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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-termed 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 gastrulation[1]. 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 machinery[2-4]. In marked contrast to *Eomes/ Nodal* signalling interactions that cooperatively regulate anterior-posterior (A-P) axis patterning and allocation of the DE cell lineage[1, 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 gastrulation[9]. 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

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Author Contributions

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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[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[7]. The highest level of *Nodal/Smad2/3* signaling is required to specify APS derivatives, namely the DE, node and notochord[5, 6, 8]. The transcription factor *Smad4*, and its DNA-binding partner the forkhead transcription factor *Foxh1*, also play essential roles in APS specification[6, 12, 13]. Additionally, the T-box transcription factor *Eomes* acts cooperatively with the *Nodal/Smad2/3* pathway to promote delamination of nascent mesoderm and specification of APS fates[1].

Eomes expression is initiated in the prospective posterior aspect of the epiblast at embryonic day 5.75 (E5.75)[14]. During gastrulation expression is maintained in the distal PS[14, 15], encompassing the same region where cranial, cardiac and paraxial mesodermal sub-cell populations are generated[10]. Inactivation of *Eomes* in the epiblast results in a severe block in EMT and arrests development at gastrulation[1]. To further characterise *Eomes* functional contributions within the mesodermal cell lineages we generated an $Eomes^{Cre}$ reporter allele. *Cre* mRNA expression recapitulates endogenous expression (Supplementary Information, Fig. S1a) allowing derivation of a fate map of *Eomes* expressing cells in later stage embryos (Fig. 1). $Eomes^{Cre/+}$ males were mated to females carrying the $ROSA26^{R}$ reporter strain[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 $Eomes^{Cre}$; $ROSA26^{R}$ 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[1], the DE and gut tube are exclusively comprised of LacZ marked $Eomes^{Cre}$+ descendants. At later stages endodermal but not mesodermal components of developing organs derived from the gut tube are LacZ positive (Supplementary Information, Fig. S1c). *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 *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 *Eomes* function is required for specification of cardiovascular progenitors we analysed E7.5 embryos carrying an epiblast-specific *Eomes* deletion ($Eomes^{CA/N}$;*Sox2.Cre*)[1] by whole mount *in-situ* hybridization (WISH). Embryos lacking *Eomes* function strongly express mesodermal marker genes, including *Brachyury*, *Fgf8* and *Snail*[1]. However in striking contrast expression of early cardiac marker genes, *Myl7* (also known as *Mlc2a*), *Wnt2* and *Nkx2.5* was absent (Fig. 2a). Moreover we observe severely reduced expression of early vascular marker genes such as *Agtrl1*, *Rasgrp3* and *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[1] were injected into wild type blastocysts carrying the *ROSA26*^{LacZ} allele[17] and the resulting embryos analysed by X-Gal staining (Fig. 2b). As expected[1] 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 *Eomes*^{GFP} reporter allele[18] 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)[1] and *Smad2* null (KT15)[7] 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[19] 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[20] (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[21-23]. Genetic fate mapping experiments demonstrate

that *Mesp1* expression marks cardiac progenitors that give rise to the myocardial and endocardial derivatives[22]. Moreover *Mesp1* activity has also been implicated in the early steps of cardiac lineage specification *in vitro*[2-4]. *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[22]. *Mesp1* mutants form cardiac mesoderm but heart morphogenesis is highly compromised leading to cardia bifida[21, 22]. *Mesp1* and *Mesp2* are functionally interchangeable *in vivo*[21]. 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[23]. *Mesp1; Mesp2* double mutant embryos block at gastrulation, and display an EMT phenotype that closely resembles the *Eomes* loss of function phenotype[1, 23]. 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[24]. 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[25]. The *cis*-acting regulatory elements responsible for controlling temporally and spatially restricted expression patterns are well characterized[25-27]. An evolutionary conserved early mesoderm enhancer (EME, Fig. 4c, Supplementary Information, Fig. S3b, d) recapitulates *Mesp1* expression in nascent mesoderm[25], while regulatory sequences adjacent to the *Mesp2* TS site direct mesodermal expression in the PS, presomitic mesoderm and developing somites[26]. Both elements contain T-box consensus binding sites[25-27] (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[28]. 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[25] 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-somitic mesoderm[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[26]. Additional T-box family members are also known to be expressed in the early gastrulation stage embryo[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[1]. We wondered whether dose-dependent *Nodal* signals could potentially regulate Eomes-dependent *Mesp1* activation. To test this possibility first we examined *Smad4*[6] and *Foxh1* mutant embryos[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[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[23]. Lowering Nodal[5, 34] or Smad2/3[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-somitic/paraxial and lateral plate mesoderm emerge at more posterior levels at late streak stages[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*, *Nxk2.5* and *Gata4*, and represses genes governing pluripotency and mesodermal and endodermal cell fates[2]. Additionally *Mesp1* promotes EMT via selective up-regulation of the zinc-finger repressor *Snail*[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[1] 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[35].

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*[2]. *Mesp1/2* expression also activates expression of the Nodal antagonist *Lefty2*[23] 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[36].

Eomes activation of *Mesp1/2* in cardiac progenitors is only transient due to a *Mesp1/2* autoregulatory negative feedback loop[2, 23, 25]. At late streak stages expression of *Mesp1/2* is re-activated in pre-somitic mesoderm by Tbx6 occupancy of the conserved T-box sites[27] (Fig. 5c). In contrast during streak elongation *Eomes* expression is maintained by high levels of *Nodal/Smad2* signaling downstream of a *Wnt3/Lef1* feed-forward loop[8]. Acting together with *Nodal*-dependent *Smad2/3/4/Foxh1* transcription complexes[5-7, 12, 34], *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 *Eomes*^{Cre} reporter allele

The *Eomes*^{Cre} allele was generated using the same strategy as previously described for the EGFP knock-in allele[18]. 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 SphI (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

using Cre specific primers (Cre-fw: 5′-GCATAACCAGTGAAACAGCATTGCTG-3′, Cre-rev: 5′-GGACATGTTCAGGGATCGCCAGGCG-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*^{R} reporter[16], *ROSA26* gene-trap[17], *Foxh1*^{12}[,] *Smad4*$^{CA\ 6}$[,] *Eomes*$^{CA/N}$*;Sox2.Cre*^{1} and *Lhx1*^{32} 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[1] 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[37] using probes for *Agtrl1*,*Cre*, *Eomes*, *Klhl6*, *Mesp1*, *Myl7*, *Nkx2.5*, *Rasgrp3*, and *Wnt2*. *LacZ* staining was performed as described[37]. 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, to promote embryoid body (EB) formation. 50 ng/ml Activin A (R&D systems) was added after 48hrs. For titration of ActivinA effects, the medium was changed after 72 hrs and new medium added with 5 or 50 ng/ml ActivinA. For cardiac differentiation, ES cells were re-suspended at 1×10^4 cells/ml in IMDM (Invitrogen) supplemented with 20% fetal calf serum, 1% non-essential amino acids, and 0.1 mM β–mercaptoethanol in hanging drops (10 μl) plated on the inside lids of bacteriological dishes. After 48hrs EBs were transferred in 10 ml medium to 10 cm bacteriological dishes. At day 4 EBs were plated on tissue culture dishes, allowed to adhere and scored at d7. EBs were plated on fibronectin-coated 15 μ-Slide 2×9well (Ibidi) and cultured for additional 96 hrs prior to imaging and immunostaining. The number and percentage of beating EBs was counted in three independent experiments. Live cell imaging was recorded on a Zeiss Observer Z.1 microscope equipped with an Axiocam MRm camera at frame rates of 10/s and 36/s.

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 3.7×10^5 cells/6 cm dish in media containing 1% DMSO (Sigma). Cells were electroporated with linearised pCAGEomesER-

IRESPuro vector[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 RNeasy 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 ΔΔCt method, as described previously[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

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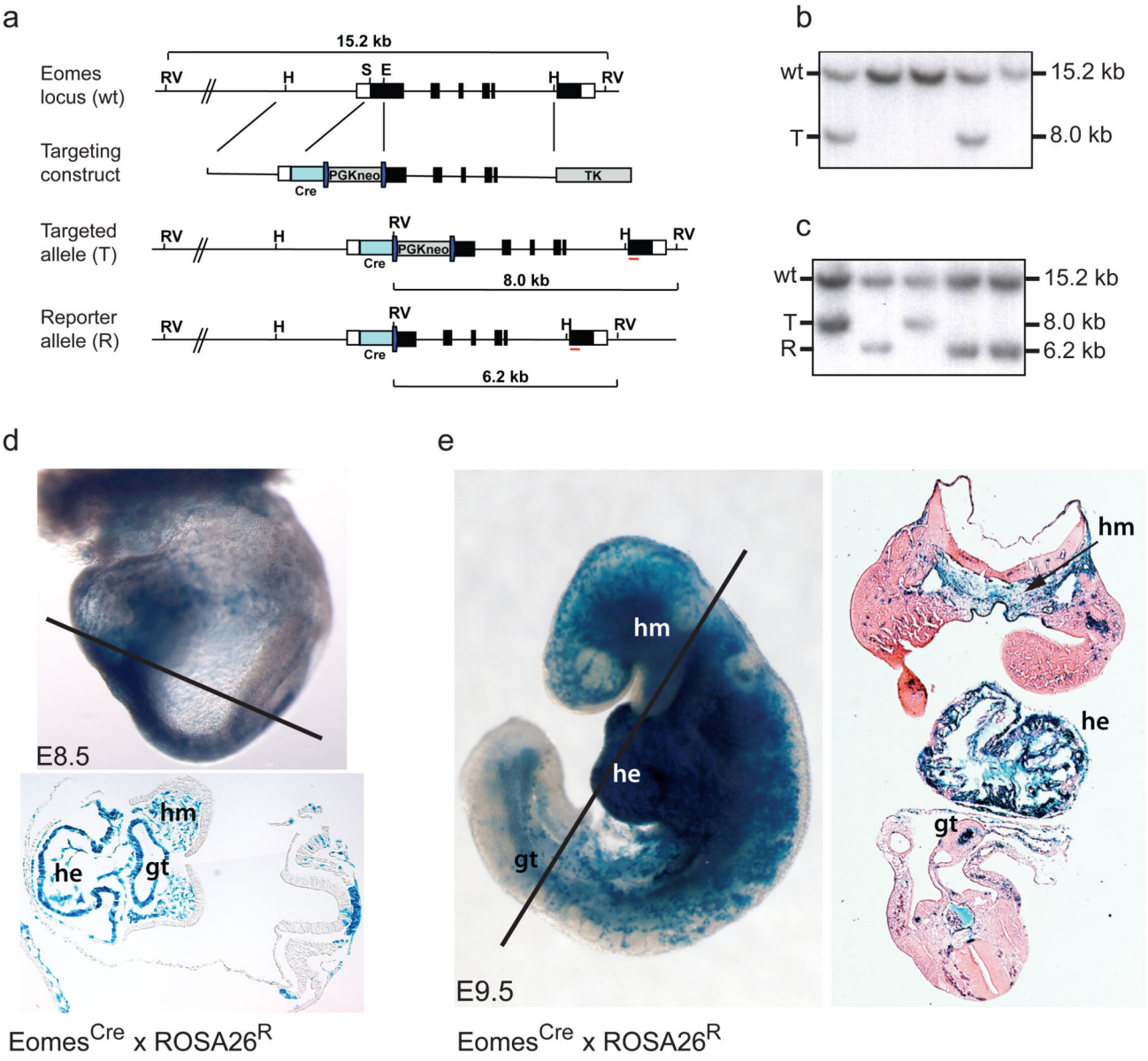

Figure 1.

Fate mapping *Eomes*^{Cre} expressing cells reveals selective contributions to DE and cardiovascular cell lineages.

(a) Targeting strategy used to generate the *Eomes*^{Cre} reporter allele. Cre recombinase coding sequences were inserted into Exon1 of the Eomes locus. **RV**, EcoRV; **H**, HpaI; **S**, SphI; **E**, EagI; **Cre,** 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*^{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.

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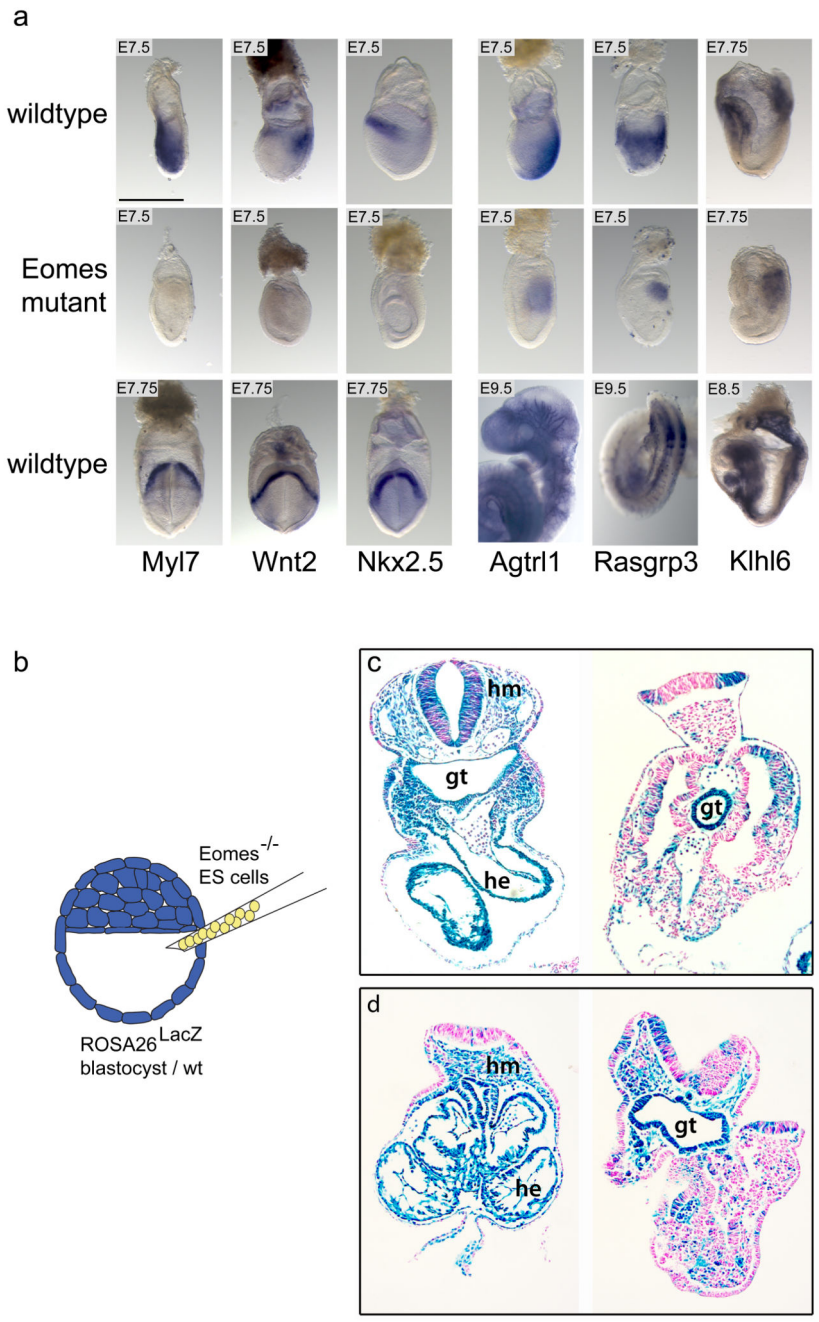

Figure 2.

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 $Eomes^{N/CA}$;*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.

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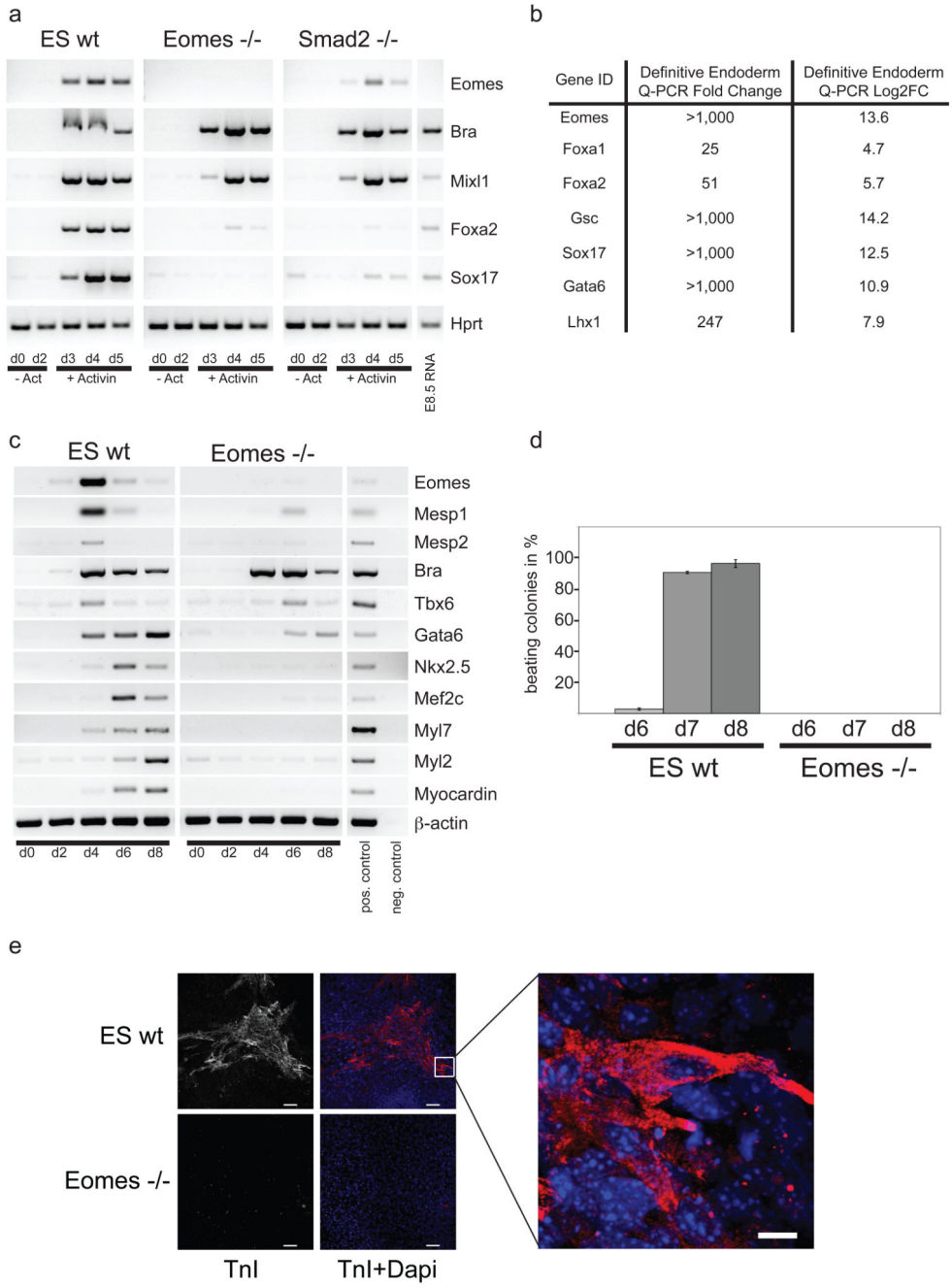

Gene ID	Definitive Endoderm Q-PCR Fold Change	Definitive Endoderm Q-PCR Log2FC
Eomes	>1,000	13.6
Foxa1	25	4.7
Foxa2	51	5.7
Gsc	>1,000	14.2
Sox17	>1,000	12.5
Gata6	>1,000	10.9
Lhx1	247	7.9

Figure 3.

Eomes null ES cells fail to give rise to cardiomyocytes.

(a) Wild-type, *Eomes*$^{-/-}$ and *Smad2*$^{-/-}$ ES cells were cultured in the presence of high-doses of ActivinA. Semi-quantitative RT-PCR analysis shows that *Eomes*$^{-/-}$ and *Smad2*$^{-/-}$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*$^{-/-}$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.

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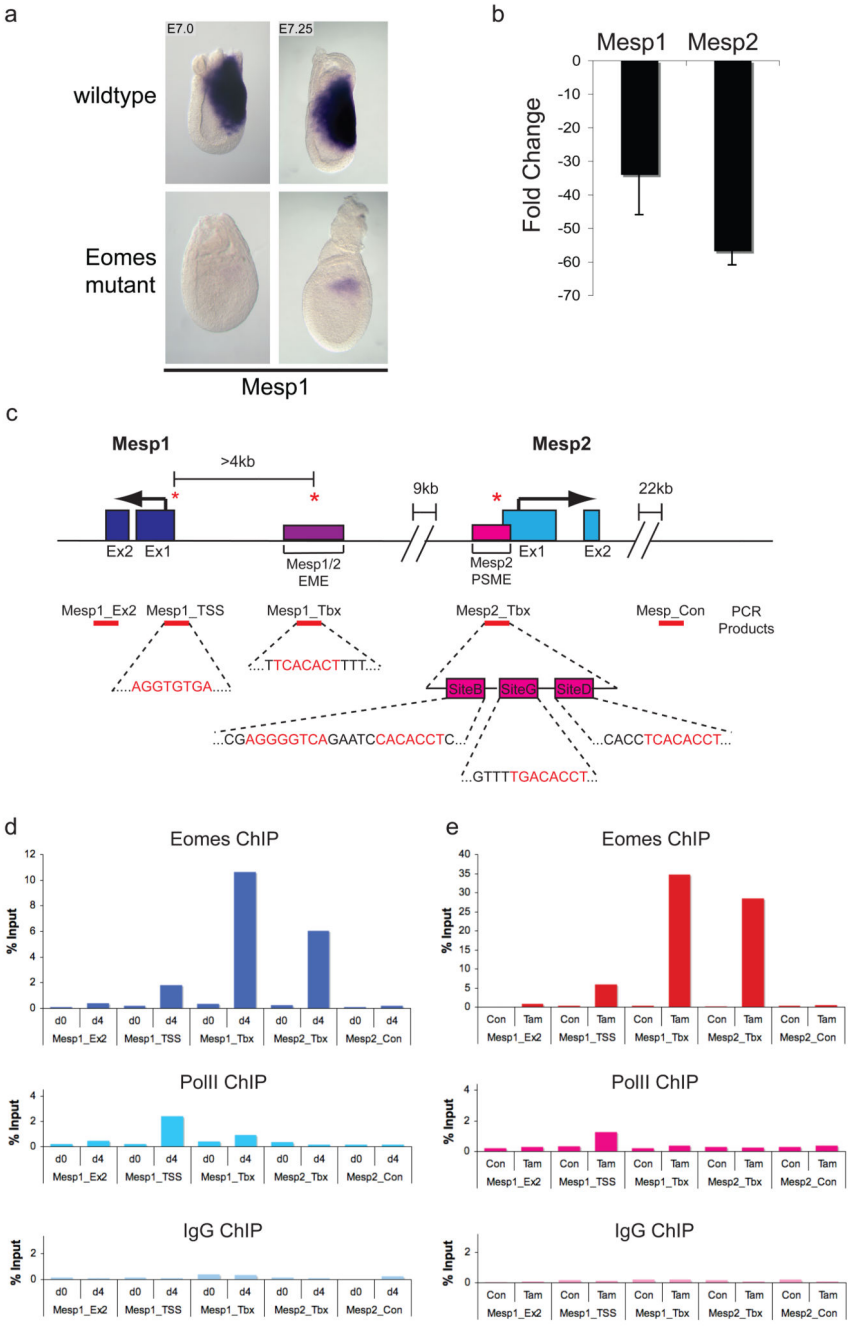

Figure 4.

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*$^{N/CA}$;*Sox2Cre* mutants. At slightly later stages (E7.25) *Eomes*$^{N/CA}$;*Sox2Cre* embryos occasionally show weak expression that likely reflects activity of *Tbx6*, known to be expressed in E7.5 *Eomes* mutants[1]. (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.

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The positions of previously identified T-box sites within the *Mesp1/2* EME and *Mesp2* PSME (nomenclature according to ref[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**, day4 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.

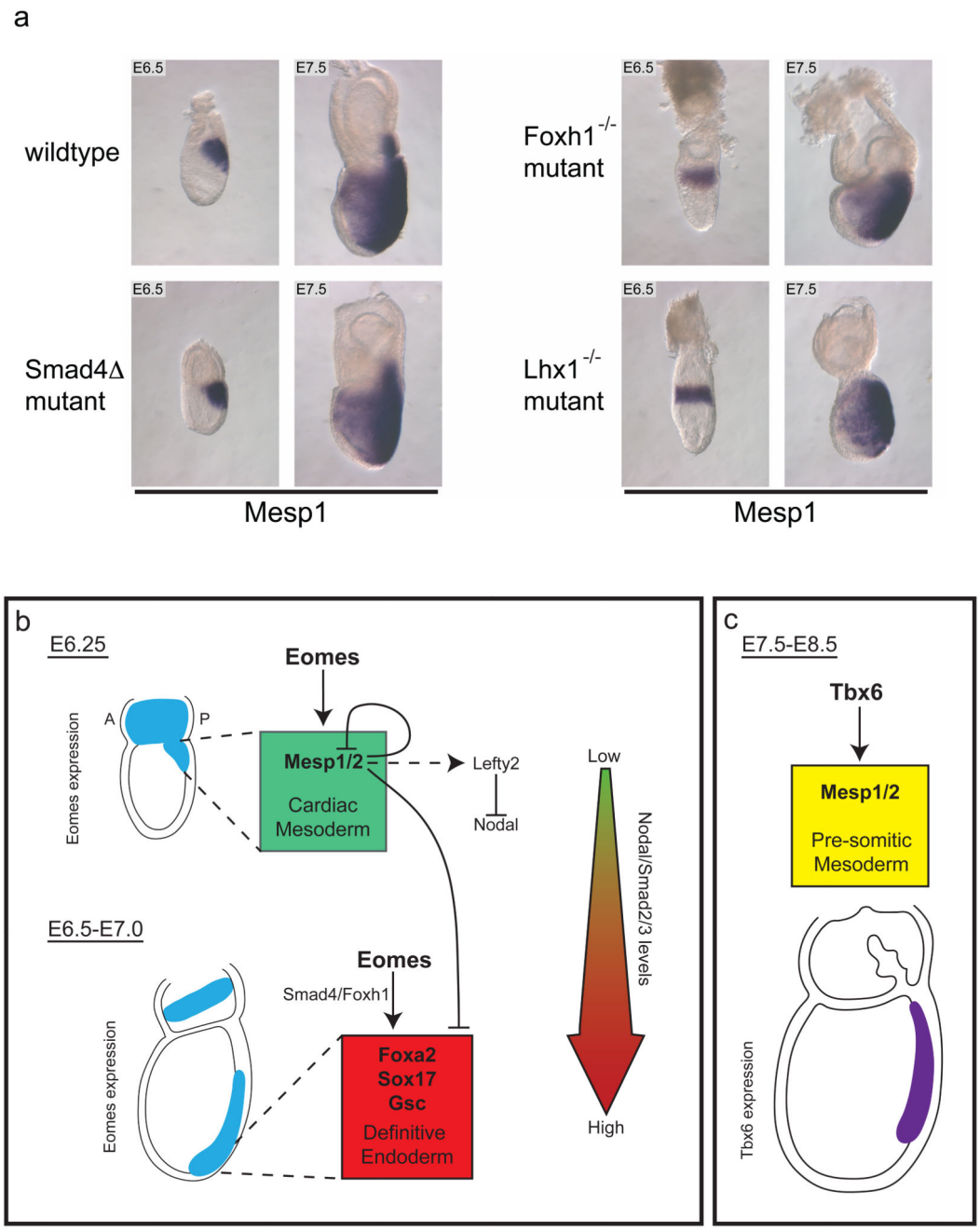

Figure 5.

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[6, 12, 13]. *Mesp1* is expressed normally in E6.5 and 7.5 *Foxh1* null embryos and in embryos lacking *Smad4* in the epiblast only (*Smad4*Δ). *Mesp1* 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 *Mesp1* 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*[23] and direct repression of DE genes[2]. As cells begin to migrate away from the PS *Mesp1/2* expression is down-regulated via a negative feedback loop[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.