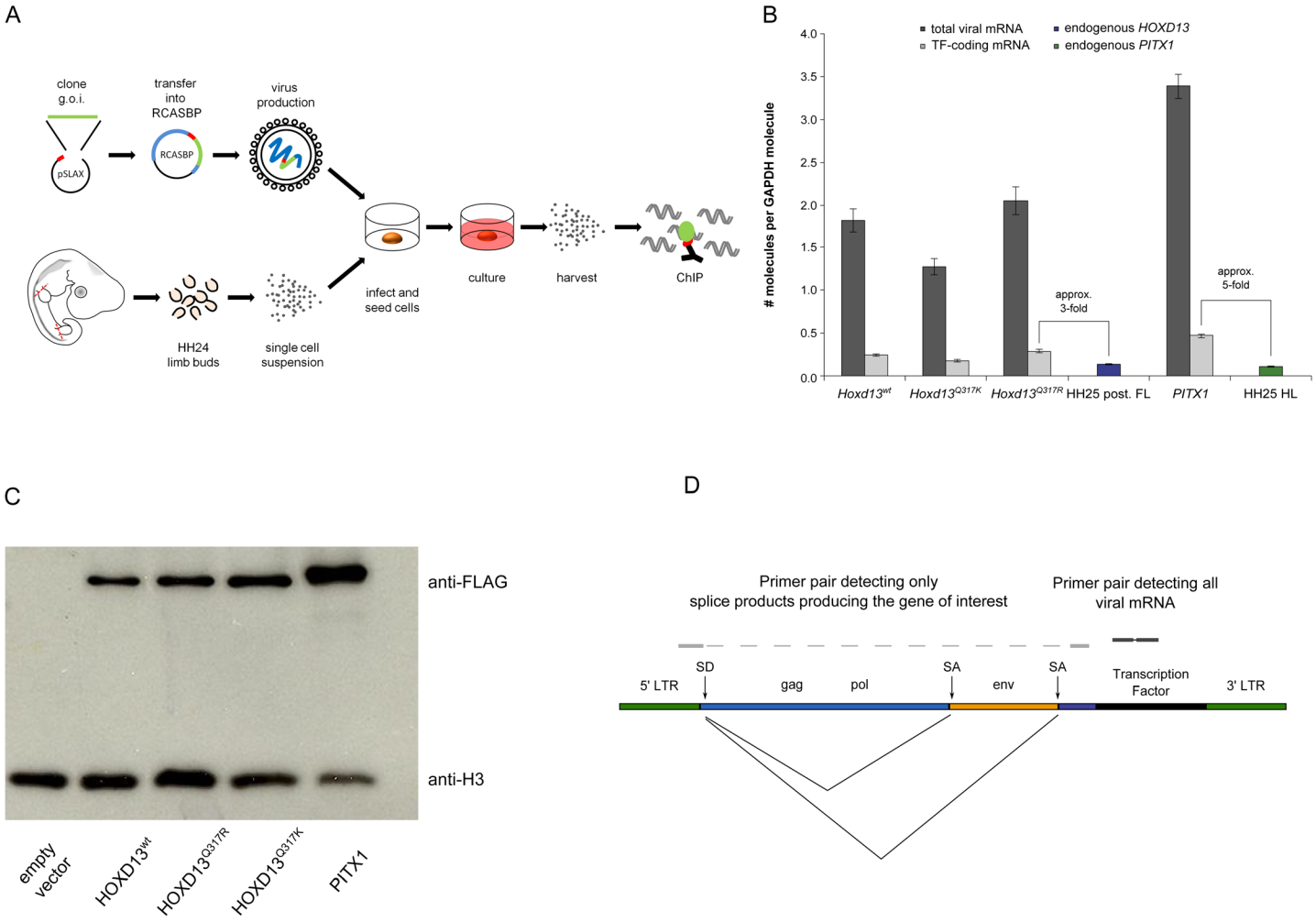


Supplemental Figure S1

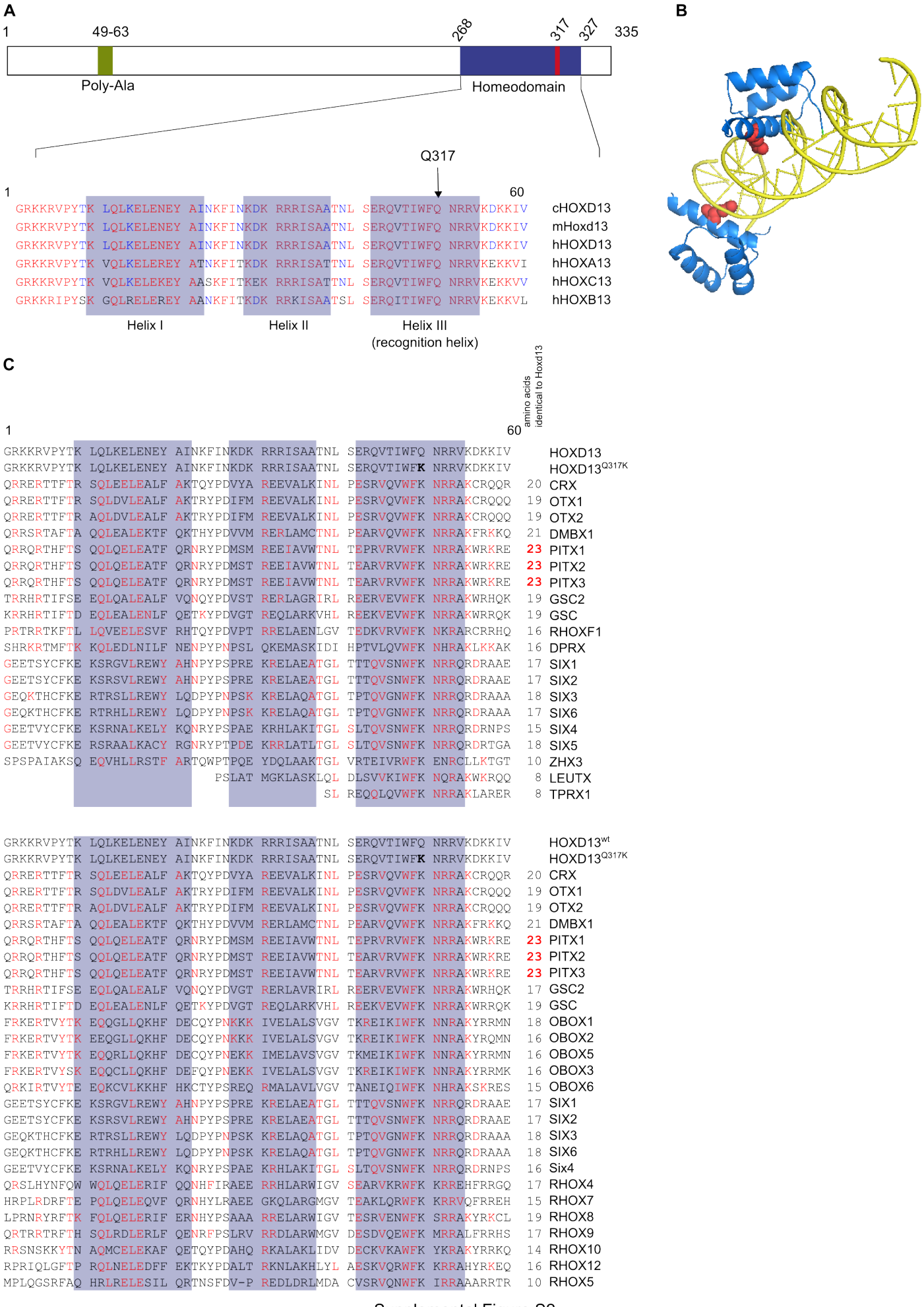
Supplemental Figure S1. Overview of the method and assessment of RCASBP-mediated overexpression

(A) *In vitro* investigation of genome wide TF binding sites using chick mesenchymal cells and RCASBP retrovirus. The coding sequence of a gene of interest (g.o.i.) is introduced into the pSLAX vector in frame with a triple FLAG tag sequence. This is transferred into an RCASBP virus using standard procedures. Single cell suspensions are isolated from the limb buds of chicken embryos at stage HH24 and are infected with the virus, leading to overexpression of the protein of interest. After the desired culture time, cells are harvested and ChIP-seq is performed using an antibody against the FLAG tag sequence.

(B) Only 15-20% of the viral mRNA is spliced into TF-coding transcript. RNA from *Hoxd13*^{wt}, *Hoxd13*^{Q317K}, *Hoxd13*^{Q317R}, or *PITX1* infected chMM-cultures was used for absolute quantification of either all viral RNA (dark gray bar) or specific splice products (light gray bar, see Supplemental Methods). The absolute transcript numbers were normalized to the number of *GAPDH* transcripts in the respective samples. The error bars indicate the S.D. from one representative example. Additionally, endogenous *HOXD13* expression levels in HH25 posterior forelimb buds (blue bar) or *PITX1* expression levels in HH25 whole hindlimb buds (green bar) are shown. The expression of virally expressed HOXD13 transcripts is approximately 3-fold higher in chMM than endogenous expression in limb buds. Viral *PITX1* expression in chMM cultures is approximately 5-fold higher than in limbs. Absolute transcript numbers were normalized to the number of *GAPDH* transcripts in the respective samples. The error bars indicate the measurement S.D. from one representative example.

(C) Western Blot for virally expressed proteins (anti-Flag M2, Sigma F1804) and Histone H3 (Abcam, ab1791) in chMM cultures.

(D) Schematic drawing of the primers used for absolute quantification of the transcripts. One primer pair amplifies a part of the 3xFLAG-tag and thus, detects all spliced and unspliced viral RNAs. The other primer pair spans the splice site just upstream of the gene of interest. Therefore it detects only the HOXD13 or PITX1 expressing viral RNAs.



Supplemental Figure S2

Supplemental Figure S2.

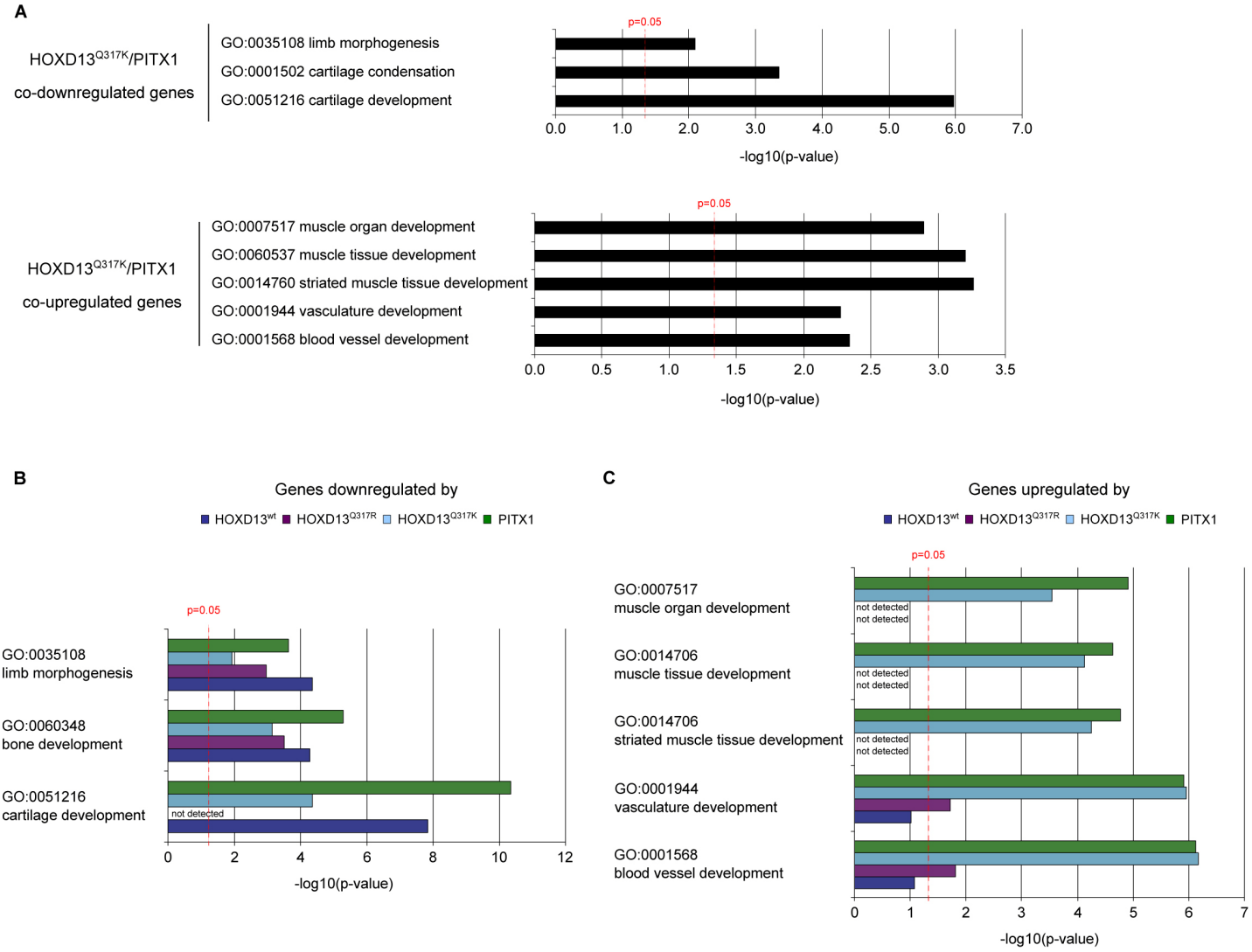
(A) Protein structure and location of the HOXD13^{Q317K} mutation in the HOXD13 protein highlighted in red. Alignment between homeodomain sequences of chicken, murine and human HOXD13, as well as the human HOXA13, HOXB13 and HOXC13 sequences.

(B) Three-dimensional structure of a homeodomain binding to DNA (*Drosophila* engrailed-homedomain (PDB ID: 3HDD, Fraenkel et al. 1998). The mutated glutamine is shown in spheres and highlighted in red.

(C) Alignment between the human (top) or murine (bottom) HOXD13 homeodomain sequence and all bicoid-type homeodomains in human or mouse respectively (Uniprot). All *identical* positions are highlighted in red. For better alignment, the HOPX sequence was omitted (13 identical amino acids).

Reference:

Fraenkel, E., et al., *Engrailed homeodomain-DNA complex at 2.2 å resolution: a detailed view of the interface and comparison with other engrailed structures.* Journal of molecular biology, 1998. **284**(2): p. 351-361.



Supplemental Figure S3

Supplemental Figure S3

GO analysis of genes differentially regulated by the four factors individually and genes co-regulated by HOXD13^{Q317K} and PITX1. For each factor a list of differentially regulated genes, compared to mock-infected cultures was generated (Supplemental Table 2) and used for GO-analysis with DAVID (Huang et al. 2008; Huang et al. 2009). The bars represent the negative log10 of the p-values of the selected GO terms.

(A) Genes associated with limb morphogenesis, cartilage development, and cartilage condensation were enriched in the set of genes downregulated by HOXD13^{Q317K} and PITX1 (top). Genes associated with GO terms related to muscle development and vasculature development were enriched in the set of genes upregulated by HOXD13^{Q317K} and PITX1 (bottom).

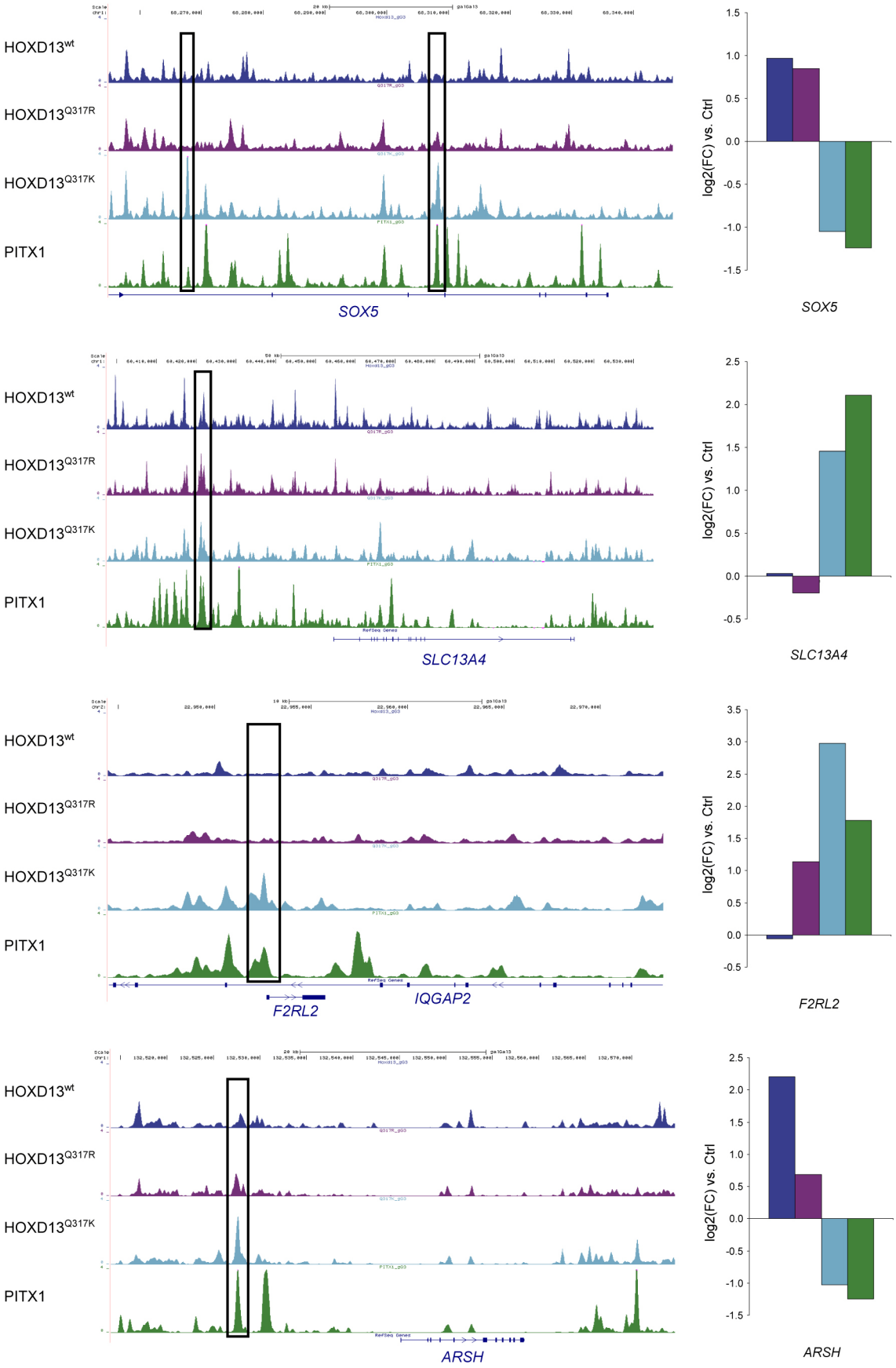
(B) Genes associated with GO terms limb morphogenesis, bone development and cartilage development were enriched in the set of downregulated genes for all four factors (except chondrocyte differentiation for HOXD13^{Q317R}). The colored bars represent the negative log10 of the p-value found for selected GO terms found in each of the sets of downregulated genes

(C) In the set of upregulated genes, genes associated with GO terms related to muscle differentiation and vasculature development were strongly enriched among the upregulated genes in HOXD13^{Q317K} and PITX1 chMM cultures, but not for HOXD13^{wt} and HOXD13^{Q317R}. The colored bars represent the negative log10 of the p-value for selected GO terms found in each of the sets of upregulated genes.

References

Huang da W, Sherman BT, Lempicki RA. 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocols* **4**(1): 44-57.

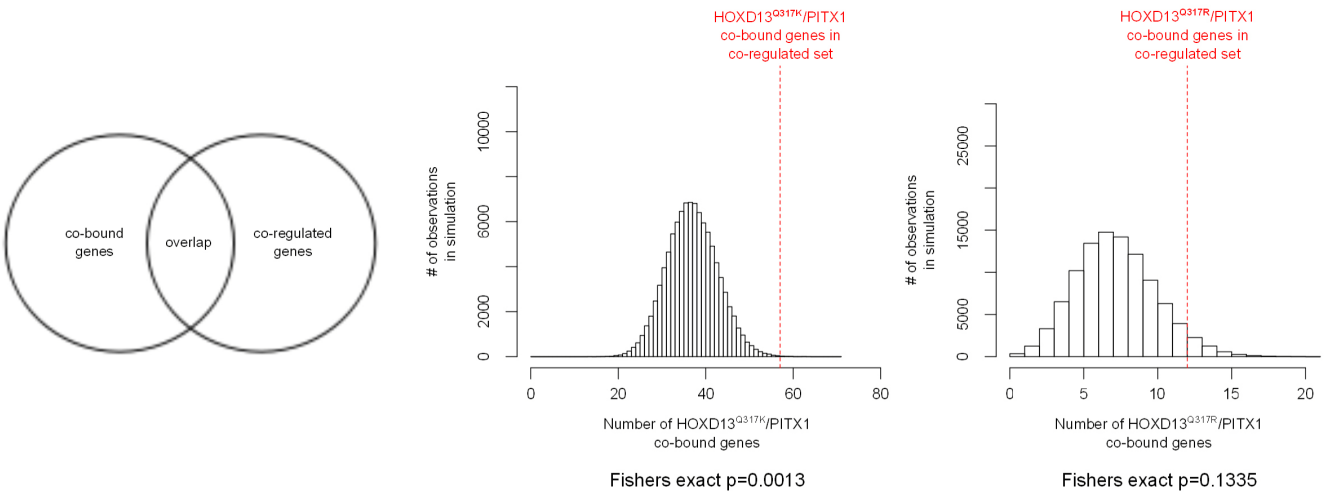
Huang da W, Sherman BT, Lempicki RA . 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research* **37**(1): 1-13.



Supplemental Figure S4

Supplemental Figure S4

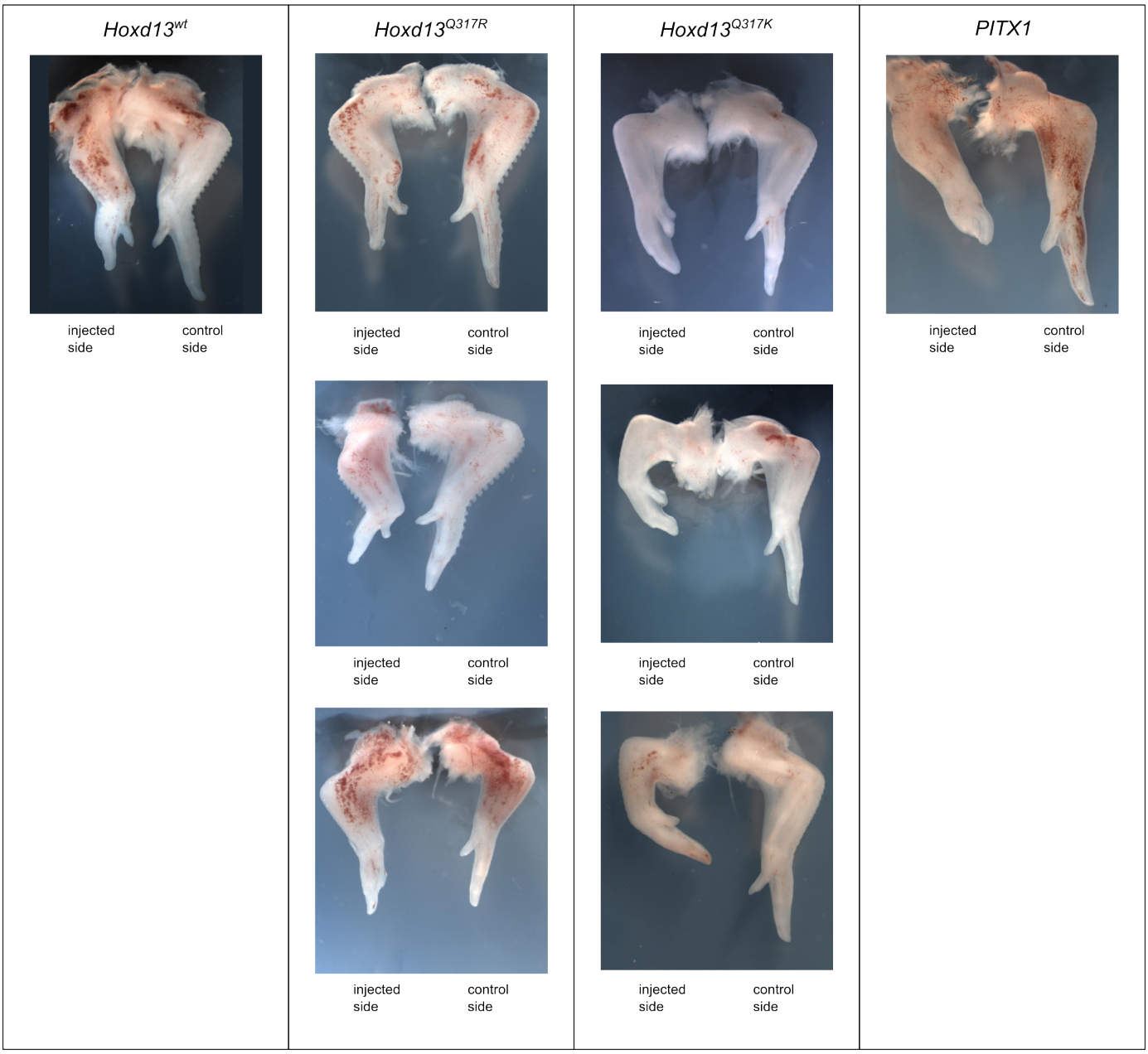
(left) Coverage profiles surrounding genes (*SOX5* (ENSGALG00000013204), *SLC13A4* (ENSGALG00000012908), *F2RL2* (ENSGALG00000023379), *ARSH* (ENSGALG00000016636) that are co-regulated and co-bound by HOXD13^{Q317K} and PITX1. Black boxes highlight the peaks that were detected by the filtering to identify shared peaks in the vicinity of co-regulated genes. (right) Log2(FC) expression differences compared to control chMM shows the specific co-regulation of the genes by HOXD13Q317K and PITX1.



Supplemental Figure S5

Supplemental Figure S5

13.1% (57 of 436) of HOXD13^{Q317K}/PITX1 co-regulated genes were also co-bound (red line), significantly more than expected by chance (Fishers exact p=1.295x10^{-3}). The histogram shows the distribution of co-bound genes when randomly selecting 346 genes (100.000 iterations).
5.1% (12 of 237) of HOXD13^{Q317R}/PITX1 co-regulated genes were also co-bound (red line), not significant (Fishers exact p=0.1335). The histogram shows the distribution of co-bound gene numbers when randomly selecting 237 genes (100.000 iterations).



Supplemental Figure S6

Supplemental Figure S6

Injected and control sides of the *Hoxd13*^{wt}, *Hoxd13*^{Q317R}, *Hoxd13*^{Q317K}, and *PITX1* infected chicken wings. The extent of phenotypic effects varies due to variability of infection efficiency. For *Hoxd13*^{Q317R} and *Hoxd13*^{Q317K} specimens encompassing the variability are presented.

SUPPLEMENTAL METHODS

1. Assessment of the RCASBP(A)-mediated overexpression

The RCAS-system is a retroviral system that leads to the integration of only one virus per cell. The time of cell culture (nine days) was chosen to assure that all cells are infected and variation in viral expression due to differences in infection efficiency (also between replicates) is minimized.
The virus produces a polycistronic mRNA that is alternatively spliced and produces three alternative splice products that code for the GAG and POL proteins, the ENV protein, or the TF of choice respectively (see Supplemental Fig. 1). Thereby only a fraction of the virally expressed RNA produces the transcription factor.
To estimate how big this fraction is, we generated a control plasmid, which can be used simultaneously for absolute quantification of all virally expressed RNA molecules and absolute quantification of splice products that code for the TF. Using qPCR absolute quantification, we measured for all viruses the relation of TF-spliced to total viral RNA. For all three viruses, only 15-25% of the virally produced RNA codes for TF of choice (Supplemental Fig. 1). Similar values were also determined for other RCASBP(A) constructs by others (Morgan and Fekete 1996).

Ectopic overexpression of *Hoxd13* by the viral construct sustains high expression levels comparable to endogenous expression in limb buds (Supplemental Figure 1). In empty vector infected cultures *HOXD13* expression levels are reduced during the course of differentiation but are still present at clearly detectable levels.
To assess how high the overexpression is in comparison with those tissues that express *HOXD13* or *PITX1* in embryonic development, we again used absolute quantification of mRNA-transcripts. We normalized viral and endogenous *Hoxd13* and *PITX1* transcript levels, from chMM cultures and chicken embryonic forelimb buds (HH stage 25) respectively, to *GAPDH* transcript levels.
We found that the transcripts of virally expressed *Hoxd13*-mRNA coding for the TF isolated from *Hoxd13*^{wt}-infected chMM RNA are approximately 3-fold overexpressed in comparison with *HOXD13*-transcripts in RNA isolated from posterior HH25 chicken forelimb buds and *PITX1* to be approximately 5-fold overexpressed compared to HH25 chicken hindlimb buds.

To estimate the amount of variation in overexpression between viruses we performed Western Blots from chMM protein extracts infected with the four different viruses respectively. The three *Hoxd13* (wt and mut) viruses produced comparable protein levels. The PITX1 protein levels were in the same range but somewhat stronger.

List of Primers used for absolute quantification of mRNA-levels

Oligo	Sequence (5' → 3')
cGAPDH_RT_F	CCA TTC CTC CAC CTT TGA TG
cGAPDH_RT_R	CAC GGT TGC TGT ATC CAA AC
cPITX1_RT_F	AAC CGC TAC CCC GAT ATG AG
cPITX1_RT_R	ACA GGT CCA TCT GCT GGT TC
cHOXD13_RT_F	AGC TCG CAC TTC TGG AAA TC
cHOXD13_RT_R	TAA GCT GGA GCT TGG TGT AGG
3xFLAG_F	GAC TAC AAA GAC CAT GAC GGT GAT TA
3xFLAG_R	CTT GTC ATC GTC ATC CTT GTA ATC
RCASsplice_RT_F	AAC TCA GAG GGT CGT CGG AAG
RCASsplice _RT_R	TCA CCG TCA TCG TCT TTG TAG TC

2. Cloning and Expression of Recombinant Homeodomains

Homeodomain sequences of *Hoxd13*^{wt}, *Hoxd13*^{Q317K} and *Hoxd13*^{Q317R} were amplified from plasmids containing the mutated coding sequence using primers HOXD13-HD5' (GAT CGA TAT CGG GAA GGA AGA AAA GGG TGC CTT A) and HOXD13-Stop (GAT CCT CGA GTC AGG AGA CAG TGT CTT TGA GCT). PCR products were cloned via EcoRV and XhoI into pET41c in frame with the GST- and His-Tag of the vector. Tagged homeodomains were expressed in *E. coli* Rosetta (DE3) pLysS and at 42 °C after induction at an OD600 of 0.3. Proteins were purified under native conditions with NiNTA-Superflow Agarose Qiagen) using manufacturer's instructions.

3. List of Primers used for EMSA

Oligo	Sequence (5' → 3')
Cy-D13-f-5' Cy-D13-f-3'	CY3-ggatcCCAATAAAAtcggc (labeled oligo) CY3-ccgaTTTTATTGGgatcc (labeled oligo)
D13-f-5' D13-f-3'	ggatcCCAATAAAAtcggc (unlabeled competitor) ccgaTTTTATTGGgatcc (unlabeled competitor)
D13mut-f-5' D13mut-f-3'	ggatcCCcAgcAcAtcgg (mutant unlabeled competitor) ccgaTgTgcTgGGgatcc (mutant unlabeled competitor)
Cy-PITX-f-5'	CY3-ggatcAGGGATTAACtcgg (labeled oligo)
Cy-PITX-f-3'	CY3-ccgaGTTAATCCCTgatcc (labeled oligo)
PITX-f-5'	ggatcAGGGATTAACtcgg (unlabeled oligo)
PITX-f-3'	ccgaGTTAATCCCTgatcc (unlabeled oligo)
PITXmut-f-5'	ggatcAGGGcggAACtcgg (mutant unlabeled competitor)
PITXmut-f-3'	ccgaGTTccgCCCTgatcc (mutant unlabeled competitor)

Morgan BA, Fekete DM. 1996. Chapter 10 Manipulating Gene Expression with Replication--Competent Retroviruses. In *Methods in Cell Biology*, Vol Volume 51 (ed. B-F Marianne), pp. 185-218. Academic Press.

4. List of Primers used for Mutation Detection in Human *ROR2* and *HOXD13*

Oligo	Sequence (5' → 3')
HOXD13-Exon1-F	GAA CCA GAG AGA AAG GAG AGG
HOXD13-Exon1-R	AAC TCC CAC TCC CAA GTA GG
HOXD13-Exon2-F	AGC TAG GTG CTC CGA ATA TCC
HOXD13-Exon2-R	ACA ACC GAA TGG CTT CTA AGC
ROR2-Exon1-F	CAT CGT AGA AAG GGG TGG TG
ROR2-Exon1-R	CAT AGT GGC GGC GGA AG
ROR2-Exon2-F	GTC TTA TCC CTC TGT GTT TTC TA
ROR2-Exon2-R	CAG GCA ATG GCA GTG CAA G
ROR2-Exon3-F	AAA TCG AAA CCT TCC CTT GG
ROR2-Exon3-R	TGC TGA CTG GTG TGT GTT CA
ROR2-Exon4-F	TGG CCT GAT TTG AAG AAG GT
ROR2-Exon4-R	AAA CCC TCA GAG CAG CAG AA
ROR2-Exon5-F	TGG ATC GCA AGA TGC TGG
ROR2-Exon5-R	TAA ACA TAC AGG CCA GGA AC
ROR2-Exon6-F	GTG GGG ACT GGA TGA ATG TC
ROR2-Exon6-R	CCC CCA TAC ACA TTT CAA GG
ROR2-Exon7-F	TAG TTT GGG CAT GTG TAG G
ROR2-Exon7-R	CCG TAC AGA GGC ACA CC
ROR2-Exon8-F	GGT TGG TAG AGA ACT TAG AGT
ROR2-Exon8-R	ATA ATT ATG TGC TAT GTA TCA AG
ROR2-Exon9-1-F	GGC TGC GGT GAC AGT GAT G
ROR2-Exon9-1-R	GCG GTC ATC ATC GGT GCT G
ROR2-Exon9-2-F	AGC CCC TGA GCA TGA TCT TC
ROR2-Exon9-2-R	GGA TCA TCT CCA CCA CAT CC
ROR2-Exon9-3-F	TCA GAC ATC TGG TCC TAC GGT GTG
ROR2-Exon9-3-R	CCA TCT GCA TTG GGA TCT GC
ROR2-Exon9-4-F	ACC AGC CCA GTG AGC AAT G
ROR2-Exon9-4-R	GTG ACT GAG GTC CCT GTG G