The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation

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Abstract

Instructive programs guiding cell fate decisions in the developing mouse embryo are controlled by a few so-called master regulators. Genetic studies demonstrate that the T-box transcription factor Eomesodermin (Eomes) is essential for epithelial to mesenchymal transition (EMT), mesoderm migration and specification of definitive endoderm (DE) during gastrulation¹. Here we report that Eomes expression within the primitive streak marks the earliest cardiac mesoderm and promotes formation of cardiovascular progenitors by directly activating the bHLH transcription factor Mesp1 upstream of the core cardiac transcriptional machinery²-⁴. In marked contrast to Eomes/Nodal signalling interactions that cooperatively regulate anterior-posterior (A-P) axis patterning and allocation of the DE cell lineage¹,⁵-⁸, formation of cardiac progenitors requires only low levels of Nodal activity accomplished via a Foxh1/Smad4 independent mechanism. Collectively our experiments demonstrate that Eomes governs discrete context dependent transcriptional programmes that sequentially specify cardiac and DE progenitors during gastrulation.

Keywords

Eomesodermin; Mesp1/2; cardiac specification; definitive endoderm; gastrulation

Much has been learned about the coordinated activities of key regulatory networks of transcription factors and growth factor signalling pathways governing cell fate decisions during gastrulation⁹. Nascent mesoderm is induced as epiblast cells ingress through the primitive streak (PS) and undergo EMT. Distinct mesodermal sub-populations become allocated according to the timing and order of these cell movements. Thus extra-embryonic...
mesoderm arises from the posterior PS, while cardiac, paraxial and lateral plate precursors emerge sequentially as the PS elongates towards the distal tip of the embryo. Fate mapping experiments demonstrate that DE progenitors are specified in the most anterior region of the PS (APS), in close proximity to the cardiovascular progenitors\textsuperscript{10, 11}.

Mesoderm formation and patterning along the proximodistal axis of the PS is known to be regulated by dose-dependent Nodal/Smad2/3 activities\textsuperscript{7}. The highest level of Nodal/Smad2/3 signaling is required to specify APS derivatives, namely the DE, node and notochord\textsuperscript{5, 6, 8}. The transcription factor \textit{Smad4}, and its DNA-binding partner the forkhead transcription factor \textit{Foxh1}, also play essential roles in APS specification\textsuperscript{6, 12, 13}. Additionally, the T-box transcription factor \textit{Eomes} acts cooperatively with the Nodal/Smad2/3 pathway to promote delamination of nascent mesoderm and specification of APS fates\textsuperscript{1}.

\textit{Eomes} expression is initiated in the prospective posterior aspect of the epiblast at embryonic day 5.75 (E5.75)\textsuperscript{14}. During gastrulation expression is maintained in the distal PS\textsuperscript{14, 15}, encompassing the same region where cranial, cardiac and paraxial mesodermal sub-cell populations are generated\textsuperscript{10}. Inactivation of \textit{Eomes} in the epiblast results in a severe block in EMT and arrests development at gastrulation\textsuperscript{1}. To further characterise \textit{Eomes} functional contributions within the mesodermal cell lineages we generated an \textit{Eomes}\textsuperscript{Cre} reporter allele. \textit{Cre} mRNA expression recapitulates endogenous expression (Supplementary Information, Fig. S1a) allowing derivation of a fate map of \textit{Eomes} expressing cells in later stage embryos (Fig. 1). \textit{Eomes}\textsuperscript{Cre/+} males were mated to females carrying the ROSA26\textsuperscript{R} reporter strain\textsuperscript{16} and the resulting embryos stained for LacZ activity (Fig. 1d, e, Supplementary Information, Fig. S1b). Surprisingly, we found in E8.5 and E 9.5 \textit{Eomes}\textsuperscript{Cre+}; ROSA26\textsuperscript{R} embryos that LacZ expressing cells are mostly absent from the somites, intermediate and lateral plate mesoderm and largely restricted to the head mesenchyme, cardiac mesoderm and vasculature (Fig. 1d, e). As expected\textsuperscript{1}, the DE and gut tube are exclusively comprised of LacZ marked \textit{Eomes}\textsuperscript{Cre+} descendants. At later stages endodermal but not mesodermal components of developing organs derived from the gut tube are LacZ positive (Supplementary Information, Fig. S1c). \textit{Eomes} expressing cells thus give rise to two discrete progenitor cell populations during gastrulation, namely the prospective cranial and cardiac mesoderm that emerge from the PS at early stages, and APS derivatives giving rise to the DE, node and notochord. In striking contrast \textit{Eomes}+ cells are excluded from the majority of mesodermal tissues derived from the paraxial and lateral plate mesoderm. These observations strongly suggest that a discrete sub-population of cells within the pre-gastrulation epiblast preferentially ingress and migrate anteriorly as a cohort to form the cardiac crescent and prospective head mesoderm.

To directly examine whether \textit{Eomes} function is required for specification of cardiovascular progenitors we analysed E7.5 embryos carrying an epiblast-specific \textit{Eomes} deletion (\textit{Eomes}\textsuperscript{CA/N};\textit{Sox2.Cre})\textsuperscript{1} by whole mount \textit{in-situ} hybridization (WISH). Embryos lacking \textit{Eomes} function strongly express mesodermal marker genes, including \textit{Brachyury}, \textit{Fgf8} and \textit{Snail}\textsuperscript{1}. However in striking contrast expression of early cardiac marker genes, \textit{Myl7} (also known as \textit{Mlc2a}), \textit{Wnt2} and \textit{Nkx2.5} was absent (Fig. 2a). Moreover we observe severely reduced expression of early vascular marker genes such as \textit{Agtr1l}, \textit{Rasgrp3} and \textit{Klhl6} (Fig. 2a).
To test whether loss of cardiac gene expression reflects a cell autonomous *Eomes* requirement we examined the developmental potential of *Eomes* null embryonic stem (ES) cells in the context of chimeric embryos. *Eomes* null ES cells were injected into wild type blastocysts carrying the *ROSA26lacZ* allele and the resulting embryos analysed by X-Gal staining (Fig. 2b). As expected at E8.5 and E9.5 *Eomes* null cells efficiently contribute to the majority of mesodermal tissues but are entirely excluded from the forming gut tube. Strikingly in all cases examined (n=10), the myocardium and endocardium of the developing heart were also exclusively comprised of wild type LacZ+ cells (Fig. 2c, d). Thus we conclude that *Eomes* plays an essential cell autonomous role during allocation of both the DE and cardiovascular progenitors.

To further evaluate *Eomes* contributions we exploited culture protocols that promote ES cell differentiation towards either DE or cardiac fates. To elicit DE formation, ES cells harboring an *EomesGFP* reporter allele were cultured in the presence of high doses of ActivinA (50ng/ml, Supplementary Information, Fig. S2a). After 4 days, as judged by GFP expression or staining with an Eomes antibody, >95% of cells were strongly positive (Supplementary Information, Fig. S2b, and data not shown). Next we compared wild type (CCE), *Eomes* null (6A6) and *Smad2* null (KT15) ES cells and monitored DE marker gene expression by RT-PCR. *Eomes* expression was detectable 24hrs after ActivinA treatment in wild type cells, severely reduced in *Smad2*-deficient cells and absent in *Eomes* null cells (Fig. 3a). In chimera studies *Smad2*-deficient cells robustly contribute to all mesodermal lineages but are excluded from DE derivatives and as shown here only inefficiently up-regulate expression of *Eomes* or DE markers.

Differentiating *Eomes* null ES cells strongly express PS and mesodermal marker genes, including *Brachyury* and *Mixl1* but expression of DE marker genes including *Sox17* and *Foxa2* was drastically reduced. Similar conclusions were reached in Q-RT-PCR experiments examining a panel of key DE-associated genes including *Gsc*, *Gata6* and *Lhx1* (Fig. 3b).

Next we examined the development of cardiac progenitors via embryoid body (EB) differentiation in hanging drops with high serum-stimulation (Supplementary Information, Fig. S2c, d). In wild type cultures *Eomes* transcripts are detectable by RT-PCR on day 2, peak around day 4 and are down-regulated by day 6 (Fig. 3c). Coincident with highest levels of *Eomes* expression, early cardiac markers *Mesp1* and *Myl7* are upregulated beginning at around day 4. In *Eomes* null EBs the pan-mesodermal marker *Brachyury* is robustly induced (Fig.3c), but in marked contrast expression of cardiac-specific genes including *Mesp1*, *Myl7*, *Myl2*, *Myocardin*, *Nkx2.5* and *Mef2c* was entirely absent. As judged by microscopic observation and staining for the cardiac protein TroponinI (TnI), close to 100% of wild type EBs contain clusters of contractile cardiomyocytes after day 7 of differentiation (Fig. 3d, e, Supplementary Information, Movie SM1). *Eomes* null EBs fail to differentiate into cardiomyocytes by these criteria (Fig. 3d, e, Supplementary Information, Movie SM2). Collectively these results suggest that *Eomes* acts upstream of the transcriptional hierarchy that specifies cardiac fates during gastrulation.

The bHLH transcription factor *Mesp1* has been described a master regulator of multipotent cardiovascular progenitor specification. Genetic fate mapping experiments demonstrate
that Mesp1 expression marks cardiac progenitors that give rise to the myocardial and endocardial derivatives. Moreover Mesp1 activity has also been implicated in the early steps of cardiac lineage specification in vitro. Mesp1 is strongly expressed at the onset of gastrulation (E6.5) along the forming PS, marking the prospective cardiac mesoderm and is then rapidly down-regulated starting around E7.5. Mesp1 mutants form cardiac mesoderm but heart morphogenesis is highly compromised leading to cardia bifida. Mesp1 and Mesp2 are functionally interchangeable in vivo. Thus premature up-regulated expression of the closely related family member Mesp2 in the loss of function mutants is sufficient to generate cardiac progenitors and partially rescue heart morphogenesis. Mesp1; Mesp2 double mutant embryos block at gastrulation, and display an EMT phenotype that closely resembles the Eomes loss of function phenotype. At E7.0 Mesp1 is completely absent in Eomes mutants (Fig. 4a). At slightly later stages (E7.25) a few exceptional Eomes mutants display weak Mesp1 staining. At E7.25 Mesp2 expression is undetectable in WISH experiments. However Q-RT-PCR analysis demonstrates that Mesp1 and Mesp2 expression are both dramatically down-regulated (Fig. 4b). As shown above differentiating Eomes null ES cells lack the ability to upregulate Mesp1 and Mesp2 (Fig. 3c). Collectively these experiments strongly suggest that Eomes acts upstream of Mesp1/2 to regulate EMT and control allocation of cardiac progenitors.

Mesp1 and Mesp2 are closely linked on chromosome 7, and arranged in opposite orientation with the two transcriptional start (TS) sites separated by approximately 17 kb. The cis-acting regulatory elements responsible for controlling temporally and spatially restricted expression patterns are well characterized. An evolutionary conserved early mesoderm enhancer (EME, Fig. 4c, Supplementary Information, Fig. S3b, d) recapitulates Mesp1 expression in nascent mesoderm, while regulatory sequences adjacent to the Mesp2 TS site direct mesodermal expression in the PS, presomitic mesoderm and developing somites. Both elements contain T-box consensus binding sites (Supplementary Information, Fig. S3). Additionally we have identified a further conserved T-box binding element in close proximity to the Mesp1 TS site (Fig. 4c, Supplementary Information, Fig. S3b, c).

To further investigate Mesp1/2 activation during cardiovascular lineage commitment, we used the P19Cl6 embryonal carcinoma (EC) cell line that efficiently differentiates into beating cardiomyocytes in the presence of 1% DMSO. Transient Eomes expression seen initially at day 2 is down regulated by day 6, associated with increased Mesp1 expression levels (Supplementary information, Fig. S4a). Activation of Eomes estrogen-receptor fusion protein in the presence of tamoxifen (EomesER, Supplementary Information, Fig. S4b) also efficiently induces strong Mesp1 expression within 24 hours as assayed by Q-PCR (Supplementary Information, Fig. S4g). To directly evaluate Eomes occupancy at the Mesp1/2 locus we performed chromatin immunoprecipitation (ChIP) analysis with day 4 DMSO-treated P19Cl6 cells as well as tamoxifen-treated P19Cl6EomesER (P19EoER) cells. Eomes binding to all three conserved T-box site-containing regions within the Mesp1/2 locus was clearly observed (Fig. 4d, e, Supplementary Information, Fig. S5). The EME, which controls early Mesp1 expression in nascent mesoderm, gave the strongest signal. Occupancy at other genomic regions, or in uninduced cells was undetectable. The T-
box sites adjacent to the Mesp2 TS site are known to be occupied by Tbx6 in pre-somatic mesoderm\textsuperscript{27}. This cis-acting regulatory element, as well as the minimal 220 bp Mesp1 EME T-box element, regulates Mesp2 and Mesp1 expression respectively at later stages during somitogenesis\textsuperscript{26}. Additional T-box family members are also known to be expressed in the early gastrulation stage embryo\textsuperscript{14, 15, 29-31}. However only Brachyury and Eomes are exclusively present in the posterior epiblast and nascent mesoderm overlapping with sites of Mesp1 expression. Specification of early heart progenitors proceeds normally in T mutant embryos. Therefore a strong argument can be made that the earliest Mesp1 expression domain marking the cardiac progenitors is directly activated by Eomes occupancy at these T-box sites.

Genetic studies analyzing double heterozygous mutant embryos demonstrate that Eomes and Nodal function cooperatively in A-P axis patterning and formation of APS derivatives\textsuperscript{1}. We wondered whether dose-dependent Nodal signals could potentially regulate Eomes-dependent Mesp1 activation. To test this possibility first we examined Smad4\textsuperscript{6} and Foxh1 mutant embryos\textsuperscript{12} at E6.5 and E7.5. Interestingly Mesp1 expression is unaffected by loss of either Smad4 or Foxh1 (Fig. 5a). The lim-homeodomain transcription factor Lhx1 is also required for specification of DE and anterior axial midline tissues\textsuperscript{32, 33}. Similarly we observe that compromised DE development has no noticeable impact on specification of Mesp1+ cardiac progenitors. Thus Lhx1 loss of function embryos display strong expression of both Mesp1 and Eomes (Fig. 5a and data not shown). Conversely it is known that expression of the APS markers Lhx1 and Gsc is unperturbed in Mesp1/2 double mutant embryos\textsuperscript{23}. Lowering Nodal\textsuperscript{5, 34} or Smad2/3\textsuperscript{7} levels during gastrulation also selectively disturbs DE specification. Next, we manipulated ActivinA concentrations in differentiating ES cells and confirmed that low levels (5ng/ml) are sufficient for robust Mesp1 expression, while conversely maintaining cultures in high ActivinA concentrations (50ng/ml) leads to induction of Sox17 (Supplementary Information, Fig. S6a, b). Collectively, these results strongly suggest that specification of both cardiac and DE progenitors requires Eomes, but these lineages arise independently, dependent on local Nodal/Smad/Foxh1 signalling levels.

Multipotent mesodermal progenitor cells that give rise to diverse tissues of the emerging body plan become progressively allocated as epiblast cells transit the PS. Fate mapping and grafting studies have shown cranial and cardiac mesoderm derive from the earliest wave of cells that exit at the mid streak stage, whereas pre-somatic/paraxial and lateral plate mesoderm emerge at more posterior levels at late streak stages\textsuperscript{10, 11}. Remarkably the present experiments identify a sub-set of proximal posterior epiblast cells already committed to adopt a cardiac fate many hours prior to mesoderm induction and overt PS formation. The early Eomes expression domain marks cardiac progenitors programmed to activate Mesp1, previously identified as the master regulator that acts instructively to specify cardiovascular cell fates. Recent studies demonstrate that Mesp1 initiates global changes in gene expression by directly binding to regulatory sequences at the promoters of many key genes in the core cardiac transcriptional machinery. Mesp1 upregulates expression of Hand2, Myocardin, Nsk2.5 and Gata4, and represses genes governing pluripotency and mesodermal and endodermal cell fates\textsuperscript{2}. Additionally Mesp1 promotes EMT via selective up-regulation of the zinc-finger repressor Snail\textsuperscript{4}, allowing the nascent cardiac progenitors to migrate anteriorly to
underlie the developing headfolds where they coalesce to form the cardiac crescent. Acting a few hours later during PS elongation Eomes/Nodal signalling results in specification of DE that gives rise to the entire gut endoderm lineage. Eomes was recently shown to be a key player in the transcriptional network upstream of DE specification during human ES cell differentiation.

How does a single transcription factor Eomes govern allocation of two independent, non-overlapping, multipotent progenitor cell populations as epiblast cells sequentially transit the PS? We suggest that the key parameter controlling cardiac versus DE cell fate is the timing and duration of exposure to Nodal signaling (Fig. 5b). Low levels of Nodal activity in the posterior epiblast are sufficient to activate Eomes and induce cardiac mesoderm formation at early post-implantation stages (Supplementary Information, Fig. S6d). Eomes expression is necessary and sufficient to activate Mesp1/2, promote EMT and migration of a discrete mesodermal sub-population that gives rise to the cardiac crescent. Within this early sub-set Mesp1 directly represses genes required for formation of DE including Foxa2, Gsc and Sox17. Mesp1/2 expression also activates expression of the Nodal antagonist Lefty2 to further insulate cardiac progenitors as they migrate away from the source of Nodal signaling (Supplementary Information, Fig. S6e). Consistent with this idea, up-regulated Nodal signalling in Tgif1/2 double mutant embryos inhibits Mesp1 expression, whereas decreased levels of Nodal activity rescues Mesp1 expression.

Eomes activation of Mesp1/2 in cardiac progenitors is only transient due to a Mesp1/2 autoregulatory negative feedback loop. At late streak stages expression of Mesp1/2 is re-activated in pre-somatic mesoderm by Tbx6 occupancy of the conserved T-box sites (Fig. 5c). In contrast during streak elongation Eomes expression is maintained by high levels of Nodal/Smad2 signaling downstream of a Wnt/Lef1 feed-forward loop. Acting together with Nodal-dependent Smad2/3/4/Foxh1 transcription complexes, Eomes promotes formation of APS progenitors that give rise to the DE, node and notochord. It will be interesting to learn more about developmental regulation of Eomes transcriptional partnerships and cell type specific changes in chromatin architecture governing T-box site occupancy at the Mesp1/2 locus and selection of target genes in the DE cell lineage.

**Methods**

**Generation of the EomesCre reporter allele**

The EomesCre allele was generated using the same strategy as previously described for the EGFP knock-in allele. The targeting vector encompasses a 8.25 kb HpaI fragment of the Eomes locus. Cre coding sequences were introduced into the Exon1 start site followed by a LoxP-flanked neomycin resistance cassette between the Sphl (translational start) and EagI sites, resulting in removal of ~500 bp of the endogenous 5′ coding region. The 3′ homology arm was flanked by a pMC1.TK negative selection cassette. Linearised targeting vector was electroporated into CCE ES cells and drug resistant ES cell colonies screened by Southern blot using a 3′ external probe on EcoRV digested DNA (wt allele; 15.2 kb; targeted allele; 8.0 kb). Correctly targeted clones were transfected with pMC1.Cre and resulting subclones screened by Southern blot to detect the reporter allele (6.2 kb). Two independent excised cell clones were used to generate germline chimeras. Offspring were genotyped by PCR.

*Nat Cell Biol. Author manuscript; available in PMC 2015 August 11.*
using Cre specific primers (Cre-fw: 5′-GCATAACCAGTAAACAGCATTGCTG-3′, Cre-rev: 5′-GGACATGTTCAAGGATGGCCAGCG-3′). The strain was maintained on a 129SvEv/C57Bl/6 mixed genetic background.

Mouse strains, genotyping and generation of chimeric embryos

All animal procedures were approved by the Ethical Review Committee of the University of Oxford. The ROSA26<sup>16</sup> reporter<sup>16</sup>, ROSA26 gene-trap<sup>17</sup>, Foxh1<sup>12</sup>, Smad4<sup>CA</sup> 6, Eomes<sup>CA/N</sup>, Sox2, Cre<sup>1</sup> and Lhx1<sup>32</sup> strains were genotyped as previously described. For generation of chimeric embryos blastocysts recovered from matings of ROSA26 males to CD1 outbred females were injected with 12-14 Eomes null ES cells<sup>1</sup> and transferred into E2.5 pseudopregnant foster females.

Whole-mount in situ hybridisation, LacZ staining and histology

Whole-mount in situ hybridisation analysis of E6.5-9.5 embryos dissected and fixed with 4% PFA overnight at 4 °C was performed according to standard protocols<sup>37</sup> using probes for Agtr1, Cre, Eomes, Klh6, Mesp1, Myl7, Nkx2.5, Rasgrp3, and Wnt2. LacZ staining was performed as described<sup>37</sup>. For histology, embryos were post-fixed in 4% PFA, dehydrated through an ethanol series, embedded in paraffin, sectioned at 8 μM and Eosin-counterstained.

Definitive endoderm and cardiomyocyte differentiation assays

Wild-type (CCE), Eomes null (6A6) and Smad2 null (KT 15) feeder-depleted ES cells were cultured in DMEM (Invitrogen) with 15% FCS, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol and 1000 U/ml recombinant LIF (Millipore). To induce DE differentiation, ES cells were cultured in serum-free ESGRO Complete clonal grade medium (Millipore) on 0.1% gelatin-coated dishes for a minimum of 2-3 passages before seeding at low density (18,000 cells/ml) in ESGRO Complete basal medium (Millipore) in bacteriological grade dishes. Whole-mount in situ hybridisation analysis of E6.5-9.5 embryos dissected and fixed with 4% PFA overnight at 4 °C was performed according to standard protocols<sup>37</sup> using probes for Agtr1, Cre, Eomes, Klh6, Mesp1, Myl7, Nkx2.5, Rasgrp3, and Wnt2. LacZ staining was performed as described<sup>37</sup>. For histology, embryos were post-fixed in 4% PFA, dehydrated through an ethanol series, embedded in paraffin, sectioned at 8 μM and Eosin-counterstained.

P19Cl6 cell culture, differentiation and generation of EomesER expressing sub-clones

P19Cl6 EC cells were routinely cultured in α-MEM (Invitrogen) supplemented with 10% FCS. To induce differentiation, cells were seeded at 3.7×10<sup>5</sup> cells/6 cm dish in media containing 1% DMSO (Sigma). Cells were electroporated with linearised pCAGEomesER-
IRESPuro vector\textsuperscript{38} and selected in 1 µg/ml puromycin, to generate P19EoER sub-clones. Expression of the EomesER fusion protein was confirmed by Western blot analysis. For EomesER activation 1 µg/ml of 4-Hydroxytamoxifen (Sigma, H7904) was added to the culture media.

**RNA isolation, One-step & quantitative RT-PCR analysis**

RNA was isolated using the RNasy Kit (Qiagen) according to the manufacturer’s instructions, using on-column DNase treatment. cDNA was generated using the SuperScriptIII kit (Invitrogen) with oligo-dT primers. Q-RT-PCR was performed using the Quantitect SYBRGreen PCR kit and a Rotar-gene Q (Qiagen) and analysed using the $\Delta\Delta$Ct method, as described previously\textsuperscript{39}. For one-step RT-PCR analysis, the One-step RT-PCR kit (Qiagen) was used with gene specific primers according to manufacturer’s instructions. Primer sequences are provided in Supplementary Information Table S1.

**Chromatin Immunoprecipitation**

Cells were cross-linked for 10 minutes at RT with 1% (v/v) formaldehyde and quenched with 125 mM glycine. Prepared chromatin was sonicated to 200-500 bp and immunoprecipitated with 15 µg of anti-Eomes (Abcam, ab23345), anti-PolII N-terminal (Santa Cruz, sc-899x) or normal rabbit IgG (Santa Cruz, sc-2027). Eluted DNA was recovered by phenol-chloroform extraction, precipitated with ethanol and resuspended in TE buffer. ChIP material was analysed using a Rotar-Gene Q (Qiagen) and SYBRGreen master mix (Qiagen). The amount of precipitated DNA was compared to the starting input material, as percentage of input. Each ChIP experiment was performed at least three times on separate biological samples. Q-PCR was performed in triplicate. ChIP primer sequences are provided in Supplementary Information Table S2.

**Immunofluorescence**

ES or P19Cl6 cultures were fixed in 4% PFA for 10 min at RT and permeabilised with 0.2% TritonX/PBS for 20 min before blocking for 1 hr in 10% FCS, 0.3% BSA, 0.3% TritonX in PBS. Primary antibodies used include rabbit anti-Eomes (Abcam, ab23345, 1:100), rat anti-Eomes (eBioscience, 14-4876, 1:100), goat anti-Gata4 (Santa Cruz, sc-1237, 1:100), mouse anti-Troponin I (Abcam, ab19615, 1:200) and mouse anti-smooth muscle actin (Dako, M0851, 1:100). Primary antibodies were applied in blocking solution overnight at 4 °C. Cells were washed with 0.1% TrionX/PBS and incubated in blocking solution for 1 hr at RT with appropriate conjugated secondary antibodies; goat anti-rabbit Alexa Fluor 555; goat anti-rat Alexa Fluor 488; donkey anti goat Alexa Fluor 488, goat anti-mouse Alexa Fluor 488 (all Molecular Probes/Invitrogen, 1:500) and donkey anti-mouse Cy3 (Jackson Immuno Research, 1:2000). Coverslips were mounted with Vectashield mountant containing DAPI (Vector Labs, H-1200). Fluorescent images were captured with a Zeiss epifluorescence microscope or an inverted laser scanning microscope (LSM 510 Meta Duo Live) equipped with a 25x/63x Plan-Apochromate objective.
Western blot analysis

Cell lysates were prepared using RIPA buffer and subjected to SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% milk in TBS-T, incubated in primary antibodies overnight including rabbit anti-Eomes (Abcam, ab23345, 1:1000), rabbit anti-phospho-Smad2 (Cell Signaling, 3101, 1:1000) and mouse anti-γ-tubulin (Sigma, T6557, 1:3000). Secondary antibodies were donkey anti-rabbit-HRP (Amersham NA934V, 1:2000) and goat anti-mouse (Dako, P0447, 1:2000). Blots were developed by chemiluminescence using ECL plus (Amersham).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Nicole Hortin, Ahmed Salman and Mihael Pavlovic for technical assistance, Christopher Böhlke and Alexis Hofherr for help with imaging techniques, Sonia Stefanovic for Q-PCR primer optimization, and Hitoshi Niwa and Yumiko Saga for plasmids. This work was supported by the Emmy Noether Programme and SFB850 of the German Research Council (DFG) to S.J.A. and a Programme Grant from the Wellcome Trust to E.J.R.

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Figure 1.
Fate mapping \(Eomes^{\text{Cre}}\) expressing cells reveals selective contributions to DE and cardiovascular cell lineages.
(a) Targeting strategy used to generate the \(Eomes^{\text{Cre}}\) reporter allele. Cre recombinase coding sequences were inserted into Exon1 of the Eomes locus. \(\text{RV}, \text{EcoRV}; \text{H}, \text{HpaI}; \text{S}, \text{SphI}; \text{E}, \text{EagI}; \text{Cre}, \text{Cre recombinase}\). (b) ES clones were screened by Southern blot on EcoRV digested DNA using a 3' probe (red line in a) to detect wildtype (wt) and targeted (T) alleles. (c) Correctly targeted ES cells were transiently transfected with Cre to excise the PGK-neo selection cassette and generate the reporter allele (R), as shown by Southern blot. (d, e) Fate mapping experiments demonstrate that descendants of \(Eomes^{\text{Cre}}\) expressing cells contribute to the myocardium and endocardium of the heart (he), the head mesenchyme (hm), vasculature and the endoderm of the primary gut tube (gt), but rarely colonize other
mesoderm tissues formed from paraxial and lateral plate mesoderm. Sections were counterstained with eosin to highlight non-labelled cells.
**Figure 2.**

*Eomes* functional loss disrupts specification of cardiovascular progenitors.

(a) Whole-mount *in situ* hybridisation analysis of cardiac mesoderm (*Myl7, Wnt2, Nkx2.5*) and vascular (*Agtrl1, Rasgrp3, Klhl6*) markers in control and *Eomes<sup>NCA</sup>;Sox2.Cre* mutant embryos. *Eomes* mutants entirely lack expression of cardiac marker genes and show significantly reduced expression of vascular markers. In contrast in wild type embryos vascular markers broadly delineate the embryonic and extra-embryonic vasculature at E8.5-9.5. (b) Schematic diagram illustrating the protocol for generation of chimeric
embryos, *Eomes*-null ES cells were introduced into wild type ROSA26^LacZ_ blastocysts. (c, d) Histological sections of two independent LacZ stained E9.5 chimeric embryos were counterstained with Eosin to identify *Eomes* mutant cell populations (pink). The myocardium and endocardium of the heart (he) and endoderm of the gut tube (gt) are exclusively comprised of LacZ positive wild type cells. Relatively few *Eomes* null cells colonize the head mesenchyme (hm), whereas the majority of other tissues are comprised of mixed populations of *Eomes* null and wild-type cells. Scale bar is 500 μm.
Figure 3.
Eomes null ES cells fail to give rise to cardiomyocytes.
(a) Wild-type, Eomes<sup>−/−</sup> and Smad2<sup>−/−</sup> ES cells were cultured in the presence of high-doses of ActivinA. Semi-quantitative RT-PCR analysis shows that Eomes<sup>−/−</sup> and Smad2<sup>−/−</sup> cultures strongly express mesoderm marker genes such as Brachyury and Mixl1, but lack expression of the DE marker genes, Foxa2 and Sox17. (b) Quantitative RT-PCR (qRT-PCR) analysis confirms the dramatically reduced DE marker transcript levels in Eomes<sup>−/−</sup> at day4 of ActivinA-induced differentiation. (c) Wild-type and Eomes mutant embryoid bodies (EBs)...
were induced to form cardiomyocytes in hanging drop cultures. Semi-quantitative RT-PCR analysis reveals that cardiac-specific transcription factors (Mesp1, Mesp2, Gata6, Nkx2.5, Mef2c and Myocardin), as well as structural proteins (Myl7, Myl2) are significantly down-regulated in Eomes null EBs while expression of the panmesodermal marker Brachyury is unaffected. (d) Clusters of beating cardiomyocytes are readily detectable in wild-type EB outgrowths but absent in Eomes null cultures at day 7. Error bars represent standard error of the mean (s.e.m.) of three independent experiments. (e) At day 8 TroponinI (TnI)-positive cardiomyocytes are detectable in wild type outgrowths but are entirely absent in Eomes mutant cultures. Higher magnification reveals characteristic cross striation of myofibrils. Scale bar, 100μm for the overview and 10μm for the higher magnification image.
Figure 4.
Eomes directly binds conserved T-box sites within the *Mesp1* locus to activate expression. (a) *Mesp1* robustly expressed in wild-type embryos at E7.0, is absent in *Eomes<sup>N/CA</sup>;Sox2Cre* mutants. At slightly later stages (E7.25) *Eomes<sup>N/CA</sup>;Sox2Cre* embryos occasionally show weak expression that likely reflects activity of *Tbx6*, known to be expressed in E7.5 *Eomes* mutants<sup>1</sup>. (b) As judged by qRT-PCR *Mesp1* and *Mesp2* transcripts are dramatically reduced in E7.25 *Eomes* mutant embryos. Error bars represent standard deviation (s.d.), n= 5 per genotype. (c) Diagrammatic representation of cis-regulatory elements in the *Mesp1/2* locus.
The positions of previously identified T-box sites within the Mesp1/2 EME and Mesp2 PSME (nomenclature according to ref\textsuperscript{26, 27}) and a novel putative T-box site identified near the Mesp1 TSS are indicated. The Mesp2 PSME contains 3 binding elements (Sites B, G, D) that contain T-box binding motifs. T-box consensus sequences are indicated in red. Red bars indicate areas amplified by qPCR after ChIP using different antibodies. **Ex1, Exon1; Ex2, Exon2; EME, early mesoderm enhancer; PSME, pre-somitic mesoderm enhancer; TSS, transcriptional start site; Tbx, T-box site.** (d) ChIP analysis of P19Cl6 cells treated for 4 days with DMSO using antibodies specific for Eomes, RNA-Polymerase II (PolII) or an IgG control. Specific enrichment for genomic loci containing T-box sites (Mesp1_TSS, Mesp1_Tbx, Mesp2_Tbx) was observed using the Eomes-specific antibody. The PolII antibody gave specific enrichment of the Mesp1_TSS, but not other tested regions of the Mesp1/2 locus. Specific binding to T-box sites (indicated by *) in the Mesp1/2 EME, the Mesp2 PSME and the Mesp1 TSS was detectable after 4 days, but not at day 0. **d0, day 0; d4, day 4 of DMSO differentiation of P19Cl6 cells.** (e) Cells expressing a tamoxifen inducible Eomes fusion construct (P19EoER cells), also shows Eomes binding to these T-box sites at day 4 of tamoxifen treatment. **Con, non-tamoxifen induced control P19EoER cells; Tam, day 4 tamoxifen treated P19EoER cells.** The best representative plots of three independent experiments are shown.
Figure 5.

_Eomes_ and dose-dependent _Nodal/Smad2/3_ signalling levels control cardiac mesoderm and definitive endoderm specification during gastrulation.

(a) _Smad4_ and _Foxh1_ are critical Nodal pathway components for transducing high levels of signalling. _Mespl_ is expressed normally in E6.5 and 7.5 _Foxh1_ null embryos and in embryos lacking _Smad4_ in the epiblast only (Smad4Δ). _Mespl_ expression is also efficiently induced in _Lhx1_ mutant embryos, which display DE and midline mesoderm defects. However the failure of A-P axis rotation results in induction of _Mespl_ throughout the
proximal epiblast. (b) Eomes activity regulates formation of both cardiac mesoderm and DE progenitors during gastrulation. Eomes+ epiblast cells confined to the posterior side of the embryo prior to overt streak formation are exposed to low levels of Nodal signalling. Eomes-dependent activation of Mesp1/2 marks the earliest cardiac progenitors induced in the forming PS. Mesp1/2 expression leads to activation of the Nodal antagonist Lefty2\textsuperscript{23} and direct repression of DE genes\textsuperscript{2}. As cells begin to migrate away from the PS Mesp1/2 expression is down-regulated via a negative feedback loop\textsuperscript{2, 23, 25}. In contrast the Eomes expression domain extends distally and overlaps with increased Nodal signalling levels as the PS elongates. Eomes, acting cooperatively with Nodal/Smad4/FoxH1 dependent signals in the APS, induces DE. (c) At later stages from E7.5 onwards Tbx6 expression in the pre-somitic mesoderm activates a second wave of Mesp1/2 expression in the pre-somitic mesoderm via occupancy of the conserved T-box regulatory elements.