The T-box transcription factor Eomesodermin governs haemogenic competence of yolk sac mesodermal progenitors

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Extra-embryonic mesoderm (ExM)—composed of the earliest cells that traverse the primitive streak—gives rise to the endo- thelium as well as haematopoietic progenitors in the developing yolk sac. How a specific subset of ExM becomes committed to a haematopoietic fate remains unclear. Here we demonstrate using an embryonic stem cell model that transient expression of the T-box transcription factor Eomesodermin (Eomes) governs haemogenic competency of ExM. Eomes regulates the acces- sibility of enhancers that the transcription factor stem cell leukaemia (SCL) normally utilizes to specify primitive erythrocytes and is essential for the normal development of Runx1^{+} haemogenic endothelium. Single-cell RNA sequencing suggests that Eomes loss of function profoundly blocks the formation of blood progenitors but not specification of Flk-1^{+} haematoendothelial progenitors. Our findings place Eomes at the top of the transcriptional hierarchy regulating early blood formation and suggest that haemogenic competence is endowed earlier during embryonic development than was previously appreciated.

The process of gastrulation generates the three primary embryonic germ layers; namely, the mesoderm, ectoderm and definitive endoderm. Beginning at embryonic day 6 (E6.0), in response to local signalling cues, pluripotent epiblast cells on the prospective posterior side of the embryo undergo a process of epithelial-to-mesenchymal transition, allowing them to delaminate and migrate within the primitive streak[1]. The first cells that traverse the primitive streak include progenitors of the ExM that migrate proximally to generate the developing yolk sac[1–3]. ExM subsequently differentiates into endothelial cells that form the yolk sac vasculature as well as blood progeni- tors that sustain growth and development of the post-implantation embryo[3,4]. Haematopoietic progenitors initially form at E7.5 to gener- ate nucleated primitive erythrocytes within the distally located yolk sac blood islands[3]. One day later, a second wave of blood progenitors arise from a subset of endothelial cells present within the developing yolk sac vasculature[5]. This so-called haemogenic endothelium undergoes an endothelial-to-haematopoietic transi- tion (EHT) whereby cells round up, detach from the endothelial layer and enter the blood stream[6–8]. Haematopoietic progenitors derived from yolk sac haemogenic endothelium at E8.25 have restricted erythro-myeloid potential and generate enucleated eryth- rocytes[9] and are therefore designated as definitive. Subsequently, at E10.5, a third wave of haematopoietic progenitors includ- ing definitive haematopoietic stem cells arise from haemogenic endothelium in vascular beds of the dorsal aorta and vitelline/ umbilical arteries[7,10]. Understanding the transcriptional hierarchy that guides hae- matopoiesis during embryogenesis is essential for the generation of haematopoietic progenitors from pluripotent stem cell sources in vitro[11,12]. Of particular interest is how haemogenic endothelium is specified, as this represents a critical early step in the generation of definitive haematopoietic cells including haematopoietic stem cells. Only a few transcriptional regulators that impact haemato- poietic output from haemogenic endothelium have been identified, including the transcription factors SCL (in the mouse, T-cell acute lymphoblastic leukemia 1; TAL1) and Runx1 that play key roles during embryonic haematopoiesis. SCL is required for specification of the blood fate and generation of haemogenic endothelium and all haematopoietic cells[13–17]. In contrast, Runx1 is non-essential for the generation of haemogenic endothelium or primitive erythrocytes/ megakaryocytes but is essential for EHT and the production of all definitive haematopoietic stem and progenitor cells[18].

Here we report that the T-box transcription factor Eomes is transiently expressed in ExM progenitors that generate virtually all yolk sac haematopoietic and endothelial cells. Using an embry- onic stem cell (ESC) differentiation system, we find that Eomes is essential for the production of primitive erythrocytes and Runx1^{+} haemogenic endothelium. Eomes is expressed before both SCL and Runx1 during mesoderm patterning.

Single-cell RNA sequenc- ing (scRNA-seq) comparisons with in vivo haematoendothelial development strongly suggest that the block in haematopoietic development in Eomes loss-of-function cultures occurs after the specification of Flk-1$^+$/SCL$^+$ haematoendothelial progenitors. Assay for transposase-accessible chromatin using sequencing (ATAC-seq) experiments have revealed that Eomes governs the accessibil- ity of Runx1 enhancers as well as *cis*-regulatory regions that SCL normally utilizes to specify primitive erythrocytes. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) experi- ments have shown that Eomes occupies Runx1 *cis*-regulatory regions and coordinates the development of haemogenic competent mesoderm in the context of Activin/Nodal and Tead–Yap signal- ling. Finally, re-expression of Runx1 in Eomes-null endothelial cul- tures is sufficient to rescue the block in EHT and definitive blood production. Collectively, these experiments show that Eomes sits at the top of the transcriptional hierarchy, functioning upstream of Runx1 expression and SCL functional activity, to promote haemo- genic competence of the yolk sac mesodermal lineage.

Results

Eomes expression transiently marks the proximal epiblast, primitive streak and Flk-1$^+$ ExM progenitors that give rise to haematopoietic and endothelial lineages of the yolk sac. The first wave of cells to traverse the primitive streak give rise to ExM, which migrates proximally, displacing the overlying extra-embryonic ectoderm (ExE) to form the inner layer of the developing yolk sac[2,3]. Subsequently, these cells generate the blood islands contain- ing primitive erythrocytes and endothelial cells that give rise to the yolk sac vascular network[3] (Fig. 1a). The T-box transcription factor Brachyury is expressed in the primitive streak and nascent meso- derm, including the haemangioblast—a multipotent progenitor that generates haematopoietic and endothelial cells[19]. Flk-1 also marks the haemangioblast[19] and in vivo fate-mapping studies have shown that yolk sac haematopoietic and endothelial cells are derived from Flk-1$^+$ ExM[20].

Here we observed that Eomes expression is detectable in proxi- mal epiblast/primitive streak cells at early/mid-primitive streak stages (E6.5) before widespread Brachyury expression (Fig. 1b,c). Additionally, at mid-streak (E6.5) stages, a population of Flk-1$^+$ migratory mesoderm co-expressed Eomes in the extra-embryonic region (Extended Data Fig. 1a). To test whether Eomes-expressing progenitors contribute to the ExM at later stages, we performed short-term lineage-tracing experiments using an Eomes$^{GFP/+}$ reporter line (a reporter line expressing green fluorescent protein (GFP) from the Eomes locus)[21]. At E7.5, Eomes-GFP$^+$ cells detected within the yolk sac blood islands co-expressed Flk-1 (Fig. 1d) and the ExM/haematopoietic marker Runx1 (Fig. 1e). However, this reflected GFP perdurance as endogenous Eomes protein was no longer detectable in Eomes-GFP$^+$ (Fig. 1f) or Flk-1$^+$ cells in the yolk sac at this stage (Extended Data Fig. 1b).

To examine contributions made by Eomes-expressing cells to yolk sac vascular and haematopoietic lineages, we generated an *Eomes*^{iCre} reporter allele (Extended Data Fig. 2a–e) and per- formed long-term lineage-tracing experiments. *Eomes*$^{iCre/+}$ males were mated with females carrying the *ROSA26*^{R} allele[22] and the resulting embryos were stained for LacZ expression. At E8.5 and E9.5, virtually all of the yolk sac haematopoietic and endothelial

cells in *Eomes*^{iCre};*ROSA26*^{R} embryos were LacZ$^+$ (Fig. 1g). Thus, we conclude that transient Eomes expression marks ExM progenitors that give rise to the yolk sac haematopoietic and endo- thelial compartments.

To assess Eomes' functional contributions, we analysed E7.5 embryos carrying an epiblast-specific *Eomes* deletion (*Eomes*$^{CA/N}$; Sox2.Cre)[23] that disrupts delamination of nascent mesoderm[23]. In contrast with wild-type (WT) embryos, *Eomes*$^{\Delta Epi}$ mutant embryos fail to induce the expression of *Flk-1* and *ER71*—genes that are essential for yolk sac haematopoiesis and vasculogenesis[24–26] (Extended Data Fig. 2f). Thus, Eomes expression in the epiblast is essential for the generation of ExM.

Eomes is expressed transiently in haematovascular progeni- tors before the onset of SCL and Runx1 expression during haematopoietic differentiation. To circumvent in vivo morpho- genetic defects caused by

Eomes functional loss, we exploited an in vitro ESC differentiation protocol[27,28] to promote the formation of yolk sac-like haematopoietic and endothelial progenitors via the staged addition of growth factors to embryoid bodies (EBs) under serum-free conditions (Fig. 2a–c). Eomes[21], SCL[29] and Runx1 (Extended Data Fig. 3a,b) expression was analysed utilizing ESC reporter lines. Dissociated EBs were stained for Flk-1 and PdgfRa—markers that distinguish between haematovascular mesoderm (Flk-1^{hi}/PdgfRa^{-}), primitive/cardiac mesoderm (Flk-1^{+}/PdgfRa^{+}) and paraxial mesoderm (Flk-1^{-}/PdgfRa^{+})—and the haematopoietic marker CD41 (refs. [26,30–34]) (Fig. 2d,e). Eomes-GFP was detectable at day 3 before expression of Flk-1/PdgfRa. The majority of cells at day 4, including the Flk-1^{hi}/PdgfRa^{-} haematovascular mesoderm com- partment, expressed Eomes-GFP (Fig. 2d). After 24 h, Eomes-GFP^{+} cells comprised roughly half of the Flk-1^{hi}/PdgfRa^{-} compartment (Fig. 2d). In contrast, developing CD41^{+} haematopoietic cells were predominantly Eomes-GFP$^{lo/-}$ (Fig. 2e).

At day 4, SCL-mCherry was exclusively expressed within the Flk-1^{hi}/PdgfRa^{-} compartment (Fig. 2d). At day 5, haematopoietic cells co-expressed CD41 and SCL-mCherry and downregulated Flk-1 expression (Fig. 2d,e). Runx1-Venus was also expressed in CD41^{+} haematopoietic cells (Fig. 2e). However, in contrast with SCL-mCherry, Runx1-Venus expression was restricted to cells that weakly expressed Flk-1 within the Flk-1^{hi}/PdgfRa^{-} compartment at day 4/5 (Fig. 2d).

Consistent with the above results, differentiation trajectories con- structed using scRNA-seq data from E6.5 to E8.5 mouse embryos[15] revealed dynamic Eomes expression in mesoderm/haematoendo- thelial progenitors that gave rise to SCL- and Runx1-expressing haematopoietic and endothelial cells (Fig. 2f). Thus, we conclude that Eomes is transiently expressed, before SCL and Runx1, in hae- matovascular mesoderm progenitors.

Eomes functional loss disrupts primitive and definitive hae- matopoiesis but not endothelial development. Next, we exam- ined the ability of Eomes-null ESCs[23] to generate haematovascular mesoderm (Fig. 3a). Eomes-null EBs contain a detectable but decreased number of Flk-1^{hi}/PdgfRa^{-} cells at day 4/5 and lack Flk-1^{+}/PdgfRa^{+} progenitors at day 4, reflecting Eomes requirements for cardiac mesoderm specification[35,36] (Fig. 3b). RNA-seq experi- ments performed at day 4 revealed that Eomes-null Flk-1^{hi}/PdgfRa^{-} cells express SCL, ER71, Gata2, Lmo2, Tek, Cdh5, Fli1 and CD31 (Fig. 3c), suggesting that Eomes is non-essential for the specifica- tion of haematovascular mesoderm. In contrast, EHT regulators (including Runx1 and Gfi1b) and genes expressed in erythroid cells (Gata1 and Hbb-bh1) were downregulated (Fig. 3c). To further examine Eomes functional requirements, we cultured EBs for three more days under pro-haematopoietic conditions (Fig. 3a, red). WT cultures robustly formed haematopoietic pro- genitors co-expressing c-Kit and/or CD41 and CD45 and generated primitive/definitive erythrocyte and myeloid/mixed haematopoi- etic colonies (Fig. 3d,e). In contrast, Eomes-null cultures lacked the ability to form CD41^{+}/CD45^{+} cells and haematopoietic colonies (Fig. 3d,e). These cultures contained CD31^{+}/Cdh5^{+} endothelial cells (Fig. 3f) and expressed normal levels of the endothelial marker genes *Cdh5*, *Flk-1*, *Flt-1* and *Fli-1* (Fig. 3g). However, *SCL*, *Runx1*, *PU.1*, *Gata1* and *Hbb-bh1* transcripts at day 6 were markedly down- regulated, confirming a block in haematopoiesis (Fig. 3g). To further explore Eomes functional contributions, Flk-1^{hi} cells were isolated at day 5 and plated on Matrigel under conditions known to promote haemogenic endothelium development[37] (Fig. 3a, blue). By day 8, WT Flk-1^{hi} cells gave rise to patches of adherent Cdh5^{+}/c-Kit^{+} haemogenic endothelium cells that actively under- went EHT to generate semi-adherent and floating CD41^{+}/CD45^{+} haematopoietic cells (Fig. 3h,i). Eomes-null Flk-1^{hi} cells gener- ated Cdh5^{+}/c-Kit^{+} endothelium but these cells failed to efficiently undergo EHT and lacked expression of the haematopoietic markers CD41 and CD45 (Fig. 3i). Thus, we conclude that Eomes is dispens- able for the generation of endothelial cells but is essential for both primitive and definitive haematopoietic development.

Eomes is essential for the generation of Runx1^{+} haemogenic endothelium. The defects observed above in Eomes-null ESCs closely resemble those reported for SCL mutants[13]. Therefore, next, we regenerated the Eomes-null allele in ESCs that contained an SCL reporter allele[29] (Extended Data Fig. 4a–c), to directly test

Eomes requirements for SCL expression. At day 5 of differentiation, many Eomes-null Flk-1^{hi}/PdgfRa^{-} cells were SCL-mCherry^{+} (Fig. 4a), and one day later a high proportion of c-Kit^{+}/Cdh5^{+} cells expressed WT levels of SCL-mCherry (Fig. 4b). Thus, the block in primitive and definitive haematopoiesis observed in Eomes-null cultures cannot be explained simply by a loss of SCL expression.

Runx1 expression is required for EHT and the generation of definitive haematopoietic cells[18]. To assess whether the block in definitive haematopoiesis in Eomes-null cultures reflects the absence of haemogenic endothelium, we disrupted Eomes expres- sion in the Runx1-Venus reporter ESCs (Extended Data Fig. 4a–c). At day 5, Eomes-null EBs almost entirely lacked Runx1-Venus expres- sion (Fig. 4c). At day 8, Runx1-Venus expression normally marked a CD41$^{-/lo}$ haemogenic endothelium subset within the c-Kit^{+}/ Cdh5^{+} compartment (Fig. 4d, blue gate). Upon EHT, cells upregu- lated CD41, generating Runx1-Venus^{+}/CD41^{+} haematopoietic cells (Fig. 4d, green gate). Strikingly, Eomes-null EHT cultures lacked Runx1-Venus^{+}c-Kit^{+}/Cdh5^{+} haemogenic endothelium (Fig. 4d, blue gate). Eomes-null Runx1-Venus day 5 EBs and day 8 EHT cul- tures lacked clonogenic progenitors for primitive erythrocyte and definitive erythro-myeloid lineages, respectively (Fig. 4e), as well as Cdh5^{-}/CD41^{+}/Runx1-Venus^{+} cells (Fig. 4d, orange gate) and bud- ding cells (Fig. 4f). We conclude that the block in definitive haema- topoiesis in Eomes-null EHT cultures is associated with the loss of Runx1^{+} haemogenic endothelium.

To test whether Eomes regulates Runx1 expression in a cell- autonomous fashion, we performed co-culture experiments. WT ESCs were mixed with Runx1-Venus or Eomes-null Runx1-Venus ESCs (Extended Data Fig. 3c). WT:Runx1-Venus co-cultures at day 6 contained CD41^{+} haematopoietic cells that were Runx1-Venus^{+}. In contrast, Runx1-Venus expression in CD41^{+} cells was barely detect- able in Eomes-null Runx1-Venus:WT co-cultures. These results strongly suggest that Eomes promotes robust induction of Runx1 expression in a cell-autonomous fashion during haematopoiesis.

Eomes influences chromatin accessibility at SCL-bound enhancers in haematovascular mesoderm. To investigate Eomes-dependent chromatin accessibility, we performed ATAC-seq[38] analysis of day 4

WT and Eomes-null Flk-1^{hi}/PdgfRa^{-} haematovascular mesoderm. We identified changes at 4,180 genomic locations corresponding to ~7% of all accessible sites (Fig. 5a). The majority of sites (>85%) showing reduced chromatin accessibility in Eomes-null cells were located in distal intergenic regions (Fig. 5a) and were enriched for binding motifs for haematopoietic regulators such as Gata1/2, SCL, Erg, Ets1, Fli1, Runx1, Meis1 and Gfi1b (Fig. 5b). Strikingly, when these peaks were compared with published ChIP-seq datasets from Flk-1^{+} cells generated using a haematopoietic differentiation pro- tocol[39], we found that the majority of Eomes-dependent sites with reduced accessibility were enriched for enhancer marks (H3K4Me1 and H3K27Ac) and SCL occupancy (Fig. 5c). Moreover, sites show- ing reduced accessibility that correlated with SCL occupancy were enriched for genes related to haematopoietic development (Fig. 5d). Of these, 231 were associated with downregulated genes in Eomes-null Flk-1^{hi}/PdgfRa^{-} cells, including *Gata1* and *Nfe2* (which are critical for primitive erythrocyte development) and those gov- erning EHT and definitive haematopoiesis (namely *Runx1*, *Gfi1/1b*, *Ikzf1* and *Myb*) (Fig. 5e). Many of the changes in chromatin accessi- bility mapped to previously identified enhancer regions at these loci (Fig. 5f)[40,41]. Consistent with the results above, there was no notice-able impact on chromatin accessibility at the SCL locus (Fig. 5f). These results show that Eomes regulates accessibility at SCL-bound *cis*-regulatory elements.

Sites co-occupied by Eomes, Tead4 and Smad2/3 are transiently marked by H3K27Ac during early stages of haematopoietic development. To facilitate the identification of Eomes target genes, we generated ESCs expressing carboxy (C)-terminally V5-tagged Eomes (Eo-V5) (Extended Data Fig. 5a–c,f). Intracellular flow cytometry showed that Eo-V5 expression peaked at day 4 during haematovascular mesoderm development (Extended Data Fig. 5d). Eo-V5 is expressed broadly in primitive/cardiac mesoderm (Flk-1^{+}/ PdgfRa^{+}) as well as a subset of developing haematovascular (Flk-1^{hi}/ PdgfRa^{-}) mesodermal cells (Extended Data Fig. 5e).

Homozygous Eo-V5 EB cultures generate Flk-1^{+}/PdgfRa^{+} primitive/cardiac mesoderm and c-Kit^{+}/CD41^{+} haematopoietic progenitors, confirm- ing that Eomes-V5 functions normally (Extended Data Fig. 5g).

We used two independent Eo-V5 ESC clones (CL A and CL B) for ChIP-seq analysis. V5-Eomes-bound genomic regions common to both clones (Fig. 6a) were highly enriched for the Eomes bind- ing motif[42] and were predominantly located >5 kilobases (kb) from transcriptional start sites (TSSs) (Fig. 6b). In total, 30% of genes linked to ChIP peaks were found to be misregulated in day 4 Flk-1^{hi}/ PdgfRa^{-} Eomes-null cells, including those transcribing transcrip- tional regulators (Runx1, Mixl1, Klf5, Pbx1, Tbx3, Foxf1 and Meis2) and signalling molecules (Dkk1, Gli2, Fzd7 and Lefty2) controlling haematovascular development[27,43–48] (Fig. 6c).

Next, we examined published ChIP-Seq datasets[49,50] to assess co-occupancy by transcriptional regulators, DNase hypersensitivity sites and local histone marks during haematopoietic differentiation (Fig. 6d). The majority of Eomes-occupied sites were marked by H3K27Ac and displayed DNase hypersensitivity in haemangioblasts (Brachyury^{+}/Flk-1^{+}) but not haematopoietic progenitors (CD41^{+}) haemogenic endothelium[52] and several Runx1 enhancers (+110, +171, +204 and −327) known to regulate expression during haema- topoiesis[40,53] (Extended Data Fig. 6). Eomes induction also rescues Runx1 expression in this context[51], suggesting that Eomes directly regulates Runx1 expression.

Finally, we overlaid our day 4 Eomes ChIP-seq peaks with the ATAC-seq peaks with reduced accessibility in day 4 Flk-1^{hi}/PdgfRa^{-} Eomes-null mesoderm, as well as SCL ChIP-seq peaks from day 4 WT Flk-1^{+} EBs[39]. Very few of the ATAC peaks (33/3,629) or SCL ChIP peaks (19/4,393) were bound by Eomes at day 4 (Extended Data Fig. 7a). Interestingly, Eomes ChIP peaks were accessible at early mesodermal (Brachyury^{+}/Flk-1^{-}) stages of development and became marked by H3K27Ac after the onset of Flk-1 expression[49] (Extended Data Fig. 7b). In contrast, Eomes-dependent ATAC peaks normally bound by SCL only became accessible in the hae- mangioblast (Brachyury^{+}/Flk-1^{+}) population[49] (Extended Data Fig. 7b). These results suggest that Eomes-dependent SCL-bound *cis*-regulatory regions become accessible only subsequent to Eomes functional activity at earlier stages in the haematopoietic differen- tiation pathway.

scRNA-seq reveals the stage at which haematopoietic develop- ment is blocked in Eomes loss-of-function cultures. To character- ize the stage when haematopoietic development is blocked in Eomes loss-of-function cultures, we performed scRNA-seq on Eomes-null and WT Flk-1^{hi}/PdgfRa^{-} populations at days 4 and 5 (Extended Data Fig. 8a). An integrated analysis[54] was performed that allowed uniform manifold approximation and projection (UMAP) and the identification of 13 clusters (Extended Data Fig. 8). Cluster identi- ties were determined by comparing marker genes conserved across genotypes (Supplementary Table 1) with those previously used to document discrete cell populations present in the E6.5 to E8.5 mouse gastrulation atlas[15]. Additionally, we were able to map the in vitro-derived cells onto the mouse gastrulation atlas[15], allowing us to transfer cell identities and embryonic stages onto our scRNA-seq dataset (Fig. 7a–d).

Cells from day 4 EBs were found mainly in clusters 2–6 (Extended Data Fig. 8b,c) and resembled in vivo mixed mesoderm and hae- matoendothelial progenitors (Fig. 7a) expressing Brachyury, Mixl1, ER71 and SCL (Fig. 7b and Extended Data Fig. 8e). In contrast, day 5 cells mainly contributed to clusters 7–13 (Extended Data Fig. 8b,c), resembling in vivo haematoendothelial progenitors, endothelium, allantois and blood progenitors 1 and 2 (Fig. 7a) and expressing varying levels of Runx1, Gata1, Cdh5, Pecam1 and Spin2c (Fig. 7b and Extended Data Fig. 8e). Day 4 Eomes-null and WT cells con- tributed relatively equally to mesodermal/haematoendothelial progenitor clusters 2–6 (Extended Data Fig. 8c). In contrast, day 5 Eomes-null cells predominately contributed to the haematoendo- thelial progenitor, endothelial and allantoic clusters (9–13) at the expense of blood progenitor clusters (7 and 8; Extended Data Fig. 8c). The exceptional Eomes-null cells contributing to clusters 7 and 8 expressed the haematovascular genes *SCL*, *ER71*, *Cdh5* and *CD31* but had reduced expression of the EHT regulators *Runx1*/*Gfi1b* (Extended Data Fig. 8e,f). Interestingly, many Eomes-null Flk-1^{hi}/ PdgfRa^{-} cells displayed transcriptional profiles similar to endo- thelial cells and haematoendothelial progenitors from E7.75–E8.5 mouse embryos (Fig. 7d). Mapping cells onto the in vivo haema- toendothelial differentiation trajectory of

the mouse gastrula- tion atlas[15] (see Fig. 2f) suggested that the developmental block in Eomes-null cultures reflects the failure of haematoendothelial pro- genitors to transition into blood progenitors 1/2 (Fig. 7e).

Runx1 re-expression in Eomes$^{-/-}$ EHT cultures rescues the production of definitive haematopoietic progenitors. Day 5 Eomes-null Flk-1^{hi}/PdgfRa^{-} cultures contained endothelial cells (Fig. 7a,e and Extended Data Fig. 8c) but could not form Runx1^{+} hae- mogenic endothelium (Fig. 4d). Both Runx1-null and Eomes-null ESCs generated a Cdh5^{+}/c-Kit^{+}/CD41^{lo} population (Extended Data Fig. 9a,b). However, this population was absent from equivalent SCL-null cultures (Extended Data Fig. 9a,b). To further character- ize Eomes functional contributions in relation to Runx1, we deleted Eomes in the context of a Runx1-inducible ESC line[14,52] (iRunx1; Runx1-null with a doxycycline (dox)-inducible Runx1b comple- mentary DNA inserted into the ROSA26 locus) (Extended Data Fig. 4d,e). This strategy enabled us to drive Runx1 expression in Eomes-null EHT cultures and perform rescue experiments.

As expected, day 5 iRunx1 Eomes-null EBs generated a Flk-1^{hi}/PdgfRa$^{lo/-}$ population (Fig. 8a) but lacked primitive erythro- cyte progenitors (Fig. 8b), highlighting differences in the require- ments for Runx1 and Eomes during primitive erythropoiesis. As expected[52], rescuing EHT and the generation of functional hae- matopoietic progenitors via enforced Runx1 expression was highly dose dependent (Fig. 8c,d). Titration experiments established that the addition of 90 ng ml^{-1} dox during days 6–8 in iRunx1 Eomes$^{+/+}$ EHT cultures efficiently rescued the generation of definitive erythro-myeloid clonogenic progenitors and Cdh5^{+}/CD41^{+} and Cdh5^{+}/CD45^{+} cells (Fig. 8c,d and Extended Data Fig. 9c). Strikingly, these conditions also rescued, albeit less efficiently, the generation of Cdh5^{+}/CD41^{+} and Cdh5^{+}/CD45^{+} cells and restored haemato- poietic colony formation in iRunx1 Eomes-null cultures (Fig. 8c,d and Extended Data Fig. 8c,d). These findings show that Eomes acts upstream of Runx1 expression during definitive haematopoiesis.

Discussion

Here we show, in the absence of Eomes function, that Flk-1^{+} hae- matoendothelial progenitors are correctly specified. However, upon further differentiation, these progenitors cannot transition into primitive erythrocytes or haemogenic endothelium capable of expressing Runx1 and undergoing EHT (Fig. 8e). As judged by scRNA-seq analyses, these in vitro cells closely resemble those formed in vivo when haematopoiesis predominates in the murine yolk sac (E7.5–E8.5). These results highlight the validity of using ESC differentiation cultures as a model of yolk sac haematopoiesis and have allowed us to uncover an essential role for Eomes in this process.

Our ATAC-seq analysis of Eomes-mutant haematoendothelial progenitors suggests that the block in primitive erythrocyte devel- opment occurs because SCL can no longer access the enhancer network through which it normally specifies this lineage. SCL itself is not responsible for governing the accessibility of key enhancers that guide this process[39]. These enhancers only become accessible during normal haematopoietic differentiation after the onset of Flk-1 expression. Eomes expression precedes Flk-1/SCL expression during haematoendothelial development. Thus, Eomes potentially functions as a pioneer factor opening SCL enhancers to direct prim- itive erythrocyte development. However, our ChIP-seq results argue against this idea, since the majority of SCL-bound enhancers are not occupied by Eomes.

Recent experiments have shown that haemogenic compe- tency of yolk sac endothelial progenitors is actively restrained via BMI1-dependent silencing of Runx1 expression[55]. Additionally, re-expression of Runx1 in non-haemogenic endothelium cells is sufficient for their conversion into haemogenic endothelium[56,57]. Therefore, Runx1 is sufficient to promote conversion of yolk sac endothelium towards a haemogenic fate. Here we demonstrate that Eomes acts upstream of Runx1 expression in the haemogenic endothelium lineage. Moreover, Runx1 re-expression in Eomes-null cultures rescues the formation of definitive haematopoietic progeni- tors. ChIP-seq in day 4/5 EBs reveals Eomes occupancy at the Runx1 proximal promoter P2, as well as previously described[40,53] (+110, +171, +204 and −327) and potentially unappreciated (−171/−181) Runx1 enhancers. Furthermore, ATAC-seq has shown that hae- matoendothelial progenitors formed in the absence of

Eomes lack chromatin accessibility at enhancers known to drive Runx1 expres- sion in the haemogenic endothelium lineage/sites of definitive hae- matopoiesis[40,58] (+3, +23, +110, −322 and −327). Considering that yolk sac endothelial cells are derived from Eomes-expressing precur- sors, Eomes probably endows haemogenic competence via its ability to allow Runx1 induction at later developmental stages within the endothelial lineage. Thus, Eomes directs the emergence of an epigen- etic landscape that primes haemogenic endothelium specification.

The majority of Eomes ChIP-seq peaks are located at *cis*-regulatory regions that are active transiently during mesodermal stages of development. Strikingly, many of these Eomes-bound sites are co-occupied in similar-stage EBs by Tead4 and Smad2/3—tran- scription factors that act downstream of Hippo/YAP and Activin/Nodal signalling, respectively, and have been shown previously to regulate haematopoietic development[27,28,31,49,59]. Computational analysis has shown that sites co-bound by this triad of transcrip- tion factors are associated with genes regulating both mesoderm and blood cell development. Interestingly, disruption of Tead–YAP complex formation profoundly disrupts EHT and the generation of $CD41^{+}$ cells[49]. Additionally, Tead signalling is essential during mesodermal stages of development, coincident with the onset of Eomes functional activity[49]. It therefore seems likely that Eomes guides haematopoietic mesoderm development in the context of active Activin/Nodal and Tead/YAP signalling.

Fate-mapping studies previously suggested that yolk sac haema- topoietic and endothelial lineages become segregated within the

proximal epiblast, before ingression through the primitive streak[2,60]. The first cells to ingress through the streak predominantly gener- ate primitive haematopoietic cells while the second wave generates endothelial precursors[2] that subsequently acquire haemogenic endo- thelium or non-haemogenic endothelium fates. Conditional inacti- vation of Eomes in the primitive streak using a *T*.Cre deleter strain has no noticeable impact on embryonic development[23]. Therefore, Eomes is probably only required for yolk sac haematopoiesis within a narrow developmental time window at the very outset of gastru- lation in the proximal epiblast/primitive streak. The present study makes Eomes the earliest-known transcriptional regulator of spe- cifically haematopoietic but not endothelial development, placing it at the top of a transcriptional hierarchy that governs haemogenic competence in the developing mouse gastrula.

Online content

Any methods, additional references, Nature Research report- ing summaries, source data, extended data, supplementary infor- mation, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41556-020-00611-8.

References

1. Arnold, S. J. & Robertson, E. J. Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. *Nat. Rev. Mol. Cell Biol.* **10**, 91–103 (2009).

2. Kinder, S. J. et al. The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. *Development* **126**, 4691–4701 (1999).

3. Ferkowicz, M. J. & Yoder, M. C. Blood island formation: longstanding observations and modern interpretations. *Exp. Hematol.* **33**, 1041–1047 (2005).

4. McGrath, K. E., Frame, J. M. & Palis, J. Early hematopoiesis and macrophage development. *Semin. Immunol.* **27**, 379–387 (2015).

5. Frame, J. M., Fegan, K. H., Conway, S. J., McGrath, K. E. & Palis, J. Definitive hematopoiesis in the yolk sac emerges from Wnt-responsive hemogenic endothelium independently of circulation and arterial identity. *Stem Cells* **34**, 431–444 (2016).

6. Kissa, K. & Herbomel, P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**, 112–115 (2010).

7. Boisset, J. C. et al. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**, 116–120 (2010).

8. Bertrand, J. Y. et al. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**, 108–111 (2010).

9. McGrath, K. E. et al. Distinct sources of hematopoietic progenitors emerge before HSCs and provide functional blood cells in the mammalian embryo. *Cell Rep.* **11**, 1892–1904 (2015).

10. De Bruijn, M. F., Speck, N. A., Peeters, M. C. & Dzierzak, E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J.* **19**, 2465–2474 (2000).

11. Keller, G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev.* **19**, 1129–1155 (2005).

12. Murry, C. E. & Keller, G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* **132**, 661–680 (2008).

13. Porcher, C., Chagraoui, H. & Kristiansen, M. S. SCL/TAL1: a multifaceted regulator from blood development to disease. *Blood* **129**, 2051–2060 (2017).

14. Lancrin, C. et al. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* **457**, 892–895 (2009).

15. Pijuan-Sala, B. et al. A single-cell molecular map of mouse gastrulation and early organogenesis. *Nature* **566**, 490–495 (2019).

16. Porcher, C. et al. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* **86**, 47–57 (1996).

17. Shivdasani, R. A., Mayer, E. L. & Orkin, S. H. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432–434 (1995).

18. De Bruijn, M. & Dzierzak, E. Runx transcription factors in the development and function of the definitive hematopoietic system. *Blood* **129**,
2061–2069 (2017).

19. Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J. & Keller, G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625–630 (2004).
20. Lugus, J. J., Park, C., Ma, Y. D. & Choi, K. Both primitive and definitive blood cells are derived from Flk-1^{+} mesoderm. *Blood* **113**, 563–566 (2009).
21. Arnold, S. J., Sugnaseelan, J., Groszer, M., Srinivas, S. & Robertson, E. J. Generation and analysis of a mouse line harboring GFP in the Eomes/Tbr2 locus. *Genesis* **47**, 775–781 (2009).
22. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70–71 (1999).

23. Arnold, S. J., Hofmann, U. K., Bikoff, E. K. & Robertson, E. J. Pivotal roles for Eomesodermin during axis formation, epithelium-to-mesenchyme transition and endoderm specification in the mouse. *Development* **135**, 501–511 (2008).
24. Shalaby, F. et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62–66 (1995).
25. Lee, D. et al. ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. *Cell Stem Cell* **2**, 497–507 (2008).
26. Koyano-Nakagawa, N. & Garry, D. J. Etv2 as an essential regulator of mesodermal lineage development. *Cardiovasc. Res.* **113**, 1294–1306 (2017).
27. Nostro, M. C., Cheng, X., Keller, G. M. & Gadue, P. Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. *Cell Stem Cell* **2**, 60–71 (2008).
28. Irion, S. et al. Temporal specification of blood progenitors from mouse embryonic stem cells and induced pluripotent stem cells. *Development* **137**, 2829–2839 (2010).
29. Chagraoui, H. et al. SCL/TAL1 cooperates with Polycomb RYBP-PRC1 to suppress alternative lineages in blood-fated cells. *Nat. Commun.* **9**, 5375 (2018).
30. Mikkola, H. K., Fujiwara, Y., Schlaeger, T. M., Traver, D. & Orkin, S. H. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood* **101**, 508–516 (2003).
31. Kattman, S. J. et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* **8**, 228–240 (2011).
32. Izumi, N., Era, T., Akimaru, H., Yasunaga, M. & Nishikawa, S. Dissecting the molecular hierarchy for mesendoderm differentiation through a combination of embryonic stem cell culture and RNA interference. *Stem Cells* **25**, 1664–1674 (2007).
33. Mitjavila-Garcia, M. T. et al. Expression of CD41 on hematopoietic progenitors derived from embryonic hematopoietic cells. *Development* **129**, 2003–2013 (2002).
34. Ferkowicz, M. J. et al. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* **130**, 4393–4403 (2003).
35. Costello, I. et al. The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation. *Nat. Cell Biol.* **13**, 1084–1091 (2011).
36. Van Den Ameele, J. et al. Eomesodermin induces Mesp1 expression and cardiac differentiation from embryonic stem cells in the absence of Activin. *EMBO Rep.* **13**, 355–362 (2012).
37. Clarke, R. L. et al. The expression of Sox17 identifies and regulates hemogenic endothelium. *Nat. Cell Biol.* **15**, 502–510 (2013).
38. Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y. & Greenleaf, W. J. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position.
Nat. Methods **10**, 1213–1218 (2013).
39. Org, T. et al. Scl binds to primed enhancers in mesoderm to regulate hematopoietic and cardiac fate divergence. *EMBO J.* **34**, 759–777 (2015).
40. Schutte, J. et al. An experimentally validated network of nine haematopoietic transcription factors reveals mechanisms of cell state stability. *eLife* **5**,
e11469 (2016).

41. Onodera, K. et al. GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. *Proc. Natl Acad. Sci. USA* **94**, 4487–4492 (1997).

42. Teo, A. K. K. et al. Pluripotency factors regulate definitive endoderm specification through Eomesodermin. *Genes Dev.* **25**, 238–250 (2011). 43. Vijayaragavan, K. et al. Noncanonical Wnt signaling orchestrates early developmental events toward hematopoietic cell fate from human embryonic stem cells. *Cell Stem Cell* **4**, 248–262 (2009). 44. Ng, E. S. et al. The primitive streak gene *Mixl1* is required for efficient haematopoiesis and BMP4-induced ventral mesoderm patterning in differentiating ES cells. *Development* **132**, 873–884 (2005).

45. DiMartino, J. F. et al. The Hox cofactor and proto-oncogene *Pbx1* is required for maintenance of definitive hematopoiesis in the fetal liver. *Blood* **98**, 618–626 (2001).

46. Fleury, M., Eliades, A., Carlsson, P., Lacaud, G. & Kouskoff, V. FOXF1 inhibits hematopoietic lineage commitment during early mesoderm specification. *Development* **142**, 3307–3320 (2015).

47. Wang, M. et al. MEIS2 regulates endothelial to hematopoietic transition of human embryonic stem cells by targeting TAL1. *Stem Cell Res. Ther.* **9**, 340 (2018).

48. Davenport, T. G., Jerome-Majewska, L. A. & Papaioannou, V. E. Mammary gland, limb and yolk sac defects in mice lacking *Tbx3*, the gene mutated in human ulnar mammary syndrome. *Development* **130**, 2263–2273 (2003). 49. Goode, D. K. et al. Dynamic gene regulatory networks drive hematopoietic specification and differentiation. *Dev. Cell* **36**, 572–587 (2016).

50. Wang, Q. et al. The p53 family coordinates Wnt and nodal inputs in mesendodermal differentiation of embryonic stem cells. *Cell Stem Cell* **20**, 70–86 (2017).

51. Tosic, J. et al. Eomes and Brachyury control pluripotency exit and germ-layer segregation by changing the chromatin state. *Nat. Cell Biol.* **21**, 1518–1531 (2019).

52. Lie, A. L. M. et al. Regulation of RUNX1 dosage is crucial for efficient blood formation from hemogenic endothelium. *Development* **145**, dev149419 (2018). 53. Fitch, S. R. et al. Gata3 targets Runx1 in the embryonic haematopoietic stem cell niche. *IUBMB Life* **72**, 45–52 (2020).

54. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* **36**, 411–420 (2018).

55. Eliades, A. et al. The hemogenic competence of endothelial progenitors is restricted by Runx1 silencing during embryonic development. *Cell Rep.* **15**, 2185–2199 (2016).

56. Tober, J., Yzaguirre, A. D., Piwarzyk, E. & Speck, N. A. Distinct temporal requirements for Runx1 in hematopoietic progenitors and stem cells. *Development* **140**, 3765–3776 (2013).

57. Yzaguirre, A. D., Howell, E. D., Li, Y., Liu, Z. & Speck, N. A. Runx1 is sufficient for blood cell formation from non-hemogenic endothelial cells in vivo only during early embryogenesis. *Development* **145**, dev158162 (2018). 58. Swiers, G. et al. Early dynamic fate changes in haemogenic endothelium characterized at the single-cell level. *Nat. Commun.* **4**, 2924 (2013).

59. Obier, N. et al. Cooperative binding of AP-1 and TEAD4 modulates the balance between vascular smooth muscle and hemogenic cell fate. *Development* **143**, 4324–4340 (2016).

60. Padron-Barthe, L. et al. Clonal analysis identifies hemogenic endothelium as the source of the blood–endothelial common lineage in the mouse embryo. *Blood* **124**, 2523–2532 (2014).