Cooperative binding of AP-1 and TEAD4 modulates the balance between vascular smooth muscle and hemogenic cell fate

Nadine Obier$^1$, Pierre Cauchy$^1$, Salam A. Assi$^1$, Jane Gilmour$^1$, Michael Lie-A-Ling$^2$, Monika Lichtinger$^1$, Maarten Hoogenkamp$^1$, Laura Noailles$^1$, Peter N. Cockerill$^1$, Georges Lacaud$^2$, Valerie Kouskoff$^2$ and Constanze Bonifer$^1$

$^1$ Institute of Biomedical Research, College of Medicine and Dentistry, University of Birmingham, Birmingham, UK

$^2$ CRUK Manchester Institute, University of Manchester, UK

*Corresponding author. Tel: +44 121 4148881; E-mail: c.bonifer@bham.ac.uk

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Summary statement:

This article shows that AP-1 activates the expression of vascular genes in hemogenic endothelial cells and that AP-1 is required for *de novo* binding of the Hippo signaling transcription factor TEAD4.

Abstract:

The transmission of extracellular signals into the nucleus involves inducible transcription factors, but how different signaling pathways act in a cell type-specific fashion is poorly understood. Here, we studied the regulatory role of the AP-1 transcription factor family in blood development using embryonic stem cell differentiation coupled with genome-wide transcription factor binding and gene expression analyses. AP-1 factors respond to MAP kinase signaling and are comprised of dimers of FOS, ATF and JUN proteins. To examine genes regulated by AP-1 and to examine how it interacts with other inducible transcription factors we abrogated its global DNA-binding activity using a dominant negative FOS peptide. We show that FOS and JUN bind to and activate a specific set of vascular genes and that AP-1 inhibition shifts the balance between smooth muscle and hematopoietic differentiation towards blood. Further, AP-1 is required for *de novo* binding of TEAD4, a transcription factor connected to Hippo signaling. Our bottom-up approach demonstrates that AP-1 and TEAD4 associated cis-regulatory elements comprise hubs for multiple signaling responsive transcription factors and defines the cistrome regulating vascular and hematopoietic development by extrinsic signals.
**Introduction**

The hematopoietic system has been a long-standing model for general principles driving the transcriptional control of cell fate decisions. During vertebrate embryonic development the emergence of definitive hematopoietic stem cells (HSCs) occurs in the dorsal aorta (Medvinsky and Dzierzak, 1996), where cells of a specialized hemogenic endothelium (HE) undergo an endothelial-to-hematopoietic transition (EHT), lose adherence and as newborn HSCs move to other sites of the embryo (Boisset et al., 2011; Eilken et al., 2009; Kissa and Herbomel, 2010; Lancrin et al., 2009). Each of these developmental transitions is regulated by an orchestrated interplay of stage-specific transcription factors (TFs). For example, SCL/TAL1 is required for the differentiation of HB cells to HE cells (D’Souza et al., 2005; Lancrin et al., 2009), while RUNX1 is essential for the EHT (Chen et al., 2009; Lancrin et al., 2009). These TFs are connected to common and distinct target genes within a dynamic transcriptional network (Goode et al., 2016). However, while the roles of transcriptional regulators of hematopoietic differentiation are beginning to be understood, it is less clear how outside signals direct their activity and drive developmental stage-specific gene expression.

The transmission of signals into the nucleus involves surface molecules, such as receptor kinases, and inducible TFs at the receiving end. Most inducible transcription factors are expressed in multiple cell types and cooperate with tissue-restricted or other inducible factors by binding to their cognate cis-regulatory elements and altering gene expression in a signaling-dependent way. How this process is coordinated and connected to the signaling network is poorly understood.
and difficult to study, because signaling pathways are highly dynamic consisting of a myriad of different components operating in a cell type-specific fashion and displaying multiple types of cross-talk. However, what is the same in all cells are the sequences that hard-wire the response to signals into our DNA. By identifying and studying the function of signalling-responsive cis-regulatory elements and their interacting factors, we are able to obtain a first insight into how signal transduction processes are coordinated at the genomic level.

A paradigm for inducible transcription factors is the activator protein 1 (AP-1) family of transcription factors which are typical targets of MAP kinases, including ERK and JNK (Chang and Karin, 2001) that enhance their transcriptional activity through phosphorylation (Angel et al., 1987; Karin and Smeal, 1992). Generally, AP-1 factors promote gene expression, often in response to stimuli such as growth factors. The AP-1 family comprises FOS (FOS, FOSB, FOSL1, FOSL2) and JUN (JUN, JUNB, JUND) proteins, but also ATF (ATFa, ATF-2, ATF-3) and JDP (JUN dimerisation proteins, JDP1, JDP2) proteins, all of which are structurally and functionally related and act as dimers (Hess et al., 2004; Jochum et al., 2001). While JUN family proteins can dimerize with FOS-, JUN-, ATF- and JDP-proteins, FOS family proteins can only heterodimerize with JUN family members. Several findings point to an important role of this TF family at early stages of hematopoietic specification: i) work from our group found AP-1 motifs enriched in open chromatin regions and co-localizing with TF binding sites specific to HE cells differentiated from mouse embryonic stem cells (ESC) (Goode et al., 2016; Lichtinger et al., 2012), ii) JUN knockout (KO) mice die around the onset of HSC emergence (Eferl et al., 1999), iii) AP-1 was reported to play a role in Xenopus hematopoiesis (Lee et al., 2012), iv) in zebrafish the transcriptional co-repressor NCoR silences Fos
transcription and NCoR knockdown leads to inhibition of HE formation (Wei et al., 2014), v) AP-1 activation is involved in the stimulation of engraftment of HSCs by epoxyeicosatrienonic acids (Li et al., 2015) and vi) FOS has been identified as a crucial factor together with GATA2, GFI1B and ETV6 in the reprogramming of mouse embryonic fibroblasts (MEFs) to blood cells (Pereira et al., 2013). However, none of these studies has identified the global genomic targets responsible for these effects. In addition, the expression of individual AP-1 family members, and thus the dimer composition, varies depending on the cellular context. Due to the redundancy in this system, the analysis of the general role of AP-1 factors has been elusive.

In this study we gained a first insight into the role of the AP-1 factor family as a whole using in vitro differentiated mouse ESCs as model. During ESC differentiation the first blood cells derive from the hemangioblast (HB), a mesodermal cell type capable to differentiate to vascular smooth muscle (SM), endothelial and hematopoietic cells (Choi et al., 1998; Huber et al., 2004; Kennedy et al., 1997; Stefanska et al., 2014). We expressed a dominant negative FOS (dnFOS) peptide from a Doxycycline (DOX)-inducible promoter and thereby abolished all AP-1 DNA binding activity (Olive et al., 1997). A surprising result of our work was that global AP-1 inhibition in spite of the near-ubiquitous expression of this factor family is compatible with hematopoietic specification as such whereby in differentiating hemangioblast cells FOS and JUN together bind to and activate a core set of vascular effector genes. Importantly, we found that at these genes AP-1 does not act alone but cooperates with TEAD4, a mediator of the Hippo signaling pathway (Meng et al., 2016) which we have previously shown to be essential for hematopoietic specification (Goode et al., 2016). AP-1 inhibition abolished TEAD4 binding at these genes, thus uncovering the mechanism of the interdependency of the two signaling
pathways. Our data therefore show how inducible transcription factors are integrated at the genomic level to form signaling hubs that modulate the balance between cell fates.

Results

Global AP-1 inhibition affects differentiation of ESCs via hemangioblast to hematopoietic cells

The family of AP-1 transcription factors consists of diverse members, most of which can heterodimerize and are likely to compensate for each other’s absence. Therefore, we inhibited all AP-1 activity directly and globally using a dominant negative version of FOS (dnFOS). This peptide contains a dimerization domain for binding to JUN family proteins and an acidic extension for blocking the DNA-binding domain of JUN, thus preventing both the formation of JUN:JUN, JUN:ATF and JUN:FOS dimers and their binding to DNA (Olive et al., 1997). In order to examine a role of AP-1 factors at different stages of hematopoietic specification, we employed in vitro ESC differentiation that has previously been used to recapitulate and investigate the different steps in hematopoietic specification (Fig S1A) (Goode et al., 2016; Lancrin et al., 2010). We constructed an ESC line carrying a Doxycycline (DOX)-inducible Flag-tagged dnFOS allele (Fig 1A) (Kyba et al., 2002). We ensured that the peptide inhibited AP-1 driven gene activation in a luciferase assay (Fig S1B), that its expression was tightly regulated (Fig S1C), that the protein was present in every cell (Fig S1D) and demonstrated that induction of the construct indeed blocked JUN binding to DNA in a genome-wide fashion (Fig 1C and Fig S1E).
Several members of the AP-1 family are expressed at one or more stages of in vitro ESC differentiation to blood (Fig S1F). To be able to study the molecular mechanism of AP-1 function in detail, we first had to examine at which stages AP-1 activity was crucially required. To this end, we induced dnFOS by addition of DOX at distinct time points of ESC differentiation.

As outlined in Fig 1B (and Fig S1A), ESCs were differentiated into embryoid bodies (EBs) and after 3.75 days HB cells were enriched by purifying cells positive for the VEGF receptor (FLK1⁺). Subsequently, FLK1⁺ cells were kept for up to 4 days in the presence of IL-6 and VEGF under blast culture (BC) conditions where they differentiated to vascular smooth muscle (SM) cells or successively to the following cell types: i) early hemogenic endothelial cells (HE1, KIT⁺TIE2⁺CD41⁻), ii) late hemogenic endothelial cells fully committed to blood but still adherent (HE2, KIT⁺TIE2⁺CD41⁺) and iii) floating hematopoietic progenitor cells that have undergone the EHT (HP, KIT⁺TIE2⁻CD41⁺). The inhibition of AP-1 activity during the establishment of HB cells by adding DOX to EB cultures led to an increase in total cell numbers within EB cultures and the proportion of FLK1⁺ cells (Fig S1G,H). To examine the role of AP-1 at later differentiation stages, we expressed dnFOS during BC by either inducing freshly purified FLK1⁺ cells (4d of induction) or we added DOX subsequently at d1, d2 or d3 of BC (Fig 1D). After 4 days complete cultures and floating cells (containing progenitor cells), respectively, were assessed for cell count and phenotypic composition. Despite no significant change in overall cell number (Fig 1E, left panel), complete blast cultures contained a higher proportion of KIT⁺ cells (Fig S1I,J) and HE2 cells after dnFOS induction (Fig 1E right panel and Fig S1K). This effect was strongest when AP-1 was inhibited at the very beginning of blast culture. Moreover, we found that an early block of AP-1 (4d DOX) led to a
significant increase in the amount of floating cells (Fig 1F left). However, myeloid commitment of such cells as measured by CD11b surface marker expression was significantly diminished (Fig 1F right panel and Fig S1L). Moreover, when AP-1 was inhibited only transiently in the first 24 h of blast culture with subsequent DOX-washout and culture medium replacement, numbers of floating cells at d3 were increased to the same extent as observed after a continuous 3-day treatment period (Fig S1M). In contrast, proportions of CD11b+ cells were decreased to a lesser extent when DOX was withdrawn after 1d (Fig S1N).

Together, these results suggest that AP-1 is involved in modulating the transitions of several distinct stages of blood development, supporting or impairing respective cell fates. However, in spite of the near-ubiquitous expression of this factor family, global AP-1 inhibition does not lead to gross disturbances in blood cell specification.

**Inhibition of AP-1 at the hemangioblast stage shifts the balance between vascular and blood cell development**

Having shown that blocking all AP-1 activity is compatible with differentiation, we next examined whether this factor was involved in regulating cell fate. To this end, we studied the differentiation of HB cells by analyzing complete blast cultures +/- DOX after 1 and 2 days of induction as well as floating cells after 3 days (Fig 2A). Although cell numbers at d1 were unaffected by AP-1 inhibition, we measured a significant reduction in the proportion of SM cells as assessed by intracellular flow cytometry using 3 different SM cell markers: SMA, SM22α and Calponin (Fig 2B,C and Fig S2D). Endothelial marker expression was unaltered with the exception of a slight increase in FLK1+ cells in DOX-treated cultures (Fig S2E). Further, when
analyzing d2 blast cultures we observed significantly increased cell numbers (Fig 2D), with overall no difference in apoptosis (Fig S2A). DOX-induced cells showed higher S- and G2/M-cell cycle phase contribution and expressed significantly lower levels of the negative cell cycle regulator gene *Cdkn2a* (p16) (Fig S2B,C), which explains enhanced proliferation. As in the time course experiment (Fig 1E), the frequency of HE2 cells was also significantly increased when AP-1 was inhibited for 2 days only (Fig 2F, Fig S2H), while SM cell proportions were significantly lower (Fig 2E, Fig S2F) and endothelial cells remained unaffected (Fig S2G). These results demonstrate that the gain of hematopoietic HE2-type cells was paralleled by a loss of SM cells. The number of non-adherent, floating cells that had undergone the EHT after a 3-day culture and dnFOS-induction period was significantly increased by more than 4-fold as compared to untreated cultures (Fig 2G). We next characterized these cells by surface marker flow cytometry. The analysis revealed that the proportions of KIT⁺, CD41⁺ and CD71⁺ cells within floating cells emerging upon AP-1 inhibition were comparable to untreated cells, demonstrating that hematopoietic commitment had happened (Fig S2I, J). However, while floating cells of untreated cultures showed expression of CD45 and CD11b, indicating their maturation and myeloid commitment, DOX-treated cells failed to express these markers and appeared to be blocked in differentiation (Fig S2I,J and Fig 2H). We further noticed a slight but significant increase in CD71⁺ cells upon AP-1 inhibition, which was reported to represent a population of primitive erythroblasts (Chao et al., 2015). To test whether floating cells derived from dnFOS-induced cultures have the potential to form normal colony as compared to untreated cultures, we performed colony-forming assays in the presence and absence of DOX. Overall we detected similar colony formation capacity, with similar numbers and sizes of colonies (Fig 2I). Although the
myeloid commitment in dnFOS-induced blast cultures was inhibited (Fig 2H and Fig S2I,J), progenitor cells derived from DOX-treated blast cultures were able to give rise to myeloid cell-containing CFU-GM and CFU-Mix type colonies when DOX was withdrawn with a slight bias towards colonies with erythroid contribution (BFU-E and CFU-Mix). Together, these experiments demonstrate that AP-1 inhibition by dnFOS in differentiating HB cells shifts the balance between smooth muscle and hemogenic cell fates and reversibly blocks myeloid commitment, suggesting a dual role of AP-1 in this developmental pathway, promoting both smooth muscle and myeloid cell types.

**AP-1 inhibition does not alter global transcriptional networks but affects expression of key stage-specific genes**

To gain insight into how AP-1 regulates cell fate we focused on the differentiation of HB cells into SM and HE cells. We investigated the transcriptional changes induced by blocking AP-1 activity using microarray gene expression analysis with RNA extracted from freshly purified FLK1+ HB cells, complete blast cultures after 5h and 1d of induction, as well as from FACS-sorted SM, HE1, HE2 and HP cells at d2 of blast culture (Fig 3A, Fig S3A,B). Principal component analysis revealed that samples of the same cell type clustered closely together with or without dnFOS induction, indicating that AP-1 inhibition did not globally alter the gene expression patterns of induced cells (Fig 3B, Fig S3C). However, we identified a number of genes that were at least 2-fold differentially expressed (Fig 3C, Fig S3D and Table S1). Consistent with AP-1 generally functioning as an activator, we found more down-regulated than up-regulated genes. K-means clustering of differentially expressed genes in the sorted cell populations SM, HE1, HE2 and HP (Fig 3D)
established a total of 15 clusters, each containing genes with similar expression fold change patterns over the 4 cell types. We also evaluated their gene ontology (GO) terms (Table S2 and Fig S3E). Clusters 3, 4 and 1 retrieved genes that were expressed at lower levels in HE2 and HP cells such as the myeloid regulators Csf1r, Sfpi1 (Pu.1) and Cebpa, which was in line with the reduced number of CD11b+ cells observed in DOX-treated blast cultures. Genes up-regulated in HP cells (cluster 13) were associated with erythrocyte differentiation and clusters 12 and 6 comprised genes that were down-regulated in SM and HE1 cells, e.g. genes like Lamc2, Myo1e, Flnb, Serpine1 or Wt1 (Fig 3D,E and Fig S3G). These gene products are associated with vascular cell types (GO terms Fig SE and (Duim et al., 2015)) and their decreased expression may reflect the lower contribution of vascular SM cells upon dnFOS-induction. We also found a group of HSC-related genes, including Hoxb4, Bmi1 and Gfi1, that were expressed at higher levels in the absence of AP-1 activity (Fig 3D,E, Fig S3G, with manual validations shown in Fig S3F). In SM and HE1 cells, blocking AP-1 activity affected vascular genes, while at later stages in HE2 and especially HP cells myeloid gene expression was reduced. In summary, despite their wide-spread expression and promiscuous involvement in multiple signaling processes, the absence of AP-1 during cell differentiation does not deregulate large sets of genes, but influences cell fate decisions by impacting on a limited set of key stage specific regulator and effector genes.

AP-1 is required for the transient activation of vascular genes in the hemogenic endothelium

The most common AP-1 complex consists of FOS and JUN proteins. To identify targets for these two factors, we performed ChIP-seq in d1 blast culture cells which
was mostly comprised of SM cells with the rest being HE1/2 cells (Fig 4A, Fig S4A).
Since AP-1 proteins only transiently bind to their templates (Biddie et al., 2011) we employed a double-crosslink procedure which greatly enhanced ChIP-signals (see materials and methods). We found a total of 4889 binding events for FOS and 2999 JUN binding sites, with the majority of them being at distal regulatory elements at either intronic or intergenic sites (Fig 4B, Table S3, Fig S4B). The integration of the two replicate JUN ChIP-experiments shown here and in Fig 1C revealed that more than 78% of the JUN peaks shown in Fig 4 overlapped (Suppl. Fig 4C). The GO terms associated with genes co-bound by FOS and JUN from both replicates produced an almost identical list of terms and p-values (Suppl. Fig 4D).

Approximately 40% of JUN peaks overlapped with FOS binding sites, while 25% of FOS peaks overlapped with JUN, suggesting that JUN and FOS interacted with different partners at unique sites. In total we identified 1227 genomic sites that were co-bound by FOS and JUN (Fig 4C). Next, we annotated the closest genes to FOS and JUN peaks, intersected both gene populations and identified 910 genes that were bound by JUN only, 2190 genes by FOS only and 1388 genes were annotated to both FOS and JUN peaks (Fig 4D, Table S4). To examine, whether the different binding configurations of JUN and FOS regulate different sets of genes, we further dissected these 1388 genes into those where FOS and JUN bound at identical sites (FOS:JUN co-bound, 1042 genes) and those where FOS and JUN bound at different sites (FOS and JUN distinct, 346 genes) (Fig 4E, Table S4). Exemplary genome browser screen shots for these different patterns are shown in Fig 4F. Gene ontology analysis revealed that FOS alone and JUN alone bound to genes associated with phosphorylation, metabolism and signaling (Fig 4G). Interestingly, in contrast to these more general GO terms, FOS and JUN bound to
heart- and muscle-linked genes, moreover, FOS:JUN dimers highly significantly co-bound vascular genes (Fig 4G, lower right panel), suggesting a direct regulation of the vascular rather than the hematopoietic cell fate.

We next integrated FOS and JUN binding patterns with transcriptional changes +/- DOX for d1 blast culture, i.e. the time point of FOS and JUN ChIP-seq by performing gene set enrichment analyses (GSEA). We found a high correlation between FOS or JUN binding and down-regulated gene expression upon dnFOS induction (Fig S5A), showing that both factors have largely activating roles. Next we investigated the general pattern of expression of genes annotated to JUN only, FOS:JUN and FOS only binding events (Fig S5B, left panel) all of which generally showed lower expression after 1 day of dnFOS induction than in control cells. This effect was strongest for FOS:JUN co-bound genes. Focusing on transcriptional changes of AP-1 target genes occurring during transitions of distinct developmental stages in the absence of DOX, we observed that AP-1 target genes were generally up-regulated from FLK1+ to SM and from FLK1+ to HE1 cells, followed by a down-regulation from HE1 to HE2 cells, but did not further change expression during the transition from HE2 to HP cells (Fig S5B, right panels). Together with the GO analysis (Fig 4G), this finding suggests that AP-1 is involved in a transient induction of vascular (endothelial and smooth muscle-related) genes in HB-derived cells which are then down-regulated during the EHT.

We next analyzed the dynamic activity of all FOS:JUN co-bound genes in more detail by conducting k-means clustering of expression patterns in control FLK1+ cells, FLK1+ cells after 5 h and 1 d of BC as well as in d2 SM, HE1, HE2 and HP cells (Fig 5A, Table S5). This analysis revealed 7 gene clusters each with similar expression patterns during the differentiation of HB to HP cells. We then compared
the averaged expression levels of each of the 7 clusters during unperturbed differentiation with averaged expression levels of dnFOS-induced cells (Fig 5B). Specific differences of expression levels between induced and control cells were apparent only in clusters 5 and 6 which contain 203 genes that are normally highly induced in d1 blast culture, SM and HE1 cells. 68 of these genes were expressed at significantly lower levels when AP-1 was blocked (Table S6), amongst them we identified collagens (*Col1a1, Col4a1, Col5a1*) and *Bmp1* (the metalloprotease that supports collagen maturation by cleaving procollagens), vascular growth factors (*Vegfa, Pdgfc, Hbegf, Ctgf*), signaling ligands (*Bmp2, Tgfb*), genes encoding integrin ITGA11 and laminin LAMC2 and the transcription factor WT1, which was recently shown to be involved in angiogenesis (Duim et al., 2015; Katuri et al., 2014). These findings strongly suggest, that during HB cell differentiation AP-1, and in particular the FOS:JUN complex, activates a specific set of genes in SM and HE1 cells that are closely linked to and important for blood vessel formation. In the subsequent transition of HE1 cells to HE2 cells, i.e. cells that are fully committed to blood, these genes are down-regulated and remain low in HP cells.

**AP-1 is required for de novo binding of the Hippo-signaling regulator TEAD4 at specific binding sites**

The developmental stage-specific response to AP-1 inhibition indicated that these factors interact with cell type-specific sets of cis-regulatory elements. In order to identify additional TFs binding to these elements, we performed a de novo motif discovery analysis of FOS only, JUN only and FOS:JUN co-bound genomic sequences. For each of the 3 groups, the AP-1 consensus sequence was retrieved as top hit as expected (Fig 5C, Fig S5C). The CREB motif was also highly enriched
and specific to JUN only peaks, reflecting the ability of JUN, but generally not FOS to dimerize with ATF proteins (Fig 5C, Fig S5C (Hai and Curran, 1991; Karin et al., 1997; van Dam and Castellazzi, 2001)). A striking result from our motif analysis was the co-association of TEAD motifs within all FOS and JUN peaks (Fig 5C). Moreover, in a motif co-occurrence clustering analysis computing enrichment against background co-localization frequencies, we found a close co-localization of AP-1 and TEAD motifs in FOS and JUN binding sites (Fig S5D). The TEAD family of transcription factors contains 4 members, TEAD1-4, all of which share the same consensus sequence and are the downstream effectors of Hippo signaling (Meng et al., 2016). In the absence of Hippo signaling TEAD factors bind to DNA, but can activate gene expression only together with co-activators such as YAP, VGLL or p160 (Pobbati and Hong, 2013). Active Hippo signaling leads to phosphorylation of LATS1/2 by MST1/2 and to subsequent phosphorylation of YAP. Consequently, phospho-YAP is restricted to the cytoplasm and fails to activate TEAD-bound genes.

Recent reports described the genomic co-localization of AP-1 and TEAD proteins in cancer cell lines (Diepenbruck et al., 2014; Liu et al., 2016; Verfaillie et al., 2015; Zanconato et al., 2015), however, how these two factors cooperate at specific genes was not studied. Thus, we investigated TEAD4 occupancy upon blocking AP-1 DNA-binding by performing ChIP-seq in d1 blast culture cells +/- dnFOS (Fig S1E, Fig 6A). For untreated cells we obtained at total of 21,422 TEAD4 peaks, while the number of peaks for DOX-treated cells was almost 2-fold diminished (11,721 peaks, Fig 6B and Fig S6A). The decrease in peak numbers did not affect the overall genomic distribution: most of TEAD4 binding was still detected in distal intergenic or intronic elements (Fig S6A). De novo motif discovery analysis showed that with or without AP-1 inhibition the TEAD motif was the top hit (Fig 6C).
However, AP-1 motifs in the DOX-treated sample were undetectable, indicating that upon dnFOS expression TEAD4 was no longer binding to sites containing AP-1 motifs (Fig 6C, right panel). To substantiate this finding, we compared all TEAD4 peaks derived from treated and untreated cells and ranked them according to the fold change of ChIP-seq signal intensity (Fig 6D) which revealed 3 classes of TEAD4 binding sites (Table S7): class 1 comprising peaks that were specific to untreated cells and lost upon AP-1 inhibition (6,787 sites, 30.6%), class 2 representing a shared class of TEAD4 binding that was not affected by dnFOS induction (14,404 sites, 65.1%) and class 3 containing a relatively small number of TEAD4 peaks that were gained (950 sites, 4.3%). The TEAD motif was found throughout all 3 classes, whereas the AP-1 motif was specific to class 1 peaks (Fig 6D shows the motif heatmap and average profiles). When annotating the closest genes and analyzing their expression fold change +/- DOX in d1 blast culture, we found significantly lower expression only in class 1 genes, showing that the interaction between TEAD4 and AP-1 is required for high-level expression (Fig 6D, right panel and box plot).

To investigate how TEAD4 binding was associated with JUN/FOS binding, we compared the ChIP-seq signals for FOS and JUN as well as the previously identified FOS:JUN co-bound, JUN only and FOS only peaks using the same ranking of TEAD4 peaks (Fig 6E). The majority of FOS and JUN binding events was contained in class 1 peaks, particularly FOS:JUN co-bound sites overlapped strongly with TEAD4 sites that were lost upon AP-1 inhibition. Intersecting the 203 FOS:JUN co-bound genes from cluster 5 and 6 (Fig 5B) with class 1 TEAD4 bound genes, revealed that 175 of them, i.e. 86%, showed loss of TEAD4 binding upon dnFOS expression. Exemplary genome browser screen shots of some of these gene loci are shown in Fig 6F and Fig S6B. We further narrowed this gene population down to a
total of 64 genes sharing the following features: transiently expressed during HB differentiation, co-bound by FOS:JUN, AP-1 dependently bound by TEAD4 and expressed at significantly lower levels upon dnFOS induction (Table S6). A high proportion of FOS and JUN peaks overlapped with TEAD4 binding without DOX (Fig S6C). We found that the average TEAD4 ChIP-seq signal centered on such FOS or JUN peaks was reduced by more than 2-fold (Fig S6D) upon AP-1 inhibition, again suggesting that a defined class of TEAD4 binding sites was AP-1 dependent.

In order to understand the dynamics of TEAD4 binding in development, we analyzed TEAD4 ChIP-seq data in FLK1+ HB cells that we had published recently (Goode et al., 2016) and in addition generated a TEAD4 ChIP-seq dataset for purified HE1 cells (KIT+Tie2+CD41) which to 75% overlapped with that of d1 BC (Fig S6A,E,G,H). In freshly purified FLK1+ HB cells TEAD4 binding was already established at class 2 sites, but not at class 1 sites (Fig S6E,G) which were specific for the HE stage. In summary, these analyses reveal that during the differentiation of HB cells into the HE cells, TEAD4 de novo binding occurs at cis-regulatory elements associated with a subset of vascular genes and that this binding is dependent on AP-1, suggesting a recruiting role for AP-1.

**AP-1 and TEAD bound regions co-localize with occupied binding motifs for multiple inducible transcription factors in open chromatin of hemogenic endothelium cells but not thereafter**

We have recently shown that the interaction between YAP and TEAD factors is required for the differentiation of hematopoietic cells from FLK1+ cells purified from ESCs and mouse embryos but not in HP cells. This was accompanied by an activation of Hippo signaling leading to the absence of TEAD and YAP in the nucleus
of HP cells (Goode et al., 2016). The analysis presented here shows that on average
all genes bound by TEAD4 showed similar expression patterns during HB
differentiation: a strong induction from FLK1+ to SM and from FLK1+ to HE1 cells (Fig
S6F). GO analysis showed that all 3 classes of TEAD4 sites were associated with
genes involved in cell adhesion and vasculogenesis (Fig S6I). However, all of these
genes were strongly down-regulated from HE1 to HE2 cells and expression did not
change in HP cells, suggesting that AP-1 cooperates with TEAD prior, but not after
the EHT. We tested this idea by performing deep DNasel sequencing in FACS-
purified HE1 (KIT+TIE2+CD41−) and HP (KIT+TIE2−CD41+) cells. We obtained 68,691
dNasel hypersensitive sites (DHSs) for HE1 and 63,712 DHSs for HP cells, 44,216
of them were shared between the 2 samples (Fig S7A,B). De novo motif discovery
analysis revealed that ETS, GATA, AP-1, TEAD and SOX motifs were enriched in
HE1 open chromatin regions, while motifs for the hematopoietic transcription factors
RUNX, ETS, CEBP, and MEIS but also AP-1 motifs were found in HP-specific DHSs
(Fig 7A,B). Upon ranking of the fold change of the union of HE1 and HP DHSs, we
identified specific patterns of motif distribution: TEAD, SOX and GATA motifs were
distinct to HE1-specific DHSs, whereas RUNX and CEBP motifs were characteristic
to HP-unique DHSs (Fig 7C, Fig S7C). The AP-1 motif was found in both HE1-
specific and HP-specific DHSs, but not in shared sites, again highlighting the
different function of these binding sites in endothelial and hematopoietic cells. When
plotting FOS and JUN ChIP-seq signal of d1 blast culture cells using the same
ranking, the highest signal was obtained in HE1-specific DHSs (Fig S7C),
concordant with binding of AP-1 in HE1 cells. We then annotated these binding sites
to the promoters of the closest genes, calculated expression fold changes for
developmental transitions and plotted the resulting heatmaps in the same ranking
Generally, genes annotated to HE1-specific DHSs were up-regulated from FLK1+ to HE1 and down-regulated from HE1 to HE2, while their expression did not change from HE2 to HP. In contrast, genes annotated to HP-specific sites showed a gradual up-regulation throughout all transitions from HB to HP. Further, HP specific sites were associated with AP-1, RUNX and CEBP motifs, as seen with FOS binding in mast cells (Calero-Nieto et al., 2014) and with reduced transcription in HP cells upon dnFOS induction (Fig 7C, Fig S7C). These observations support our hypothesis of AP-1 playing a role during the differentiation of HP cells to myelomonocytic cells. However, TEAD appears to be involved only in a HE1-specific context.

To examine the interaction between TEAD4 and AP-1 at base-pair resolution, we performed digital footprinting analysis on our DNaseI-seq data using the Wellington algorithm (Piper et al., 2013), and identified 70,522 and 53,964 regions in HE1 and HP cells that were protected from DNaseI digestion, respectively (Fig S7D). Interestingly, the overlap between those was found to be not significant ($p=1$), denoting a markedly different repertoire of occupied TF binding sites between the two stages. Both AP-1 and TEAD motif-containing footprints were enriched in HE1 cells and occupancy of these motifs in HE1 cells was strongly reduced in HP cells (Fig S7E,F,G,H). Moreover, when centered on FOS and JUN ChIP-seq peaks of d1 blast culture, AP-1 motifs were preferentially occupied in HE1 cells but not HP cells (Fig S7I), and were associated with class 1 genomic sites (see Fig 6D, AP-1-dependent TEAD4 sites) for HE1 but not for HP (Fig S7J). This assay also allowed us to investigate the co-occupancy of other transcription factor binding motifs with AP-1 and TEAD by clustering HE1-footprinted motifs using bootstrapping analysis which determines the significance of co-clustering motifs within 50 bp of DNA. We
found a significant co-occurrence of occupied AP-1 and TEAD motifs together with occupied motifs for other signaling inducible transcription factors such as SMAD, NFAT, CREB and TCF together with motifs for tissue-specific factors such as SOX and GATA (Fig 7D). Our results are supported by previous reports describing a co-association of GATA and AP-1 in mature vascular cells (Linnemann et al., 2011). In contrast, in HP cells occupied motifs for the hematopoietic transcription factors ETS, E-Box (TAL1), RUNX- and CEBP predominantly clustered together (Fig S7K). Importantly, at co-occurring AP-1 and TEAD footprinted motifs in HE1 cells, we found a bias towards a defined oriented distance between both motifs with a 7bp spacing (Fig 7E and Fig S7L). This composite motif likely indicates direct interaction between AP-1 and TEAD, as was identified from other composite motifs previously (Chen et al., 1998; Cockerill et al., 1995; Hollenhorst et al., 2009). In conclusion, these analyses show that AP-1 and TEAD co-occupy a subset of genomic sites that are accessible in HE1 cells but inaccessible in HP cells. Genes associated to these elements are transiently activated from HB to HE1 commitment but down-regulated in HE2 and HP cells.

In summary, we propose a model in which AP-1 (FOS:JUN) promotes the differentiation of vascular smooth muscle cells and also binds to vascular genes in the hemogenic endothelium (Fig S8). During the transition from HB to HE1 cells, AP-1 is needed for the transient induction of such genes which are subsequently down-regulated when cells further differentiate to blood-committed cells. A substantial proportion of these genomic sites is co-occupied by the Hippo signaling-related TF TEAD4 and we show that AP-1 is required for its de novo binding to these elements. While AP-1 is also important later for the maturation of HP cells, it does no longer co-localize with TEAD4 at this developmental stage. Altogether, our study highlights the
versatility of the ESC differentiation system to dissect the molecular mechanism of specific knockout phenotypes which would otherwise not be amenable to biochemical studies.

Discussion

Inhibition of AP-1 activity shifts the balance between vascular and hematopoietic cell fates

By identifying and studying the function of genomic regions bound by signaling responsive TFs we can obtain a first mechanistic insight into how such processes are coordinated and which signaling processes are involved. Here we used such a bottom-up approach to study the function of the AP-1 transcription factor family in hematopoietic specification. Our results uncover a role of AP-1 in the establishment of the smooth muscle and vascular program from hemangioblast cells, identify the genes involved in this process and pinpoint the developmental stage at which this occurs. Lineage tracing experiments demonstrated that SM cells and HE cells develop independently of each other from HB cells (Stefanska et al., 2014). We hypothesize that the impaired induction of the vascular gene expression program in the hemogenic endothelium after AP-1 inhibition leads to an imbalanced cell fate decision towards blood-committed cells and consequently to more HP cells. This is consistent with a previous report, stating that repression of arterial/vascular genes in HE is sufficient for hematopoietic fate acquisition (Lizama et al., 2015). Runx1 expression is not affected by dnFOS expression (Suppl. Table S1) and thus hematopoietic progenitor cells undergo the EHT and up-regulate the RUNX1-dependent CD41 marker (Lie-A-Ling et al., 2014). In the presence of DOX, the cells cannot mature to CD45\(^+\) and CD11b\(^+\) cells, indicating that further differentiation is
blocked. However, in the absence of dnFOS they resume their normal differentiation behavior and form different types of colonies, indicating that this block is reversible.

In addition to changes in the differentiation pattern, we observed enhanced proliferation throughout ESC differentiation in the presence of dnFOS. \textit{Cdkn2a} and \textit{Cdkn2b} encoding the negative cell cycle regulators p16INK4a and p15INK4B, respectively, were bound by AP-1 and expressed at lower levels after AP-1 inhibition. Both \textit{Cdkn2a} and \textit{Cdkn2b} are expressed at very high levels in d1 BC and SM cells under normal conditions (Fig 3), a lower expression of these genes upon AP-1 inhibition would preferentially enhance the proliferation of SM cells compared to HE or HP cells. However, we found that the relative proportion of HE2 cells and not SM cells was increased. Further, after only 1d of blast culture, when cell numbers +/-DOX were identical, we already observed reduced proportions of SM cells. In addition our gene expression analyses using purified cells show clearly that the shift in balance between vascular and hemogenic fate is based on true alterations in gene expression and not just a change in proliferation.

Among the genes that require AP-1 for their induction, we identified TFs, ligands, membrane receptors, cytoskeleton and extracellular matrix proteins (Table S6). However, our data also show that AP-1 is not a cell fate-deciding factor, but plays a role in modulating cell fate decisions, most likely in concert with true master regulators such as RUNX1. We suggest that signaling-responsive TFs boost the expression of genes that actually define a cell, such as metabolic genes, extracellular matrix and focal adhesion genes and other effector genes. One of these genes encodes the tyrosine kinase AXL (O'Bryan et al., 1991). AXL has been shown to be involved in vasculogenesis, both together with its ligand GAS6 and also ligand-independently through VEGFA-VEGFR2 crosstalk and subsequent PI3K activation.
Thus, reduced Axl expression leads to compromised response to VEGFA and in return to attenuated endothelial function. Another gene we have identified as AP-1 dependent encodes the transcription factor Wilms’ tumour 1 (WT1) (Call et al., 1990; Gessler et al., 1990). This zinc finger protein is known to be essential for blood vessel formation, particularly for coronary vessels (Duim et al., 2015; Katuri et al., 2014).

**TEAD requires AP-1 to activate vascular genes in the hemogenic endothelium**

We have previously shown that the interaction between TEAD and YAP is absolutely required for hematopoietic specification (Goode et al., 2016). An important finding of our study is therefore that AP-1-dependent transiently expressed vascular genes in the HE are bound by both AP-1 and TEAD4. During HB differentiation (d1 BC) and in HE1 cells, TEAD4 requires AP-1 to be recruited to joint binding sites, indicating a cross talk between AP-1 (MAPK and others) and Hippo signaling pathways. In HB cells these regulatory elements are not yet bound by TEAD4, although TEAD4 binding is present at other genomic sites at this developmental stage. We were unable to ChIP JUN in FLK1+ cells (unpublished observation) as Fos is only expressed at low levels, but is then up-regulated in the blast culture (Fig S1F), suggesting that in differentiating HB cells AP-1 becomes active, recruits TEAD4 and activates gene expression. In HE2 cells when a hematopoietic fate is acquired in response to the up-regulation of Runx1, these genes are then repressed. Expression remains low in HP cells and AP-1 and TEAD footprints are no longer detectable. In a recent report an early hemogenic precursor cell type in mammalian placentas was identified and its transcriptome was studied (Pereira et al., 2016) and it was found that the genes specific for hemogenic precursors are linked to AP-1-,
TEAD-, TCF- and GATA motifs, supporting the findings of our digital footprinting experiments which provides direct evidence that these sites are occupied.

**AP-1 and TEAD4 binding cis-regulatory elements form signaling hubs in the hemogenic endothelium**

Only recently insights have been gained into the role of signaling pathways involved in embryonic blood specification. For example, pro-inflammatory cytokines including TNFα and IFNγ were reported to promote HSC emergence by acting either upstream or downstream of Notch (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Sawamiphak et al., 2014). WNT signaling via β-catenin is essential for the generation of the hemogenic endothelium (Ruiz-Herguido et al., 2012). Catecholamines from the sympathetic nervous system are important components of the developing HSC microenvironment (Fitch et al., 2012) and BMP4 signaling leads to a SMAD1/5-mediated repression of *Erk* transcription in HE cells, implying a role for mitogen-activated protein kinase (MAPK) signaling in these cells (Zhang et al., 2014). The specification of HSCs from the hemogenic endothelium is a tightly regulated biological process that is controlled by dynamic changes of signaling and TF activity. The HE is a heterogeneous tissue in which only a fraction of cells commits to blood while others remain part of the endothelial layer and may be important to provide signals for the microenvironment to support the EHT (Thambyrajah et al., 2016). Our digital footprinting analyses suggest that HE-specific occupied binding sites form signaling hubs binding TFs responding to different signaling pathways, all of which impact on hematopoietic specification (Kim et al., 2014) and which are becoming decommissioned after the EHT. We propose a model in which various stimuli regulate the expression of the same set of genes but via
different factors: WNT signaling via TCFs, Hippo signaling through TEADs, TGFβ and BMP signaling through SMADs, calcium signaling through NFAT and MAPK / cyclic AMP signaling through AP-1, ATFs and CREB. It remains open, however, whether all of these signals are active simultaneously in one cell or if subpopulations of HE cells respond to individual signals at a given time-point.

In conclusion, our bottom-up strategy identified subtle and varied roles for AP-1 transcription factor family members during embryonic blood development. Future work will determine how different signaling pathways regulate the transcriptional activity of genes associated with signaling hubs, and how these genes are linked to pathways that are required for in vitro generation of transplantable HSCs.

**Materials and methods**

Detailed methods are available in the Supplementary Information.

**Construction of p2lox-dnFOS plasmid, ESC transfection, culture and differentiation**

The Flag-tagged dominant negative FOS (dnFOS) construct was PCR-amplified from CMV plasmid (CMV500-8584hep-fosLZ(MO), A-FOS, kindly provided by Charles Vinson (Olive et al., 1997)) and HindIII as well as Not1 sites were introduced. The GFP of the p2lox plasmid (kindly provided by Michael Kyba (Kyba et al., 2002)) was exchanged for Flag-tagged dnFOS.

A2lox ESCs (a gift from Michael Kyba (Kyba et al., 2002)) or Bry-GFP ESCs were cultured and transfected as described before (Gilmour et al., 2014; Lichtinger et al., 2012; Regha et al., 2015). ESCs were differentiated as described (Gilmour et al.,
2014; Lancrin et al., 2010; Lichtinger et al., 2012). Further details can be found in Supplementary Information.

Chromatin immunoprecipitation (ChIP) and library preparation
2-5x10^6 cells were harvested and PBS-washed before a 2-step crosslinking procedure. First, proteins were crosslinked by incubating cells for 45min at RT in PBS supplemented with 0.83 mg/ml Di(N-succinimidyl) glutarate (DSG, Sigma 80424). After 3 PBS washes, formaldehyde crosslinking of proteins and DNA was done for 10min at RT at a concentration of 1% formaldehyde (Pierce) in PBS. Formaldehyde was quenched by adding glycine to a final concentration of 100mM and crosslinked cells were washed twice in ice-cold PBS. Further, nuclei isolation, sonication and ChIP were performed as previously described (Gilmour et al., 2014; Lichtinger et al., 2012; Regha et al., 2015). Further details can be found in Supplementary Information.

RNA extraction, RT-qPCR and gene expression microarray analyses
For RNA extraction cell pellets were re-suspended in Trizol (Invitrogen) and purified according to the manufacturer’s protocol. The microarrays used were Agilent SurePrint G3 Mouse 8X60K microarrays (catalogue number: G4852A-028005). C-DNA was prepared from the mRNAs using MMLV-RT (Promega M170A) and oligo dT primers as per the manufacturer’s recommendations. Real-time PCR was performed with SYBR Green PCR master mix (Life technologies, 4309155) and in an ABI Stepone realtime PCR machine. Further details can be found in Supplementary Information.
DNaseI digestion and library preparation

DNaseI digestion and libraries were prepared as described (Ptasinska et al., 2012) and deep sequencing was done on Illumina HiSeq2000.

Data accessibility, processing and analysis

Details of the bioinformatics methodologies in this paper can be found in Supplementary information.

Public datasets

The HB DNaseI-seq and TEAD4 ChIP-seq (Goode et al., 2016) as well as mast cell FOS ChIP-seq (Calero-Nieto et al., 2014) datasets were downloaded as SRA archives from the Gene Expression Omnibus accession numbers GSM1692782, GSM1968747 and GSM1167585, respectively. These were converted to fastq via sra-toolkit 2.5.2 (Leinonen et al., 2011). These datasets were processed as other high-throughput sequencing samples from this study.

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Competing interests

The authors declare no competing interests

Author contributions
N.O., J.G., M.L., M.L-A-L. and L.N. performed the laboratory studies, P.C., P.N.C. and S.A. performed bioinformatics analyses, and G.L. and V.K. contributed materials and helped writing the manuscript, C.B. conceived and directed the study and together with N.O. designed the experiments and wrote the manuscript.

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**Data availability**

ChIP-seq, DNasel-seq and gene expression microarray datasets were deposited as subseries on the Gene Expression Omnibus database under accessions GSE79320, GSE79321 and GSE79322, respectively, under superseries GSE79323.
References


Figure 1.

AP-1 inhibition affects differentiation of ESCs to hemangioblast, to hemogenic and to hematopoietic cells

A) Schematic representation of the targeted HPRT-gene locus of the DOX-inducible dnFOS expressing A17 2lox mouse ESC line (dnFOS ESCs).

B) Overview of the \textit{in vitro} differentiation of ESCs to blood cells and the corresponding timecourse of dnFOS induction (DOX).

C) JUN ChIP-seq in uninduced and DOX-treated dnFOS cells. FLK1+ dnFOS cells were cultured under blast culture conditions for 1d +/- 1 µg/ml DOX, cells of complete cultures were double-crosslinked as described in materials and methods and chromatin was used for JUN ChIP followed by genome-wide sequencing. Shown is the JUN ChIP-seq signal in the untreated dataset and the corresponding signal detected in the DOX dataset.

D) Schematic overview of the DOX-induction timecourse approach during blast culture. FLK1+ dnFOS cells were cultured for 4d under blast culture conditions. Cells remained either left untreated (4d untreated) or DOX was added from start of culture (for 4d = 4d DOX), at d1 (3d DOX), d2 (2d DOX) or d3 (1d DOX) of culture. Subsequently, at d4 complete cultures and floating cells were analyzed for cell numbers and surface marker profiles by flow cytometry.

E) Total cell counts (left) and a representative CD41-/Tie2-specific flow cytometric analysis of pre-gated cKit\textsuperscript{pos} cells (right) of d4 complete blast cultures that were either left untreated or DOX-treated for 1d, 2d, 3d or 4d (shown are mean and SD, n=3).
F) Total cell counts (left) and a representative CD11b-specific flow cytometric analysis (right) of d4 floating cells derived from dnFOS blast cultures that were either left untreated or DOX-treated for 1d, 2d, 3d or 4d (shown are mean and SD, n=3).
Figure 2

AP-1 inhibition at the hemangioblast stage enhances cell proliferation and shifts the balance between vascular and blood cell development

A) Experimental setup: dnFOS ESCs were differentiated for 3.75d as EBs, FLK1+ HB cells were purified and subsequently cultured +/- 1µg/ml DOX under blast culture conditions for 1d, 2d or 3d, respectively, before either complete cultures (d1 and d2) or floating cells (d3) were analyzed.

B) Fold changes of cell numbers at d1 compared to d0 (= seeded cell numbers) of complete dnFOS blast cultures with and without DOX induction (shown are mean and SD, n=5, t-test).

C) Complete d1 dnFOS blast cultures with and without DOX induction were assessed by intracellular SM cell-specific Calponin staining. A representative flow cytometric analysis and the summary of 3 experiments is shown (for other smooth muscle cell markers and statistical summary see Fig S2).

D) Fold changes of cell numbers at d2 compared to d0 (= seeded cell numbers) of complete dnFOS blast cultures with and without DOX induction (shown are mean and SD, n=5, t-test).

E) Complete d2 dnFOS blast cultures with and without DOX induction were assessed by intracellular SM cell-specific Calponin staining. A representative flow cytometric analysis and the summary of 3 experiments is shown (for other smooth muscle cell markers and statistical summary see Fig S2).
F) Cell composition of complete d2 dnFOS blast cultures +/- DOX was analyzed by flow cytometry using antibodies against KIT, TIE2 and CD41. A representative contour plot of pre-gated KIT$^+$ cells is shown.

G) Shown is the number of dnFOS floating cells with and without DOX induction at d3 per FLK1$^+$ cell that was seeded at d0 (shown are mean and SD, n=4).

H) Floating cells of d3 dnFOS blast cultures with and without DOX induction were harvested and stained with a CD11b-specific antibody prior to flow cytometric analysis. A representative contour plot is shown.

I) Floating cells derived from d3 dnFOS blast cultures with and without DOX induction were harvested and plated into methylcellulose medium for a hematopoietic colony assay (in the absence of DOX). After 10 days colonies were counted and classified as BFU-E, CFU-Mix and CFU-GM (shown are mean and SD, n=3). Examples of colonies are shown at the bottom.
**Figure 3**

**dnFOS induction causes distinct changes to global gene expression programmes**

A) Experimental setup: dnFOS ESCs were differentiated for 3.75d as EBs, FLK1<sup>+</sup> HB cells were purified and subsequently cultured with and without 1µg/ml DOX under blast culture conditions for 5h, 24h or 48h. Freshly purified FLK1<sup>+</sup> cells (=0h), 5h and 1d cultures with and without DOX induction were used directly for RNA extraction, while d2 cultures with and without DOX induction were sorted by FACS into pure populations of SM, HE1, HE2 and HP cells prior to RNA extraction. RNA was used for genome-wide gene expression arrays. Each population was analyzed in duplicates.

B) Principle component analysis with 3 components for all 13 indicated cell types.

C) Pairwise comparison of microarray data from untreated and DOX treated cells. Depicted are the numbers of significantly (at least two-fold) up- and down-regulated genes. The numbers on the x-axis represent up-regulated genes between DOX and untreated samples for each time point, the numbers on the y-axis represent down-regulated genes between DOX and untreated samples for each time point. The numbers within the table show how many of the up-/down-regulated genes at each time point are also mis-regulated (green: down, red: up) at other time points.

D) SM, HE1, HE2 and HP samples with and without DOX induction were used for k-means clustering by fold change of genes that change expression at
least 2 fold upon dnFOS induction. For the resulting 15 clusters of genes a heatmap was generated and some genes of interest are indicated next their respective cluster.

E) Shown are expression values of exemplary genes over the course of differentiation with and without DOX induction based on data from microarrays. Individual replicates for each sample are depicted.
Figure 4

During hemangioblast commitment FOS and JUN bind to genes involved in blood vessel development, cell adhesion and cell signaling

A) Experimental setup: Bry-GFP WT ESCs (JUN) or dnFOS ESCs (FOS) were differentiated as indicated, FLK1^+ cells were purified and cultured for 1d under blast culture conditions in the absence of DOX. Cells of complete cultures were double-crosslinked as described in materials and methods and chromatin was used for FOS and JUN ChIP followed by genome-wide sequencing.

B) Binding and genome localization statistics of FOS and JUN peaks within the mouse genome.

C) Overlap of peaks detected in FOS and JUN ChIP-seq datasets.

D) Overlap of genes associated with FOS and JUN peaks.

E) Overlap of genes associated with intersecting FOS and JUN peaks (C) and genes associated with FOS and JUN binding events (D).

F) Representative genome browser screen shots of FOS and JUN ChIP-seq at the Lamc2 and Nrp1 loci.

G) Gene ontology analysis of genes bound by FOS only, JUN only, FOS:JUN co-bound and bound by FOS and JUN distinctly. Shown are the top GO terms and the corresponding –log p-value.
Figure 5

AP-1 binding is required in SM and HE1 for the transient activation of genes and is enriched at AP-1 and TEAD motifs

A) K-means clustering of log$_2$ expression levels of genes associated with FOS:JUN co-bound sites. Each of clusters (1-7) represents a specific pattern of gene expression levels.

B) Average mRNA microarray expression values of genes from clusters 1-7 as defined in (B), without (black) and with (red) DOX for the different cell types of the differentiation.

C) Enriched transcription factor binding motifs in FOS-only, FOS:JUN and JUN-only peaks using HOMER *de novo* motif discovery analysis.
Figure 6

**AP-1 is required for de novo TEAD4 binding during differentiation of HB to HE**

A) Experimental setup: dnFOS ESCs were differentiated into EBs FLK1+ cells were purified and cultured for 24h under blast culture conditions with and without 1µg/ml DOX. Cells of complete cultures were double-crosslinked and chromatin was used for TEAD4 ChIP followed by genome-wide sequencing.

B) Overlap of TEAD4 ChIP-seq peaks in DOX- treated and untreated d1 BC cells.

C) Enriched transcription factor binding motifs within TEAD4 ChIP-seq peaks from DOX- treated and untreated samples using HOMER *de novo* motif discovery analysis.

D) TEAD4 ChIP-seq signal from DOX- treated and untreated cells (left), TEAD and AP1 motif presence (middle) and d1 BC DOX/untreated gene expression fold change (right) ordered by increasing DOX/untreated TEAD4 ChIP-seq signal. Classes of peaks are indicated to the left and defined using cut-offs of ±1 log₂ fold change, with class-specific average profiles as well as a boxplot showing gene expression fold change at the bottom.

E) JUN and FOS signals (left) and presence of FOS:JUN intersecting peaks, JUN- and FOS-only peaks (right) ordered as in (D). Bottom: average profiles for peak presence for classes defined in (D).
F) Representative genome browser screenshot of the Lamc2 locus showing TEAD4 loss at AP-1 binding sites following dnFOS induction (red arrow).
Figure 7

AP-1 and TEAD footprints occur together in HE1 DHS peaks and are strongly reduced in HP hypersensitive sites

A) Transcription factor binding motifs enriched in HE1-specific DHSs employing HOMER \textit{de novo} motif discovery.

B) As in (A) examining HP-specific DHSs.

C) Heatmaps depicting HE1 and HP DNaseI-seq signal (left), motif (middle) and gene expression fold change (right) sorted based on increasing HP/HE1 fold change.

D) Co-occurrence clustering of factor binding motifs specifically occupied (footprinted) in HE1 demonstrating the co-occurrence of motifs for signaling responsive transcription factors. Z-sores represent enrichment over 1000 equally-sized, random sub-samplings in HP DHSs.

E) AP-1 and TEAD motifs are arranged in a specific conformation. Distribution of AP-1 motif start distances to TEAD motif starts, aligned by TEAD motif orientation (top). Bottom: composite TEAD-AP1 motif with 7bp spacer.
Figure S1 Establishment and characterization of ESCs expressing an inducible dnFOS (related to Figure 1)

A) Scheme representing setup and conditions of the *in vitro* differentiation of ESCs to FLK1+ HB and further to SM, HE1, HE2 and HP cells.

B) Shown are the results of a luciferase assay in RAW cells that were transfected with the TK control plasmid or the TK plasmid with 3 AP-1 binding sites upstream of the firefly luciferase, together with either an empty CMV vector or the CMV vector with dnFOS. The cells remained either untreated or were stimulated for 4h with 100mM PMA to enhance AP-1 activity. Firefly luciferase activity was normalized to the renilla luciferase signal (n=2).

C) Control of dnFOS induced expression. Targeted and selected dnFOS ESC clones were treated with 1µg/ml DOX for 24h, RNA was extracted and dnFOS expression was measured with Flag-tag-specific primers. Expression is shown as relative to gapdh expression.

D) Flag-specific immunostaining of dnFOS clone#8 ESCs that were DOX-treated for 24h.

E) JUN ChIP analysis of dnFOS#2 cells that were cultured for 2d in blast culture conditions +/- 1µg/ml DOX (shown are mean and SD, n=3).

F) Left: gene expression heatmap of different AP-1 family members in FLK1+ cells and sorted blast culture cell types (SM, HE1, HE2 and HP). Right: Western blot probing for JUN (and GAPDH control) protein expression in FLK1+, FLK1+, d1 blast culture cells and floating cells at d3 and d4.

G) Top: fold change of cell numbers of d3.75 dnFOS EB cultures +/-DOX (shown are mean and SD, n=4, t-test). Bottom: representative flow cytometric analysis of FLK1+ cells within d3.75 dnFOS EB cultures +/-DOX.

H) Normalized fold change of FLK1+ cells within d3.75 dnFOS EBs that were derived from either untreated or DOX-treated (1µg/ml) cultures (shown are mean and SD, n=6, t-test).

I) Representative KIT-positive cells within d4 complete blast cultures that were either untreated or DOX-treated for 1d, 2d, 3d or 4d (shown are 2 individual replicates).

J) Proportion of KIT-positive cells within d4 complete blast cultures that were either untreated or DOX-treated for 1d, 2d, 3d or 4d (shown are 2 individual replicates).

K) Proportion of KIT+CD41+Tie2+ cells within d4 complete blast cultures that were either untreated or DOX-treated for 1d, 2d, 3d or 4d (shown are 2 individual replicates).

L) Summary of normalized proportions of CD11b positive cell of d4 floating cells derived from dnFOS blast cultures that were either untreated or DOX-treated for 1d, 2d, 3d or 4d (corresponding FACS plots in Figure 1F, shown are mean and SD, n=3, t-test).

M) DOX-withdrawal experiment: shown are the normalized cell numbers of floating cells derived from d3 blast cultures which were either untreated, 3d with DOX, untreated with fresh untreated media after 1d or DOX-treated for 1d with fresh untreated media from d1 to d3 (mean and SD, n=3, t-test).

N) DOX-withdrawal experiment: shown are the normalized frequencies of CD11b+ cells within floating cells derived from cultures as described in M) (mean and SD, n=3, t-test).
Figure S2
Induction of dnFOS affects proliferation and differentiation (related to Figure 2)

A) Complete d2 dnFOS blast cultures (BC) +/- DOX were analyzed by flow cytometry for apoptosis by AnnexinV/propidium iodide staining. A representative plot is shown.

B) Complete d3 dnFOS blast cultures +/- DOX were analyzed by flow cytometry for DNA content by propidium iodide staining of ethanol-fixed cells. Percentages of cells in G1, S and G2 phase were calculated by Flow Jo software and are indicated.

C) qPCR expression data for the dnfos transgene and Cdkn2a relative to Gapdh expression of RNA obtained from complete d3 dnFOS blast culture +/- DOX (mean and SD, n=4, t-test).

D) Complete d1 blast cultures (BC) +/- DOX were analyzed by intracellular flow cytometry for smooth muscle cell markers SMA, SM22a and Calponin. Percentages of marker positive cells are shown (mean and SD, n=3, t-test) and a representative FACS analysis for SMA is shown.

E) Complete d1 blast cultures (BC) +/- DOX were analyzed by flow cytometry for endothelial markers CD31, TIE2 and FLK1. Percentages of marker positive cells are shown (mean and SD, n=3, t-test) and a representative FACS analysis for SMA is shown.

F) Complete d2 blast cultures (BC) +/- DOX were analyzed by intracellular flow cytometry for smooth muscle cell markers SMA, SM22a and Calponin. Percentages of marker positive cells are shown (mean and SD, n=3, t-test) and a representative FACS analysis for SMA is shown.

G) Complete d2 blast cultures (BC) +/- DOX were analyzed by flow cytometry for endothelial markers CD31, TIE2 and FLK1. Percentages of marker positive cells are shown (mean and SD, n=3, t-test).

H) Proportion of KIT+Tie2+CD41+ cells in d2 dnFOS blast cultures +/- DOX (mean and SD, n=7, t-test).

I) Floating cells of d3 blast cultures (BC) +/-DOX were analyzed by flow cytometry for KIT, CD41, CD71, CD45, CD11b and SCA1. Percentages of marker positive cells are shown (mean and SD, n=5, t-test).

J) Shown are representative flow cytometry data of cells as described in I).

K) Floating cells of d3 blast cultures (BC) +/-DOX were plated into methylcellulose medium for colony assays in the absence of DOX for 7-10 days. Subsequently representative colonies were picked, cytopspun and Giemsa-stained. The arrows indicate the presence of macrophage-like cells.
**Supplementary information**

**A**

**B**

**C**

**D**

**E**

**F**

**G**

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**Top GO terms:**

- **Cluster 01**
  - metabolic process
  - localization
  - transport
  - hematopoiesis

- **Cluster 02**
  - reproduction
  - gamete generation
  - nucleosome organization

- **Cluster 03**
  - signal transduction
  - response to cold

- **Cluster 04**
  - immune response
  - response to stimulus
  - cell adhesion

- **Cluster 05**
  - developmental process
  - cell differentiation
  - transcription

- **Cluster 06**
  - nucleic acid metabolism
  - transcription
  - gene expression

- **Cluster 07**
  - metabolic process
  - respiratory system development

- **Cluster 08**
  - response to stimulus
  - cell differentiation
  - development

- **Cluster 09**
  - nucleic acid metabolism
  - cell cycle

- **Cluster 10**
  - disease process
  - cell differentiation
  - development

- **Cluster 11**
  - neurological system development
  - cell differentiation

- **Cluster 12**
  - response to wounding
  - embryonic development

- **Cluster 13**
  - blood vessel morphogenesis
  - cell differentiation

- **Cluster 14**
  - cell differentiation
  - response to stimulus

- **Cluster 15**
  - cell differentiation
  - soma development

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**Gene expression relative to normalised expression**

- **Flk1+ 5h**
- **HE1**
- **HE2**
- **SM**
- **HP**

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**Gene expression changes**

- **Cdkn2b**
- **Col1a1**
- **Gfi1**
- **Flk1+ 5h**
- **Cebpa**
- **d1 BC**

---

**Gene expression localisation**

- **Flk1+ 5h**
- **HE1**
- **HE2**
- **SM**
- **HP**

---

**Gene expression changes**

- **Cdkn2b**
- **Col1a1**
- **Gfi1**
- **Flk1+ 5h**
- **Cebpa**
- **d1 BC**

---

**Gene expression changes**

- **Cdkn2b**
- **Col1a1**
- **Gfi1**
- **Flk1+ 5h**
- **Cebpa**
- **d1 BC**

---

**Gene expression changes**

- **Cdkn2b**
- **Col1a1**
- **Gfi1**
- **Flk1+ 5h**
- **Cebpa**
- **d1 BC**
Figure S3
Alteration of gene expression in dnFOS expressing differentiating cells (related to Figure 3)

A) Representative FACS contour plots demonstrating gating strategy and purity of d2 blast culture sorted cell populations SM (KIT- TIE2- CD41-), HE1 (KIT+TIE2+CD41-), HE2 (KIT+ TIE2+ CD41+) and HP (KIT+ TIE2- CD41+) that were subjected to RNA extraction for microarray analysis.

B) Complete d2 blast culture cells were first surface-stained using KIT-, TIE2- and CD41-specific antibodies followed by a subsequent SMA-specific intracellular staining. Live cells were first gated on KIT+ and KIT- populations, KIT+ cells were further subdivided into TIE2-CD41- (HE1), TIE2-CD41+ (HE2) and TIE2-CD41+ (HP) populations, while KIT- cells were further gated on TIE2-CD41- cells (triple neg). All 4 populations were analyzed for SMA-Cy3 fluorescence and a representative histogram overlay is shown.

C) PCAs for individual replicates of gene expression microarray data.

D) Hierarchical clustering of standardized gene expression for all differentially expressed genes with at least 2-fold difference after dnFOS induction.

E) Summary of top hit gene ontology terms for the respective clusters of genes obtained from k means clustering (see Fig. 3D).

F) Manual validation of gene expression microarray data. Expression of Gfi1, Sfpi1, Cdkn2a, Sox7 and Runx1 was measured relative to Gapdh by qPCR using RNA obtained from +/- DOX HE2 and HP FACS-sorted cell populations (top, individual replicates for each sample are depicted). The corresponding gene expression microarray values of the individual replicates are depicted underneath.

G) Expression values of exemplary genes over the course of differentiation +/-DOX based on the gene expression microarray data. Individual replicates for each sample are depicted.
Figure S4
Genome-wide binding of FOS and JUN (related to Figure 4)

A) Phenotypic characterisation of d1 blast culture cells by FACS. From left to right: SSC and αrb-Cy3-only intracellular staining, SSC and αSMA+αrbCy3 intracellular staining, FSC and KIT-APC, TIE2-PE and CD41-PE-Cy7 gated on KIT-positive cells.

B) Example UCSC genome browser screenshots of FOS and JUN ChIP-seq at the Flnb, Jund, Tgfbi, Col1a1 and Cdkn2a loci.

C) Venn diagram showing the overlap of peaks detected in FOS, JUN (Bry-GFP) and JUN (dnFOS) ChIP-seq datasets.

D) Gene ontology analysis of genes co-bound by FOS:JUN (dnFOS, replicate 2). Shown are the top GO terms and the corresponding –log p-value.
**A**

JUN

ES = 0.329, NES = 10.029, p/FDR-q/FWER p = 0

FOS

ES = 0.301, NES = 9.302, p/FDR-q/FWER p = 0

**B**

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**C**

JUN only

FOS:JUN

FOS only

Distance to summit

**D**

JUN

FOS
Figure S5
JUN and FOS bind to elements containing AP-1 and TEAD motifs and binding is correlated with gene expression (related to Figure 5)

A) Shown are the enrichment plots of a GSEA (left JUN, right FOS) using gene expression fold change of dnFOS blast culture cells (24h +/- DOX, left down-regulated +DOX versus -DOX in red, right up-regulated in blue) and the corresponding presence/absence of the top 1000 annotated JUN and FOS peaks (black lines). The enrichment score (ES), normalized ES score, nominal p-, FDR q- and FWER p-values are shown underneath each plot.

B) FOS and JUN ChIP-seq signal heatmaps (left) at JUN-only, FOS:JUN shared and FOS-only peaks as defined in Figure 4C, with log2 expression fold change for associated genes (right).

C) Heatmaps showing FOS and JUN signals (left) and AP-1, CREB, and TEAD motif frequencies (right) in populations described in Figure 4C.

D) Motif co-occurrence clustering (bootstrapping analysis) within 50 bp of all motifs enriched in FOS or JUN peaks in each peak dataset. Z-scores represent enrichment over 1000 equally-sized, random sub-samplings of HE1 DNase I sites.
**A**

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**B**

Peak overlap

**C**

**D**

JUN peaks

**E**

Invariant TEAD4 peaks

**F**

**G**

TEAD4 FLK1+ class1

**H**

**I**

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Figure S6
Interaction between AP-1 and TEAD factors (related to Figure 6)

A) Binding and genome localization statistics for d1BC TEAD4 ±DOX and HE ChIP-seq peaks across the mouse genome
B) Representative genome browser screenshots at the Bmp1 and Cdkn2b loci (top, bottom) showing TEAD4 loss at AP-1 peaks following dnFOS induction (red arrows). DNaseI hypersensitivity at the respective position in the same cell types is shown as the bottom red tracks.
C) Overlap of JUN, FOS and TEAD4 binding events in d1 BC cells in the absence of DOX.
D) TEAD4 ±DOX ChIP-seq heatmaps at JUN peaks by decreasing JUN signal (left), and for FOS (middle) as in Figure S6A-B. Right: TEAD4 ±DOX ChIP-seq heatmaps at invariant sites overlapping with neither JUN nor FOS peaks. Average profiles of TEAD4 ChIP-seq are shown at the bottom.
E) TEAD4 ChIP-seq signal heatmaps for d1 blast culture ±DOX and HB by increasing TEAD4 ±DOX fold change, as in Figure 7D.
F) Heatmaps depicting log2 expression fold changes of genes associated with TEAD4 peaks ranked as in (E).
G) Average profiles for TEAD4 HB and HE ChIP-seq signals per class, as defined in Figure 7D.
H) Venn diagrams highlighting intersections of all pair-wise combinations of TEAD4 HB, d1BC ±DOX and HE ChIP-seq datasets. Hypergeometric p-values are shown underneath each Venn diagram.
I) Gene ontology analysis (molecular function) on genes associated with peak classes as defined in Figure 7D.
Figure S7
AP-1 and TEAD binding at vascular genes is abolished in HP cells (related to Figure 7)

A) Binding and genome localization statistics of HE1 and HP DNasel-seq peaks within the mouse genome.
B) Overlap of HE1 and HP DNasel-seq peaks. The hypergeometric overlap p-value is shown underneath.
C) HE1 and HP DNasel-seq signal (left), motif (middle). FOS, JUN ChIP-seq in d1 BC cells and FOS ChIP-seq in mast cells (right, Caliero-Niero et al., 2015) sorted by increasing HP/HE1 fold change, as in Figure C.
D) Venn diagram overlap of HE1 and HP footprints. The hypergeometric overlap p-value is shown underneath.
E) Number of HE1- and HP- footprinted AP-1 and TEAD motifs.
F) HE1 and HP footprinting occupancy scores of HE1-footprinted AP-1 (top) and TEAD motifs (bottom) indicating a high correlation between ChIP-seq and footprinting signals.
G) Heatmaps showing DNaseI cutting strand imbalances around footprinted AP-1 motifs in HE1 and HP, by motif orientation. Red represents higher numbers of cuts on the positive strand, green on the negative strand with a gap in the middle indicating motifs protected from DNaseI cutting.
H) As in (G) for footprinted TEAD motifs.
I) HE1 and HP footprinted AP-1 (left) and TEAD motifs (right) ordered by decreasing JUN (left) and FOS (right) ChIP-signal, as in Figure S6A-B. Average profiles shown underneath.
J) D1 BC ±DOX TEAD4 ChIP-seq signal heatmaps sorted by TEAD4 ChIP-seq fold change (left) as in Figure 6D are compared to HE1- and HP- footprinted AP-1 motifs (middle) and AP-1 motif presence (right).
K) Co-occurrence clustering of HP-footprinted motifs. Z-scores represent enrichment over 1000 equally-sized, random sub-samplings in HE1 DHSs.
L) Sequences of 14 TEAD and AP-1 motifs with a fixed 7bp spacer (Figure 7E)
Figure S8
Model showing the effects of AP-1 inhibition during hemangioblast differentiation and hematopoietic specification

AP-1 (FOS) is up-regulated in differentiating HB cells, binds to de novo sites, recruits TEAD4 and activates vasculature genes (endothelial, smooth muscle). When AP-1 activity is inhibited, the balance of development is shifted towards the hemogenic endothelium.

When HE cells differentiate to blood AP-1 and TEAD binding is lost. In mature blood cells AP-1 is required to activate myeloid-specific genes. At that stage TEAD binding is no longer present.
List of Supplemental Tables

Table S1: List of all differentially expressed genes over the whole differentiation and in each cell type +/- dnFOS

Click here to Download Table S1

Table S2: List of genes in clusters 1-15 (linked to Fig 3D), their expression fold change and GO terms

Click here to Download Table S2

Table S3: List of peaks obtained from FOS and JUN ChIP-seq with genomic coordinates and closest genes annotated

Click here to Download Table S3

Table S4: List of target genes, their GO terms and KEGG pathway terms associated to JUN only, FOS only, FOS and JUN distinct and FOS:JUN co-bound sites

Click here to Download Table S4

Table S5: List of FOS:JUN co-bound genes in clusters 1-7 (linked to Fig 5B) and their averaged expression +/- DOX

Click here to Download Table S5

Table S6: List of FOS:JUN co-bound genes in cluster 5 and 6 (Fig 5B), their expression fold change in d1 BC, SM and HE1 cells +/- DOX and their affiliation to class 1 TEAD4 binding sites (linked to Fig 6D)

Click here to Download Table S6

Table S7: List of peaks obtained from TEAD4 ChIP-seq in d1 BC +/-DOX, ranked by tag count fold change, with genomic coordinates and closest genes annotated

Click here to Download Table S7
Supplementary materials and methods

ESC culture and p2lox dnFOS targeting

A2lox ESCs (a gift from Michael Kyba (Kyba et al, 2002)) or Bry-GFP ESCs were cultured as described before (Gilmour et al, 2014; Lichtinger et al, 2012; Regha et al, 2015). Briefly, cells were grown on mitomycin C-inactivated mouse embryonic feeder cells (MEFs) in DMEM medium supplemented with 15% FCS (Stem Cell Technologies), 1 mM sodium pyruvate, 1 mM glutamine, 100 units per ml penicillin and 100 µg ml−1 streptomycin, 25 mM HEPES buffer, 1 × non-essential amino acids, 0.15 mM MTG and 103 units per ml LIF (ESGRO mLIF, Millipore ESG1107). Medium was changed every day and cells were passaged every 2nd day using TrypLE Express (Thermo Fisher). For transfection with p2lox-dnFOS plasmid, 2.5x10⁶ A2lox ESCs were resuspended in 100 µl PBS (Sigma D8537), 20 µg of each p2lox-dnFOS plasmid and Cre-expressing plasmid were added and cells were electroporated at 240 V, 7 ms settings on an EPI 2500 electroporator (Fischer). Cells were plated quickly on NEO-resistant feeder cells in ESC medium and 1 day later selection was started by adding 300 µg/ml G418. Clones were picked after 6 days of selection, assessed for their differentiation ability and transgene expression upon doxycycline addition.

In vitro hematopoietic differentiation

ESCs were differentiated as previously described (Gilmour et al, 2014; Lancrin et al, 2010; Lichtinger et al, 2012). Briefly, ESCs were cultured for one day on gelatinized plates in DMEM ESC medium and for another day on gelatinized plated in IMDM ESC medium in the presence of LIF. Subsequently, cells were differentiated as embryoid bodies (EBs) in 15cm bacterial-grade dishes at 1.25x10⁶ cells per 50 ml for 3.75 days in IMDM differentiation medium without LIF. EBs were then dispersed to single cells by TrypLE Express treatment and FLK1⁺ cells were purified using MACS technology. Purified FLK1⁺ cells were cultured in gelatinized T150 tissue culture-grade flask at 1.2x10⁶ cells per flask in blast culture medium (IMDM supplemented with 10% FCS, 100 units per ml penicillin and 100 µg ml−1 streptomycin, 1 mM glutamine, 0.45 mM MTG, 0.18 mg ml−1 Human transferrin (Roche 10652202001) 25 µg ml−1 ascorbic acid, 20% D4T conditioned media, 5 µg l−1 recombinant mouse Vascular Endothelial Growth Factor (Peprotech: 450-32), 10 µg l−1 mIL-6 (Peprotech: 216–16)). For dnFOS-induction cells were treated with 1µg/ml doxycycline for the duration indicated in each experiment.
Methylcellulose hematopoietic progenitor colony assay

Floating progenitors were harvested from the supernatant of d3 blast cultures +/- DOX, counted and plated in triplicates at 10,000 cells per ml of MethoCult methylcellulose (Stem Cell Technologies: M3434) without DOX. After 7-10 days colonies were counted and classified as BFU-E, CFU-Mix or CFU-GM.

Giemsa staining

Individual colonies of methylcellulose cultures were picked, washed in PBS and immobilized on microscope slides by cytospin (4 min, 800 rpm, Shandon Cytospin 3). Cells were fixed with methanol for 1min, air-dried, Giemsa-stained for 3min (Sigma, GS500) and analyzed by microscopy.

Flow cytometry and FACS sorting

Cells at developmental stages indicated in each experiment were harvested by TrypLE Express treatment (floating progenitor were just taken together with culture supernatant) and stained on ice in 100 µl MACS buffer (PBS, 0.5% BSA, 2mM EDTA) with individual or combinations of the following antibodies at the given dilutions: 1:100 anti-mouse CD202b (TIE2) PE (eBioscience: 12-5987-81), 1:50 APC-conjugated rat anti-mouse CD117 Clone 2B8 (KIT) (BD Pharmingen: 553356), 1:100 anti-mouse CD41 PE-Cy7 (eBioscience: 12-0411-82), 1:100 anti-mouse CD11b PE (eBioscience: 12-0112-81), 1:100 CD45-APC (eBioscience: 17-0451-82), 1:100 SCA1-PE (eBioscience: 12-5981-83), 1:100 CD71-PE (eBioscience: 12-0711-82), 1:100 FLK1-PE (eBioscience: 12-5821-83), 1:100 CD31-APC (BD Biosciences: 551262).

For intracellular staining of smooth muscle cell markers, ~ 3x10^5 blast culture cells were fixed for 30 min at room temperature (RT) in 300 µl Fixation/Permeabilization buffer (eBiosciences: 00-5123 and 00-5223), washed with Permeabilization buffer (eBiosciences: 00-8333), incubated with 100 µl Permeabilization buffer and 1:50 rabbit anti-mouse SMA (abcam: ab32575), 1:100 rabbit anti-mouse Calponin (abcam: ab46794) or 1:100 goat anti-mouse SM22a (abcam: ab10135) for 1h at RT, washed twice, incubated with 1:800 anti-rabbit Cy3 (abcam: ab97075) or 1:2000 anti-goat Alexa Fluor^R 647 (abcam: ab150131) for 45min at RT in dark and washed with Permeabilization buffer and MACS buffer before analysis.

DNA content was measured in cells after fixation in 70% ice-cold ethanol by incubating cells for 30min at 37°C with 100µg/ml Rnase and 50µg/ml propidium iodide and a subsequent washing step.
Cell death-specific staining was done using Annexin V-FITC apoptosis detection kit (BD Pharmingen: 556547) by following the manufacturer’s instructions.

All stainings were measured using a Cyan ADP flow cytometre with Summit 4.3 software and analyzed with FlowJo software. Isotype controls were used to define gating strategy. FACS sorting was done on a Moflow sorter.

**RNA extraction, RT-qPCR and gene expression microarray analyses**

For RNA extraction cell pellets were resuspended in Trizol (Invitrogen) and purified according to the manufacturer’s protocol. To increase the yield of RNA from small cell numbers after FACS sorting, glycogen was added to Trizol. For microarrays, RNA was further purified using MinElute RNeasy columns (Qiagen). RNA concentration was determined by a nanodrop and its integrity checked using an Agilent Bioanalyzer. The microarrays used were Agilent SurePrint G3 Mouse 8X60K microarrays (catalogue number: G4852A-028005).

C-DNA was prepared from the mRNAs using MMLV-RT (Promega M170A) and oligo dT primers as per the manufacturer’s recommendations. Real-time PCR was performed with SYBR Green PCR master mix (Life technologies, 4309155) and in an ABI Stepone realtime PCR machine.

**RT-PCR expression primers**

- **Dnfs**
  - TAGCATGACTGGTGAGGACAGC
  - AGCTTTCGGCCTCTTTTTC
- **Gapdh**
  - ACCTGCAAGATGATGACATCA
  - GGTCCAGTGTAGCCCAAGA
- **Cdkn2a**
  - CGTGAAGGACTGCTGGAGAAG
  - ACCAGCGTGTCCAGGAAGCC
- **Gfi1**
  - GTGAGCCTGGAGCAACACAA
  - CTCTTGAAAGCTCTTGGCACCACAGA
- **Sfpi1**
  - CCA TAG CGA TCA CTA CTG GGA TTT
  - TGT GAA GTG GTT CTC AGG GAA GT
- **Sox7**
  - GAACCCGGACCTGCACAAC
  - GCTCTGCTCATTCCACATAAGG
**Runx1**

AGATTCAACGACCTCAGGT

CGGATTGTAAAGACG

**Immunofluorescence staining**

dnFOS ESCs were cultured for 2 days on glass cover slips in 24 well plates with MEFs and ESC medium. Cultures were treated for 24h with 1µg/ml doxycycline and fixed for 10min at RT in PBS + 4% formaldehyde. After two washes with PBS cells were permeabilized for 10min at RT in PBS + 0.5% TritonX-100 + 1% BSA. Blocking of unspecific epitopes was done by 30min incubation with PBS + 5% BSA + 0.2% gelatine at 37°C. Subsequently, the first antibody (anti-Flag, abcam ab1162) was incubated over night at RT at 1:500 dilution in PBS + 3% BSA + 0.2% gelatine. After three washes with PBS, 2nd antibody was added (3h, RT, PBS + 3% BSA + 0.2% gelatine, 1:800 dilution of anti-rabbit Cy3 (abcam: ab97075)). After 3 washes in PBS, the coverslip was removed from the well and inverted onto a glass slide while mounting with DAPI-containing mounting medium. Slides were imaged at a Zeiss Axioplan2 microscope with Smart Capture 3 software.

**Western Blotting**

Protein extracts in Laemmli buffer were separated on 4-20% pre-cast gradient SDS-PAGE gels (Biorad) and Western blots prepared by wet transfer onto nitrocellulose membrane. Blots were blocked with 5 % milk powder in 0.05 % TBS-Tween and incubated with anti-JUN (Santa Cruz: sc1694), and anti-GAPDH (abcam: ab8245) antibodies. Proteins were visualized using Pierce SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

**Chromatin immunoprecipitation (ChIP) and library preparation**

2-5x10^6 cells were harvested and PBS-washed before a 2-step crosslinking procedure. First, proteins were crosslinked by incubating cells for 45min at RT in PBS supplemented with 0.83 mg/ml Di(N-succinimidyl) glutarate (DSG, Sigma 80424). After 3 PBS washes, formaldehyde crosslinking of proteins and DNA was done for 10min at RT at a concentration of 1% formaldehyde (Pierce) in PBS. Formaldehyde was quenched by adding glycine to a final concentration of 100mM and crosslinked cells were washed twice in ice-cold PBS. Further, nuclei isolation, sonication and ChIP were performed as previously described (Gilmour et al, 2014; Lichtinger et al, 2012; Regha et al, 2015). Briefly, nuclei were sonicated using a Bioruptor (Diagenode), insoluble debris was centrifugated, then sheared chromatin fragments were incubated for ~4h at 4°C with antibodies coupled to 15 µl Protein G dynabeads (Invitrogen). Antibodies were used at the following concentrations per ChIP (per 2-5x10^6 cells): TEAD4 – 3 µg (abcam, ab58310), FOS – 3 µg (Santa
Cruz, sc253x), JUN – 5 µg (Santa Cruz, sc1694x). Beads were washed with low salt, high salt, LiCl and Na/TE buffers before crosslinks were reversed as described (Gilmour et al, 2014). DNA was extracted using Ampure beads (Beckman Coulter) and qPCRs were performed to validate ChIP quality using negative control primers (Chr2) and positive control primers (Tbp promoter). For library preparation, ChIP DNA of experiments was pooled (TEAD4_FLK1*: 2 ChIPs, TEAD4_HE1: 1 ChIP, TEAD4_d1BC_noDOX: 3 ChIPs, TEAD4_d1BC_DOX: 3 ChIPs, JUN_d1BC: 2 ChIPs, FOS_d1BC: 1 ChIP) and libraries were prepared using either Kapa Hyper Prep Kit (for TEAD4 libraries) or Illumina TruSeq Library preparation Kit (for FOS and JUN libraries) according to the manufacturer’s guidelines. Libraries were size-selected on a 2% agarose gel for 200-400 bp fragments and re-validated by qPCR before high throughput sequencing. Libraries were sequenced on Illumina Genome Analyzer Ix (FOS ChIP), Illumina HiSeq 2000 (FOS and JUN ChIP) and Illumina HiSeq2500 (TEAD4 ChIPs).

ChIP-PCR primers

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**DNaseI digestion and library preparation**

Freshly FACS-sorted HE1 (KIT+TIE2+CD41-) cells and HP (KIT+TIE2-CD41+) cells differentiated from E14 ESC were thoroughly counted and resuspended at 1x 10^6 cells per 100 µl DNase I buffer (60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 10 mM Tris pH 7.4, 0.3 M sucrose), mixed with an equal volume of DNaseI NP-40 buffer (60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 10 mM Tris pH 7.4, 0.3 M sucrose, 0.4% NP-40, 2 mM CaCl2) with suitable 2-fold concentration of DNaseI enzyme (final concentration ranging between 30 U for HE1 and 40 U for HP) and incubated at 22°C for 3 min. The reaction was stopped by addition of 2x volume of lysis buffer (300 mM NaAcetate, 10 mM EDTA pH 7.4, 1% SDS, 1 mg/ml proteinase K) and samples were incubated over night at 45°C. After phenol-chloroform extraction DNA was precipitated with 2x volume of 100% ethanol, 10 μg of digested DNA were run on a 1.2% agarose gel in TAE buffer, 100 - 500 bp fragments were size-selected and purified using Qiagen MinElute gel extraction kit. Before library preparation the DNaseI digested and size-selected material was validated by realtime PCR analysis using Chr2 negative control and Tbp positive control primers. Libraries were prepared as described (Ptasinska et al, 2012) and deep sequencing was done on Illumina HiSeq2000.

**Luciferase assay**

RAW cells were grown in DMEM medium supplemented with 10% FCS, Penicillin and Streptomycin and plasmids were transfected using Invitrogen Lipofectamine according to the manufacturer’s guidelines. Plasmids were used at the following concentrations: TK (pXPG-TK, as negative control, 500 ng), 3xAP1 (pXPG-AP1-3, 3x AP-1 sites upstream of firefly luciferase, 500 ng), CMV (empty control vector, 500 ng), CMV_dnFOS (CMV500-8584hep-fosLZ(MO), A-FOS, kindly provided by Charles Vinson (Olive et al, 1997), 500 ng), Renilla (control for luciferase, 20 ng). One day after transfection cells were stimulated for 2h with 320 nM Phorbol 12-myristate 13-acetate (PMA) before harvesting. Samples were prepared using Promega DnaL Luciferase Reporter Assay.

**High throughput sequencing processing, peak detection and generation of coverage tracks**

Illumina basecall outputs were converted to the fastq format via bcl2fastq 1.8.4. Sequence alignment was performed using bowtie (Langmead et al, 2009) to the mm10 genome, using --all --best --strata --v 2 --m 1 as parameters. Peak detection was carried out using MACS 1.4.2 using default peak detection parameters (Zhang et al, 2008). For ChIP-seq samples, duplicate read parameters were set to --keep-
dup=auto, while for very high read depth DNasel-seq samples this was set to --keep-dup=all, to reflect the decreasing likelihood of duplicate reads corresponding to sequencing artefacts with increasing read coverage. Peak quality filtering was performed as previously described (Bevington et al, 2016), excluding low summits corresponding to background peaks below the inflexion point of the distribution of summit heights. To remove sequencing artefacts, peak and summit files were intersected against the mouse ENCODE blacklist (ENCODE Project Consortium, 2012) as well as the UCSC browser simple repeats track (Kent et al, 2002) via bedtools intersect (version 2.2.3) using the non overlapping parameter -v (Quinlan & Hall, 2010). Coverage files were also generated via MACS using the -w -S switches.

ChIP-seq and DNasel-seq rankings, heatmaps and average profiles

For all ChIP-seq and DNasel-seq signal heatmaps, tag counts were recovered ±1 kb around summits via HOMER (version 4.7.2) annotatePeaks using -hist 10 -ghist –size 2000 -wig as parameters (Heinz et al, 2010). For FOS- and JUN- based ChIP-seq signal heatmaps, regions subsequently sorted by decreasing tag counts ±200 bp around summits. For TEAD4 and DNasel signal fold change heatmaps, unions of summits were obtained as previously described (Cauchy et al, 2015). In brief, summits were concatenated via the bash command cat, merged using bedtools merge using -d 400 as the distance parameter, sorted via bedtools sort. Tag counts were recovered ±200 bp around summits, which were used to rank regions by increasing fold change. For DHSs, since the distribution of center-scaled log2 fold changes could be approximated to a Gaussian distribution via a one-sample Kolmogorov-Smirnov test using the ks.test function in R (Team, 2014) against the pnorm cumulative distribution (p≤4.94 10-324), we used cutoff thresholds of ±1 log2 fold change to define classes. For TEAD4, since the distribution of fold changes was largely skewed towards smaller numbers, we performed Gaussian fitting using the normalmixEM function of the mixtools package in R, yielding two Gaussians corresponding to distinct populations. Classes were defined as above µ+σ for each Gaussian (where µ and σ are the mean and standard deviation, respectively), resulting in three classes. Corresponding signal tables were also retrieved via HOMER annotatePeaks using the same parameters and subsequently ranked by the reference union sorting. Heatmap images were generated using Java TreeView (Saldanha, 2004). Average profiles per class were plotted using Excel using signal tables.

Motif discovery, heatmaps and average profiles

De novo motif discovery was performed via HOMER findMotifsGenome using -size 200 as a parameter. To retrieve motif counts on regions ±1 kb around sorted summits, HOMER annotatePeaks was used with -hist 10 -ghist –size 2000 -m as
parameters. Heatmaps were generated using Java TreeView. For average profiles, a sliding window of 100 bp was used on average motif counts.

**Gene ontology analyses**

Gene ontology (GO) analyses (biological process and KEGG pathway ontology classes) were performed using DAVID (Huang da et al, 2009) for all figures except Figure 4.

**Venn Diagrams**

Venn diagrams using gene names were derived using BioVenn (Hulsen et al, 2008). For high-throughput sequencing peaks, the makeVennDiagram function of the ChIPpeakAnno R package (Zhu et al, 2010) was used, which was also used to compute hypergeometric p-values of intersections.

**Digital genomic footprinting**

Digital genomic footprinting was performed using the wellington_footprints function of the Wellington algorithm (version 0.1.7) on HE1 and HP DHSs using standard parameters (Piper et al, 2013). Footprints exhibiting p-values lower than 10-20 were retained. Specific footprints were by intersecting HE1 and HP footprints via bedtools intersect. Footprinted motifs were derived by annotating motifs to footprints using HOMER annotatePeaks -m mbed. Heatmaps and average cut profiles around footprinted motifs were computed using the dnase_to_javatreeview and dnase_average_profiles functions of the Wellington algorithm using a correction for cutting bias.

**Motif co-occurrence clustering**

Motif co-occurrence clustering was essentially performed as previously described (Bevington et al, 2016; Cauchy et al, 2015). Briefly, intersections of motifs (annotated with HOMER annotatePeaks) occurring within ±25 bp of each other in tested regions was computed via pybedtools (version 0.6.2) intersection_matrix (Dale et al, 2011). For motifs within FOS and JUN peaks, tested regions corresponded to summits ±200 bp, whereas for footprints, these corresponded to exact footprint spans. Enrichment compared to a random background was computed using similarly-sized populations of 10,000 random sub-samplings of lengths corresponding to tested regions. For motifs within FOS and JUN peaks, the random sub-samplings were computed from total HE1 DHSs as the background; for footprints, these were computed from the reciprocal population, i.e. HP footprints for HE1-specific footprints and HE1 footprints for HP-specific footprints. Z-scores were calculated using the formula Z=(x-µ)/σ, where x is the actual number of co-occurrences for a pair of motifs in the tested population, µ and σ the mean and standard deviation of intersections of co-occurrences derived for a pair of motifs in the background populations. Correlation
clustering was subsequently performed with cluster 3.0 (de Hoon et al, 2004) using -e 2 -g 2 as parameters. Heatmap images were generated using Java TreeView.

Gene set enrichment analyses

Gene-set enrichment analyses were performed with the GSEA analysis suite (Subramanian et al, 2005). Expression data was derived as a text file with gene names normalized log2 expression microarray values from this study. CLS files were generated for each comparison. GRP files were input as the top 1000 peaks for FOS and JUN ChIP-seq datasets due to the size limitations of GSEA. Classic enrichment statistics and Diff_of_classes as the metric for ranking genes were used as further parameters. The permutation type to compute the normalized enrichment score was set to gene_set.

K-means clustering

Expression values of the closest gene were recovered for FOS:JUN co-bound peaks. K-means clustering was performed aiming for 7 gene clusters using cluster 3.0 using -g 2 -k 7 -na -ng as parameters.

Motif distances

Matches for the AP-1 and TEAD HE footprinted motifs were intersected within 100bp using bedtools window -w 100. Distances to TEAD motif ends were calculated by subtracting the AP-1 motif coordinate start (adjusted by either +3bp or +9bp, if they were found on the positive or negative strand, respectively, to reflect the actual start of the core TGASTCA motif within the longer HOMER NTGACTCANN motif) by the TEAD motif coordinate start ( adjusted by +2bp or +7bp , if they were found on the positive or negative strand, respectively, to reflect the actual start of the core ATTCC motif within the longer HOMER RCATTCCWNN motif. Distributions of distances were plotted using the hist function in R. The consensus TEAD/AP-1 motif logo was derived by aligning all 14 matches showing a 7bp spacer and generated using STAMP (Mahony & Benos, 2007).

Microarray Data Analysis

The microarray gene expression scanned images were analyzed with Feature Extraction Software 10.7.1.1 (Agilent) (protocol GE1_107_Sep09, Grid: 028005_D_F_20100614 and platform Agilent SurePrint G3 Mouse GE 8x60K). The raw data output by Feature Extraction Software was analyzed using the LIMMA R
package (Smyth, 2005) with quantile normalization and background correction by using the normexp method (Ritchie et al, 2007) and an offset value of 16. Contrast matrix, lmFit and eBays function were used and a p value cut-off <= 0.001 was applied. Only genes with a minimum log$_2$ intensity value equal to or greater than 6.5 in at least one array were selected as expressed genes. Genes that changed expression at least two fold up or down with respect to -/+ DOX or genes that changed expression at least two fold up or down through differentiation were considered as differentially expressed.

The Principal Component Analysis (PCA) was carried out on the RNA expression level values of the probe set intensity within each experiment and was calculated using “prcomp” function implemented in R.

Clustering of gene expression was carried out for all differentially expressed genes associated with at least a two-fold change. Standardized values were used for clustering purposes and was obtained by converting the expression values to Z-scores ($z_{ij} = (e_{ij} - \mu_i) / \sigma_i$) where $\mu_i$, is the mean expression and $\sigma_i$ is standard deviation of the gene “i” across all the cell lines. Hierarchical clustering was used with Euclidean distance and complete linkage methods. Then the differentially expressed genes from SM, HE1, HE2, and Progenitors cells were clustered into 15 different patterns using k-means clustering with Euclidean distances. These genes within each cluster were then hierarchically clustered using the “complete linkage” agglomeration method (Figure 3D). Heatmaps were generated using Mev from the TM4 microarray software suite (Saeed et al, 2003).

Gene ontology analysis was performed using BiNGO (Maere et al, 2005) and DAVID online tools using Hypergeometric for overrepresentation and Benjamini and Hochberg (FDR) correction for multiple testing corrections.

**Supplemental references:**


