RESEARCH ARTICLE

Functional characterisation of cis-regulatory elements governing dynamic Eomes expression in the early mouse embryo

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ABSTRACT

The T-box transcription factor (TF) Eomes is a key regulator of cell fate decisions during early mouse development. The cis-acting regulatory elements that direct expression in the anterior visceral endoderm (AVE), primitive streak (PS) and definitive endoderm (DE) have yet to be defined. Here, we identified three gene-proximal enhancer-like sequences (PSE_a, PSE_b and VPE) that faithfully activate tissue-specific expression in transgenic embryos. However, targeted deletion experiments demonstrate that PSE_a and PSE_b are dispensable, and only VPE is required for optimal Eomes expression in vivo. Embryos lacking this enhancer display variably penetrant defects in anterior-posterior axis orientation and DE formation. Chromosome conformation capture experiments reveal VPE-promoter interactions in embryonic stem cells (ESCs), prior to gene activation. The locus resides in a large (500 kb) pre-formed compartment in ESCs and activation during DE differentiation occurs in the absence of 3D structural changes. ATAC-seq analysis reveals that VPE, PSE_a and four additional putative enhancers display increased chromatin accessibility in DE that is associated with Smad2/3 binding coincident with transcriptional activation. By contrast, activation of the Eomes target genes Foxa2 and Lhx1 is associated with higher order chromatin reorganisation. Thus, diverse regulatory mechanisms govern activation of lineage specifying TFs during early development.

KEY WORDS: Eomesodermin, Enhancer, Capture-C, Nodal signalling, Definitive endoderm

INTRODUCTION

Reciprocal signalling cues between the pluripotent epiblast and adjacent tissues, namely the extra-embryonic ectoderm (ExE) and visceral endoderm (VE), precisely coordinate cell fate decisions during gastrulation. Nodal/Smad signals from the epiblast are required for specification of the AVE, a discrete signalling centre that establishes anterior-posterior (A-P) polarity (Brennan et al., 2001; Robertson, 2014; Stouer and Srinivas, 2014). The A-P axis initially becomes visible at gastrulation, when proximal posterior cells undergo an epithelial-to-mesenchymal transition (EMT) at the PS to form nascent mesoderm. Shortly later, following distal extension of the streak, endoderm progenitors delaminate and emerge onto the surface of the embryo (Kwon et al., 2008).

The T-box transcription factor (TF) eomesodermin (Eomes), acting downstream of Nodal/Smad signals, is required to promote AVE formation and orientation of the A-P axis (Arnold et al., 2008a; Ciruna and Rossant, 1999; Nowotschin et al., 2013), as well as EMT of nascent mesoderm cells (Arnold et al., 2008a; Costello et al., 2011; Russ et al., 2000; van den Ameele et al., 2012). At post-implantation stages, Eomes is expressed in the ExE and embryonic-VE, robustly induced at the onset of gastrulation in the PS and maintained in the anterior PS as it extends, before being abruptly lost (coincident with node formation) (Kwon and Hadjantonakis, 2007). Fate-mapping experiments demonstrate that transient Eomes expression marks progenitors of the cardiovascular lineage, definitive endoderm (DE), node and midline (Costello et al., 2011).

Transgenic and targeted deletion approaches have provided insight into cell type-specific developmental enhancers that govern expression of key genes responsible for partitioning the pluripotent epiblast into discrete cell lineages. Proximal cis-regulatory regions within 20 kb of the transcriptional start sites (TSS) directing spatiotemporally restricted expression of Nodal, Mesp1/2 and Lhx1 have been identified. Both the ASE, an intronic autoregulatory enhancer (Adachi et al., 1999; Norris and Robertson, 1999), and the Wnt signalling responsive 5′ PEE (Ben-Haim et al., 2006) cooperatively regulate Nodal expression. Mutant embryos lacking these genomic sequences display dose-dependent defects in specification of mesoderm and DE/midline progenitors (Norris et al., 2002; Vincent et al., 2003). Similarly, the Mesp1/2 genes, which are essential for formation of nascent mesoderm, are jointly regulated by the EME, an Eomes-dependent enhancer (Costello et al., 2011; Haraguchi et al., 2001). Our recent work demonstrates that Lhx1, which is required for AVE and anterior mesendodermal specification (Barnes et al., 1994; Shawlot and Behringer, 1995), is directly controlled by Eomes binding to a proximal promoter element (Nowotschin et al., 2013).

Eomes, which is rapidly induced in the proximal-posterior epiblast coincident with the acquisition of A-P polarity (Ciruna and Rossant, 1999), is widely viewed as a master regulator of mesendodermal lineages (Costello et al., 2011; Izumi et al., 2007; Teo et al., 2011; van den Ameele et al., 2012). Thus, Eomes represents the earliest lineage-specifying gene in the embryo proper. However, relatively little is known about the cis-acting regulatory elements controlling its dynamic pattern of expression. Recent studies of mouse and human ESCs have identified a conserved switch enhancer ~7 kb upstream of the TSS (Beyer et al., 2013; Kartikasari et al., 2013; Rada-Iglesias et al., 2011) that is repressed under self-renewing conditions (Teo et al., 2011), and becomes activated during mesoderm and endoderm differentiation. However, possible functional contributions made by this genomic region have yet to be assessed in vivo.
Here, we investigate the structural features of the locus that govern *Eomes* expression during early mouse development. Gain-of-function transgenic reporter assays identified three gene-proximal *Eomes* enhancer-like sequences (PSE-a, PSE-b and VPE). However, when we engineered germline deletions to evaluate their functional contributions in vivo, surprisingly, only the VPE was found to influence expression in the early embryo. We also exploited Next Generation (NG) Capture-C technology (Davies et al., 2016) to describe the 3D structural features of the locus. The *Eomes* promoter occupies a discrete 500 kb regulatory compartment in ESCs, and this chromatin conformation is not appreciably altered during DE differentiation. However, our ATAC-seq analysis revealed that the VPE, PSE-a and four additional distal regulatory elements located within this pre-formed compartment display increased chromatin accessibility and acquire Smad2/3 occupancy during DE differentiation. This mode of 3D genome organisation probably serves to facilitate rapid Nodal/Smad-dependent activation of the locus. By contrast, developmentally regulated Foxa2 and Lhx1 promoter-promoter and promoter-enhancer interactions seem to require substantial structural changes during the shift from a transcriptionally inactive to active conformation.

RESULTS

Identification of proximal *Eomes* enhancers that are active during gastrulation

Putative enhancer elements containing DNase I hypersensitive sites and marked by H3K4me1 are considered to be active if also enriched for H3K27ac or, alternatively, viewed as poised if enriched for H3K27me3 (Rada-Iglesias et al., 2011; Zentner et al., 2011). To identify candidate enhancers at the *Eomes* locus, we examined ChIP-seq datasets from undifferentiated ESC, epiblast-like cells (EpLC) and mesodermal precursors (MES) (Alexander et al., 2015; Buecker et al., 2014; ENCODE Project Consortium, 2012) corresponding to the E4.5 epiblast (ESC), the E5.5 epiblast (EpLC) or E6.5 primitive streak (MES) cell populations.

We identified three DNase I hypersensitive sites close to the *Eomes* promoter marked by H3K4me1 that show increased H3K27ac upon differentiation, including two sites (PSE-a and PSE-b) located close together, spanning a 5 kb region between −11 kb to −6 kb upstream of the transcriptional start site (TSS), and a third candidate region (VPE) lying +8 kb downstream of the TSS (Fig. 1A, S1A). Notably, the upstream cluster contains the previously described switch enhancer (PSE-b) activated during ESC differentiation to DE and mesendoderm (Beyer et al., 2013; Kartikasari et al., 2013). Additionally, two downstream DNaseI hypersensitive sites bound by CCCTC-binding factor (CTCF) were identified in ESCs (Fig. S1A). The three proximal regions are highly conserved among mammals (Fig. S1A), associated with H3K4me1/H3K27me3 in ESCs and, thus, probably represent poised enhancers that are primed for activation. Consistent with a shift to the active state during the transition from pluripotency to lineage commitment, these regions contain increased H3K27ac and decreased H3K27me3 in EpLC and MES. The homologous regions are also associated with active enhancer marks in human DE cultures (Fig. S1B).

To test the activities of these candidate enhancers, we generated transgenic strains carrying LacZ reporter constructs and subsequently examined embryonic expression at early post-implantation stages (Kothary et al., 1989). The 5 kb upstream region was designated the PSE (primitive streak enhancer) because PSE-LacZ activity is restricted to the PS at early (ES), mid- (MS) and late-streak (LS) stages (Fig. 1B). There was no detectable LacZ expression in the ExE or VE. However, the 0.7 kb downstream enhancer, designated the VPE (visceral endoderm and primitive streak enhancer), showed activity in the proximal-posterior epiblast, and also in the AVE at pre-streak (PrS) stages (Fig. 1C). Slightly later, LacZ expression was detectable in the PS, nascent mesendoderm and the AVE, subsequently became restricted to the anterior PS, and was lost by LS stages. Collectively, these three enhancers faithfully recapitulate the endogenous *Eomes* expression patterns within both the VE and embryo proper.

The PSE is dispensable for normal embryonic development

The 5 kb PSE contains both an upstream element, PSE-a, as well as the previously described PSE-b switch enhancer reported to interact with the *Eomes* promoter during DE differentiation (Fig. S1A) (Beyer et al., 2013; Kartikasari et al., 2013). To investigate their functional activities in the context of the developing embryo, we generated discrete germine targeted deletions (Fig. 2A, Fig. S2). Surprisingly, homozygous mice lacking the 2 kb PSE-b genomic fragment −8 kb to −6 kb upstream of the TSS (ΔPSE-b) were recovered at Mendelian ratios and are indistinguishable from wild-type littermates (Table 1A). These results demonstrate that the PSE-b is dispensable in vivo. It is well known that heterozygous mice carrying null alleles (*Eomes*ΔGFP/+; *Eomes*LacZ+/−; or *Eomes*Axenom2.5−/−) are fully viable (Arnold et al., 2008a, 2009; Russ et al., 2000). To investigate whether the PSE-b deletion may compromise transcriptional output, we crossed *Eomes*ΔPSE-b/+;PSE-b−/+ mice to those carrying the *Eomes*ΔGFP+ allele (hereafter referred to as *Eomes* null; *Eomes*Δlop−/−). The resulting *Eomes*ΔPSE-b−/+ compound mutants develop normally (Table 1B).

Next, we engineered a deletion that eliminates the entire 5 kb PSE cluster (referred to as ΔPSE, Fig. S3). However, as for the PSE-b, removal of the entire PSE region in *Eomes*ΔPSE/APE mice has no noticeable effect on viability (Table 1A). Finally, crossing these deletion mutants with mice carrying the *Eomes* null allele also failed to perturb embryonic development (Table 1B). Thus, it appears that the PSE can activate expression in gain-of-function transgenic embryos. Nonetheless, this genomic region is clearly dispensable for *Eomes* expression in vivo.

Targeted deletion of the VPE leads to defective gastrulation

To investigate functional contributions made by the VPE, we generated a targeted deletion lacking this 0.7 kb region (Fig. S4). Homozygous ΔVPE mutants are viable and fertile (Table 1A). However, when we crossed *Eomes*ΔVPE/APE mice with *Eomes*−/− heterozygous animals carrying the null allele, we observed a significant under-representation of viable *Eomes*ΔVPE/− compound heterozygotes (Table 1B), with ~40% (n=18) of the expected numbers recovered at weaning (equivalent to *Eomes*ΔVPE/−, n=44). These results strongly suggest that *Eomes*ΔVPE acts as a hypomorphic allele.

Next, to determine the onset of lethality, we examined embryos from E5 onwards. Approximately one-third of *Eomes*ΔVPE/− embryos are morphologically normal. However, two distinct classes of abnormal embryos were recovered at roughly equivalent numbers. The most severely affected (class I) mutants arrest at early gastrulation stages, while a second group (class II) progress to mid-gestation (Fig. 2B).

In class I embryos, the AVE marker *Hex* is induced at E6.5 but remains localised to the distal tip. Thus, the AVE is specified but remains restricted towards the prospective anterior side of the embryo. These embryos fail to correctly orient the A-P axis and lack a discrete PS. At E7.5, mesoderm (Brachyury) and DE (Foxa2) markers are restricted proximally. Class I mutant embryos, distinguished by the accumulation of disorganised mesenchymal cells in the epiblast cavity and a constriction at the embryonic and...
extra-embryonic boundary, phenocopy those selectively lacking *Eomes* activity in the VE (Nowotschin et al., 2013). Taken together with results above that demonstrate VPE-*LacZ* expression in the VE, the simplest explanation is that these abnormalities are caused by loss of *Eomes* function in the VE.

The class II embryos, which represent approximately one-third of the *Eomes*ΔVPE/− embryos, successfully establish normal A-P polarity. However, as gastrulation proceeds they display focal defects in the anterior PS (APS) and its derivatives the DE, midline, node and notochord. Brachyury (*T*) expression in the PS fails to extend to the distal tip of the streak at E7.5. *Foxa2*-positive DE progenitors are specified but fail to migrate anteriorly. As judged by *Afp* expression, the VE is retained over the epiblast and fails to become distally restricted. These tissue disturbances probably reflect the functional loss of *Eomes* within the APS (Arnold et al., 2008a; Teo et al., 2011). APS derivatives are known to provide essential trophic signals required for patterning the anterior neurectoderm (Arkell and Tam, 2012). Consistent with this, at E9.5, class II mutant embryos display ventral closure and neural tube defects, fused or malformed somites, and loss of forebrain tissue.

The VPE is required for optimal *Eomes* expression levels

To test directly whether targeted loss of the VPE compromises *Eomes* transcriptional output, we eliminated the VPE in the context of our *Eomes*GFP reporter allele containing an EGFP-pA cassette inserted in-frame at the translational start site in exon 1 (Fig. 3A, Fig. S5) (Arnold et al., 2009) and performed flow cytometry analysis to quantify expression levels. The *Eomes*GFP reporter is robustly activated during ESC differentiation to embryoid bodies (EBs) (Costello et al., 2011) (Fig. 3B). As shown in Fig. 3C, GFP expression in *Eomes*GFPΔVPE/+ embryos recapitulates domains of the *Eomes*GFP/+ control embryos at E6.5, in the ExE, PS, nascent mesoderm and VE (Fig. 3E,F). The VPE deletion reduced...
expression levels but tissue-specific expression patterns were unperturbed. Similar conclusions were reached by whole-mount in situ hybridisation experiments examining Eomes mRNA expression in EomesΔVPE- ΔVPE embryos (Fig. S4E). Thus, reduced Eomes transcription (~50%) as in EomesΔVPE-ΔVPE embryos is sufficient to promote A-P axis specification and gastrulation. However, as shown above, further reduced expression (~25%) in EomesΔVPE-ΔVPE embryos results in gastrulation defects.

**Foxh1-independent Nodal/Smad2/3 signals regulate VPE activity**

Eomes activation in the VE and PS depends on Nodal/Smad signals (Brennan et al., 2001; Nowotschin et al., 2013). To investigate Nodal/Smad requirements in cultured EBs, we used the small molecule SB-431542 (SB), a potent inhibitor of type I activin receptor like kinases 4, 5 and 7. As expected, in control cultures, maximal Eomes expression was detectable between day (d)3.5 and d4 (Fig. 4A). Eomes expression was dramatically reduced in cultures treated with the SB inhibitor from d3, and by d4 is severely compromised to only 2% of that seen in controls (Fig. 4A). These results confirm that Nodal signalling is required to induce Eomes expression during the transition from pluripotency to lineage commitment. Additionally, when we compared Smad2/3 ChIP-seq datasets in ESC and DE cultures (Yoon et al., 2015), we found evidence for Smad2/3 occupancy at the VPE specifically in DE cultures (Fig. 4B). These observations strengthen the idea that Nodal/Smad signals controlling Eomes expression activate transcription via the VPE.

It is well known that the forkhead transcription factor Foxh1 functions as a Smad2/3 co-factor governing Nodal/Smad target gene expression (Attisano et al., 2001; Izzii et al., 2007). Foxh1 has been proposed to act as a pioneer factor and to recruit Smad2/3 complexes to switch enhancers, activated as ESCs transition to DE fates (Beyer et al., 2013; Cirillo et al., 2002; Cirillo and Zaret, 1999; Kim et al., 2011). Interestingly, the VPE Smad2/3 peak also
contains a conserved Foxh1-binding motif. Moreover, the VPE region is co-bound by FOXH1, SMAD2/3 and SMAD4 in human DE cultures (Fig. S6) (Beyer et al., 2013; Brown et al., 2011; Kim et al., 2011; Teo et al., 2011). Consistent with the idea that Foxh1 cooperatively activates Eomes expression via the VPE, homozygous null Foxh1Δ/Δ embryos phenocopy the EomesΔVPE/ΔVPE-embryos, displaying either defective AVE formation prior to gastrulation or disturbances in APS specification at later stages (Hoodless et al., 2001).}

To evaluate directly Foxh1 functional contributions, we analysed Eomes expression at E6.5 and E7.5 in the context of Foxh1Δ/Δ mutant embryos (Fig. 4C). In mutants with AVE/DVE defects at E6.5, Eomes is expressed in the thickened VE at the distal tip of the embryo, and at E7.5 in the chorion and proximal epiblast. Foxh1 mutants with APS defects express Eomes in the ExE and PS. Eomes is clearly expressed in both classes of Foxh1 mutant embryos. Slightly reduced levels in the PS can be explained by the loss of Foxh1-dependent activation of the auto-regulatory ASE Nodal enhancer (Norris et al., 2002). In striking contrast to Eomes/Nodal double heterozygotes (Arnold et al., 2008a), we found no evidence here for Eomes and Foxh1 genetic interactions. Indeed, Eomes and Foxh1 compound mutant mice are fully viable (Table 2). Finally, to confirm that VPE activity is Foxh1 independent, we examined expression of the VPE-LacZ transgene in Foxh1 mutant embryos. LacZ staining is detectable throughout the epiblast at E6.5 (Fig. 4E), and also in the thickened VE at the distal tip. Foxh1 function is nonessential for VPE-LacZ reporter activity. Thus, we conclude that Nodal/Smad signals activate Eomes expression in a Foxh1-independent manner, raising the possibility that other forkhead family members may recruit Smad2/3 complexes during Eomes induction in vivo.

Characterisation of the Eomes 3D regulatory chromatin compartment during endoderm differentiation

The finding that the VPE targeted deletion partially reduces but fails to completely eliminate Eomes expression, strongly suggests that additional regulatory elements contribute to transcriptional output of the locus. Enhancer interactions with target promoters have been

### Table 1. Genotypes of mutant weanlings

| Genotype | Expected ratios | ΔPSE | ΔPSE_Δ | ΔVPE |
|----------|-----------------|------|--------|------|
| (A) Heterozygous enhancer deletion intercrosses (EomesΔ/+ x EomesΔ/+)) | | | | |
| +/-     | 25%             | 16 (25%) | 41 (39%) | 36 (29%) |
| Δ/+     | 50%             | 34 (52%) | 42 (40%) | 57 (46%) |
| ΔΔ      | 25%             | 15 (23%) | 23 (22%) | 31 (25%) |
| Total   |                 | 65     | 106    | 124   |

| (B) Heterozygous null and homozygous enhancer deletion matings (EomesΔ/+ x EomesΔ/Δ) | | | | |
| Δ/+     | 50%             | 31 (52%) | 36 (52%) | 44*** (71%) |
| ΔΔ      | 50%             | 29 (48%) | 33 (48%) | 18*** (29%) |
| Total   |                 | 60     | 69     | 62    |

Homozygous deletion of proximal enhancers does not affect viability (ΔPSE P=0.9, ΔPSE_Δ P=0.04, ΔVPE P=0.5, Chi-square test). EomesΔVPE/ΔVPE-animals are significantly under-represented compared with littermate controls (ΔPSE, P=0.87; ΔPSE_Δ, P=0.07; ΔVPE, ***P=0.001; Chi-square test).

The number of animals with each gene-proximal enhancer-like sequence is given with percentages in brackets.
analysed by chromatin conformation capture techniques (de Wit and de Laat, 2012). We took advantage of the recently developed Next Generation (NG) Capture-C methodology (Davies et al., 2016) to screen for Eomes regulatory enhancer elements. During DE differentiation, Eomes expression increased by ∼600 fold (Fig. S7B) resulting in activation of the Eomes target genes, Lhx1 and Foxa2 (Fig. S7C) (Nowotschin et al., 2013; Teo et al., 2011).

NG Capture-C using viewpoints from the PSE_a and PSE_b exhibited promoter interactions in ESC (Fig. S8) when analysed with FourCseq (Klein et al., 2015). These interactions were marginally reduced in DE. However, the overall change was not statistically significant. By contrast, NG Capture-C revealed significant interactions between the VPE and the Eomes promoter in both ESC and DE cells (Fig. S8). Thus, the locus appears to be primed for activation prior to expression.

Next, performing Capture-C using a viewpoint from the Eomes promoter revealed that the Eomes locus, together with an upstream 300 kb gene desert and its neighbouring genes Azi2 and Cmc1, occupies a discrete ∼500 kb chromatin compartment (Fig. 5A). This region contains numerous CTCF-binding sites (Handoko et al., 2011). Consistent with CTCF-mediated chromatin loops forming the compartment boundaries, motif analysis suggests that the outermost binding sites face inwards (Fig. 5A). This compartment structure is readily detectable in both ESC and DE cells but is completely absent in control terminally differentiated erythrocytes lacking Eomes expression (Fig. 5A, Fig. S9). Comparison of the NG Capture-C data from ESC and DE, in which the Eomes locus is transcriptionally silent or active, respectively, demonstrates that the compartment is highly stable. Moreover, there were no detectable changes in long-range promoter interactions within the compartment (Fig. S10).

To map changes in regions of open chromatin associated with Eomes activation and identify potential novel DE enhancers within the compartment, we performed ATAC-seq. We identified 85,581 total peaks in ESC and DE, and of these 19% were gained and 32.5% lost during differentiation (Fig. S9). Within the Eomes compartment we identified six regions that show increased accessibility in DE, including the VPE and the PSE_a, as well as four additional sites at −93 kb, −45 kb, −38 kb and +9 kb relative to the Eomes TSS (Fig. 5B).

Next, we examined Smad2/3 binding across the compartment (Yoon et al., 2015). Smad2/3 occupancy was detectable in DE but not in ESCs at all six of the differentially accessible sites (Fig. 5B). These findings demonstrate the Eomes locus is organised into a large 3D regulatory chromatin compartment in pluripotent ESCs that is maintained upon DE differentiation. Global structural changes are not required for Eomes induction during DE differentiation. Rather, transcriptional activation seems to reflect increased chromatin accessibility and Smad2/3 recruitment at DE enhancers. The −95 kb and −45 kb regions, and to a lesser extent the −38 kb region, are associated with poised and active enhancer marks as cells transition from ES to Epi to MES states, respectively (Fig. S11). Additionally, recently published TF ChIP-seq data

Table 2. Genotypes of weanlings from Eomes+/−×Foxh1+/−

| Genotype           | Expected ratios | Observed |
|--------------------|-----------------|----------|
| Wild type          | 25%             | 6 (17%)  |
| Foxh1+/−           | 25%             | 12 (34%) |
| Eomes+/−           | 25%             | 10 (29%) |
| Eomes+/−; Foxh1+/− | 25%             | 7 (20%)  |
| Total              |                 | 35       |
demonstrate that the −45 kb ATAC-seq peak, together with the PSE_a and VPE, are co-bound by Tcf3 in DE (Wang et al., 2017), suggesting that both Nodal and Wnt signalling converge on these enhancer regions during gastrulation (Ben-Haim et al., 2006). Consistent with its activities as a key Eomes regulatory element during DE specification, the VPE is also bound by Otx2 and Lhx1 in EpiLC and mesendoderm cultures, respectively (Buecker et al., 2014; Costello et al., 2015).

Foxa2 and Lhx1 promoters form long-range interactions in polycomb bodies

The forkhead TF Foxa2 and the LIM domain homeobox TF Lhx1 function together with Eomes as master regulators of APS cell fates (Ang and Rossant, 1994; Costello et al., 2015; Perea-Gomez et al., 1999; Shawlot and Behringer, 1995). One possible model is that this pre-configured genomic structure might be a common feature shared by endoderm-specific transcriptional factors (Fig. S7C). As for Eomes, Capture-C of the Foxa2 and Lhx1 promoters demonstrates localisation within pre-formed compartments (both −350 kb) in ESCs, but not in erythrocytes where the genes are inactive (Fig. 6A,B). However, these Foxa2 and Lhx1 compartments were found to undergo significant rearrangements during DE differentiation (Fig. 6A,B). Unlike Eomes, Lhx1 and Foxa2 promoters both make long-range contacts with neighbouring developmental genes lying outside the compartment boundaries in ESCs (Fig. 6A,B). These long-range interactions range from 370 kb to 1.8 Mb in size and are almost entirely specific to gene promoters (Table S3); they are lost as cells acquire a DE fate (Fig. 6A,B).

Both Foxa2 and Lhx1 are repressed by polycomb in ESC (Leeb et al., 2010). Examination of published ESC ChIP-seq data-sets for Polycomb components Ezh2, Suz12 (PRC2) and Ring1b (PRC1) (Chen et al., 2008; Ku et al., 2008), as well as the polycomb repressive mark H3K27me3 (Yue et al., 2014), showed they are present at all of the promoters of the adjacent genes with which Lhx1

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Fig. 5. Eomes is regulated by Smad2/3 binding in a preformed compartment. (A) NG Capture-C interaction profiles of the Eomes promoter (chr9:117,683,476-118,771,067) from erythrocytes (grey), ESC (blue) and DE (green). Tracks show mean interactions of normalised biological replicates (n=3) and DESeq2 significant differences between DE and ESC (−log(Padj); P≤0.05). The Eomes compartment, as determined by boundaries of strong promoter interactions with CTCF orientation (arrowheads), is based upon binding in ESCs (Handoko et al., 2011). Histone modifications for H3K4me3 (DE, n=3) show promoter regions. (B) Enlargement of the region of the Eomes compartment showing highest association with the promoter, from chr9: 118,252,500-118,405,500. Open chromatin was generated using ATAC-seq in ESC and DE (n=3), with the addition of MACS2 called peaks annotated beneath each ATAC-seq track and Smad2/3 ChIP-seq in ESCs (blue) and DE (green) (Yoon et al., 2015). Regions of chromatin accessibility unique to ESCs (−73 kb) and those associated with Smad2/3 occupancy in DE (−93 kb, −45 kb, −38 kb, PSE_a, VPE and +9 kb) are indicated.
and Foxa2 interact (Fig. 6), suggesting that these genes are present in Polycomb bodies (Pirrotta and Li, 2012). Interestingly, these Polycomb repressive components are also present at the Eomes promoter in ESC, but we found no evidence for long-range interactions with gene promoters lying outside the compartment (Fig. S10). Collectively, the results above demonstrate that three essential TFs required for cell fate specification, Eomes, Foxa2 and Lhx1, were found to exhibit distinct modes of 3D chromatin organisation during differentiation.

**DISCUSSION**

The spatiotemporal expression of key lineage-specifying transcription factors (TF) is tightly controlled during early mouse development to ensure correct cell fate decisions. Interactions of cell type-specific cis-acting enhancer elements with gene promoters, within topologically discrete chromatin compartments, directs developmentally regulated patterns of expression (de Laat and Duboule, 2013). Our recent studies demonstrate that the T-box TF Eomes, dynamically expressed in the VE, ExE and PS during
gastulation, acts downstream of the Nodal signalling pathway as an essential master-regulator of the DE and cardiac mesoderm cell lineages. Here, we exploit transgenic reporter assays, targeted deletion and NG Capture-C strategies to investigate the regulatory landscape at the *Eomes* locus.

We demonstrate using gain-of-function experiments that conserved proximal *cis*-regulatory elements, namely the so-called PSE (comprising PSE\(^*_a\) and PSE\(^*_b\)) and the VPE, have the ability to drive reporter activity in the PS, or VE and PS, respectively. The conserved *Eomes* PSE\(^*_b\) region, which represents an archetypal poised developmental enhancer in both human and mouse ESC, was recently shown to be activated upon mesendoderm induction in response to Nodal (Smad2/3, Foxh1) and Wnt (β-cat) signalling pathways (Beyer et al., 2013; Brown et al., 2011; Buecker and Wysocka, 2012; Estarás et al., 2015; Funa et al., 2015; Kartikasari et al., 2013; Kim et al., 2011; Rada-Iglesias et al., 2011). However, surprisingly our targeted deletion experiments demonstrate that this switch enhancer, and the adjacent PSE\(^*_a\), are dispensable for correct developmentally regulated *Eomes* expression in the early embryo. Moreover, mutant mice that entirely lack this genomic region develop normally and are viable and fertile.

*Eomes* is required for the maintenance and migration of the AVE (Nowotschin et al., 2013). Additionally, robust expression in the PS is essential for formation of APS progenitors (Arnold et al., 2008a). The present results demonstrate that the VPE activates expression in both the AVE and PS, and makes important functional contributions that govern *Eomes* activities during gastrulation. We found that removal of this element halves transcriptional output from the locus as assessed *in vitro*. Moreover, *Eomes\(^{AXWPU-}\)* embryos exhibit pleiotropic tissue defects, due to compromised specification of AVE or APS, that closely resemble those caused by defective Nodal signalling or loss of the Smad2/3/4 co-factor (Nowotschin et al., 2013). Additionally, robust expression in the PS was recently shown to be activated upon mesendoderm induction in response to Nodal (Smad2/3, Foxh1) and Wnt (β-cat) signalling pathways (Beyer et al., 2013; Brown et al., 2011; Buecker and Wysocka, 2012; Estarás et al., 2015; Funa et al., 2015; Kartikasari et al., 2013; Kim et al., 2011; Rada-Iglesias et al., 2011). However, surprisingly our targeted deletion experiments demonstrate that this switch enhancer, and the adjacent PSE\(^*_a\), are dispensable for correct developmentally regulated *Eomes* expression in the early embryo. Moreover, mutant mice that entirely lack this genomic region develop normally and are viable and fertile.

**Fig. 7.** *Eomes, Foxa2* and *Lhx1* exhibit distinct modes of 3D chromatin organisation during differentiation. (A) In ESCs, *Eomes, Foxa2* and *Lhx1* are organised into pre-formed chromatin compartments. (B) Unlike *Eomes*, both *Foxa2* and *Lhx1* promoters form extra-compartmental contacts with other polycomb-repressed gene promoters. (C) Model for *Eomes* activation. The poised chromatin architecture at the *Eomes* locus is permissive for rapid transcriptional induction in response to localised Nodal signalling during gastrulation, primarily via enhancer binding of Smad2/3 complexes.

Considerable evidence suggests that stable enhancer-promoter interactions within pre-formed chromatin compartments initiate transcription through the release of paused polymerase (De Laat and Duboule, 2013; Ghavi-Helm et al., 2014; Jin et al., 2013; Williamson et al., 2016). We found that promoter-enhancer interactions are relatively stable. However, our ATAC-seq experiments reveal significant changes in open chromatin regions during DE differentiation. We identified several candidate enhancers within the *Eomes* compartment that display increased chromatin accessibility and are greatly enriched for Smad2/3 occupancy upon DE differentiation (Yoon et al., 2015). Moreover, we confirm that Smad2/3 is required for *Eomes* activation, as inhibition of receptor-mediated Nodal/Smad2/3 signalling blocks transcription. Smad2/3 associations with the histone demethylase JmjD3 are known to be required for the activation of Nodal target genes (Dahle et al., 2010; Kartikasari et al., 2013). JmjD3 activates poised developmental genes by removing promoter-proximal H3K27me3 and releasing paused polymerase (Chen et al., 2012). We propose that the poised chromatin architecture at the *Eomes* locus is permissive for rapid transcriptional induction in response to localised Nodal signalling during gastrulation, primarily via enhancer binding of Smad2/3/JmjD3 complexes to release promoter-paused polymerase.

The 3C technologies developed over the past two decades have provided important new insights into the regulatory chromatin landscapes that orchestrate tissue-specific transcription. Here, we...
characterise for the first time cis-regulatory elements that activate *Eomes* expression during gastrulation, and describe the higher order chromatin architecture of the locus. We speculate that the pre-formed chromatin compartment and the absence of additional epigenetic safeguards prior to expression facilitates the rapid induction of *Eomes* expression in response to dynamic signalling cues at the onset of gastrulation. However, the stage of embryonic development during which these compartments are established, and later dismantled, remains elusive. Future studies will investigate whether these enhancers and permissive chromatin configuration are tissue invariant and can also control cell type-specific *Eomes* expression governing cell fate decisions at other sites such as the developing cortex, and adult NK and CD8+ T-cell lineages (Arnold et al., 2008b; Gordon et al., 2012; Pearce et al., 2003).

**MATERIALS AND METHODS**

**Animals and PCR genotyping**

*Eomes<sup>GFP/+</sup>*, *Eomes<sup>APE/+</sup>*, *Eomes<sup>AE/+</sup>*, *Eomes<sup>VPE+/+</sup>* and *Eomes<sup>GPAVPE+/</sup>* strains were generated from targeted ESC clones using standard methods (Arnold et al., 2009) (Figs S2-S5, see supplementary Materials and Methods) and maintained on a mixed 129Sv/Ev/C57BL/6 background. To generate PSE.<sup>LacZ</sup> ES cell lines using standard methods (Arnold et al., 2009) (Figs S2-S5, see with Home Office (UK) regulations and approved by the University of Oxford Local Ethical Committee.

**ESC differentiation**

ESC lines were differentiated in DMEM (Invitrogen) supplemented with 15% fetal calf serum (Gibco), 1% penicillin/streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 1 mM glucose (Invitrogen), 1% MEM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Sigma), 1000 U/ml LIF (ESGRO) on gelatin-coated plates. For analysis of GFP reporter expression, wild-type (CCE), *Eomes<sup>GFP+/+</sup>*, *Eomes<sup>APE/−</sup>*, *Eomes<sup>AE/−</sup>*, *Eomes<sup>VPE−/−</sup>* and *Eomes<sup>GPAVPE−/−</sup>* ESCs were seeded as 10 μl hanging drops (1×10<sup>4</sup> cells/ml) in the absence of LIF to induce EB formation. After 2 days, EBs were transferred to suspension culture. For SB inhibition experiments, ES cells were seeded in suspension at low density (1×10<sup>4</sup> cells/ml) in the absence of LIF to form EBs. On day 3, EBs were cultured in the presence or absence of 10 μM SB431542 inhibitor (Tocris). For DE differentiation, ES cells were induced to form EBs in suspension, as described above, but were transferred on day 2 into N2B27 medium (Cellartis) supplemented with 20 ng/ml activin A (R&D systems) and 20 ng/ml EGF (Peprotech) to induce DE differentiation (Morrison et al., 2013). CTCF-motifs were identified using the FIMO function of MEME Suite (Bailey et al., 2009; Grant et al., 2011).

**RNA analysis**

RNA was isolated from using Qiashredder homogeniser (Qiagen), RNeasy mini kit (Qiagen) and RNase-Free DNase Set (Qiagen). RNA was reverse transcribed to CDNA using SuperScript III First Strand Synthesis System (Life Technologies) and qRT-PCR was carried out in triplicate using SYBR-green kit (Qiagen) on a Rotagene cycler (Qiagen) with primers listed in Table S1. Relative gene expression was normalised to *Gapdh* and calculated as 2ΔΔCt.

**in situ hybridisation, X-gal staining and immunofluorescence**

Whole-mount *in situ* hybridisation was performed according to published protocols (Behringer et al., 2013). *LacZ* activity was visualised using whole-mount X-gal staining as described previously (Behringer et al., 2013). Whole-mount *in situ* hybridisation and X-gal-stained embryos were photographed after clearing in 80% glycerol.

For immunofluorescence, embryos were fixed overnight in 1% PFA. EBs were fixed in 4% PFA for 30 min at room temperature. Samples were washed in 0.1% Triton-X in PBS, permeabilised in 0.5% Triton-X in PBS for 15 min, washed in 0.1% Triton-X in PBS, then blocked in 0.1% Triton-X, 0.2% BSA and 5% donkey serum in PBS for 2 h at room temperature. Samples were incubated with primary antibodies (Table S2) overnight at 4°C, washed, incubated with secondary antibodies or phallloidin AlexaFluor 633 stain (A22284; Invitrogen) in block solution for 2 h at room temperature, counterstained with DAPI and mounted in Vectashield (Vector Laboratories) on chamber slides (LabTek). Images were acquired using an Olympus FV1000 inverted confocal microscope.

**Flow cytometry**

Day 4 EBs were incubated in 0.25% trypsin for 5 min at 37°C and dissociated into single cells using a 20-gauge needle. FACS analysis was performed using a BD FACScalibur 4 (BD Biosciences) and data analysed using FlowJo.

**ATAC-seq**

Tagmentation and indexing of single cell suspensions of ESC, DE and erythrocytes from phenylhydrazine-treated mice (Davies et al., 2016) was performed as previously described (Buenrostro et al., 2013; Hay et al., 2016). Samples were sequenced using a 75-cycle paired-end kit on the Illumina NextSeq platform.

**ChIP-seq**

Single cell suspensions (5×10<sup>4</sup>) were cross-linked in 1% formaldehyde for 15 min at room temperature and processed using standard methods. Briefly, cells were lysed on ice for 20 min (5 mM PIPES, 85 mM KCl and 0.5% Igepal-CA 630), and pelleted nuclei were lysed (50 mM Tris-HCl, 10 mM EDTA and 1% SDS). Sonicated chromatin was incubated overnight with anti-H3K4me3 (2 μl: 07-473; Millipore) and Protein A Dynabeads (Invitrogen). Beads were washed with RIPA buffer variants (10 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS and 0.1% sodium deoxycholate) – RIPA, high salt RIPA (500 mM NaCl) and RIPA with 250 mM LiCl – and with TE buffer before RNase A (Roche) and protease K (Thermo Fisher) treatment. Phenol-chloroform-extracted DNA was indexed using NebNext Ultra II (New England BioLabs), multiplexed and sequenced using a 75-cycle paired-end kit on the Illumina NextSeq platform.

**ATAC-seq and ChIP-seq analysis**

ATAC-seq and ChIP-seq data were analysed as described previously (Hay et al., 2016) using a custom pipeline (http://userweb.molbiol.ox.ac.uk/public/teilenius/pipeSite.html). Sequenced reads were aligned using Bowtie to the mm9 build of the mouse genome. Genomic browser tracks were generated from pooled data from multiple replicates and normalised per million mapped reads using a custom Perl script. Peak detection was performed with the MACS2 (Feng et al., 2012). For differential analysis, a union set of peaks for each cell type generated from at least two peak calls per site. Peaks were filtered for high ploidy regions using MIG Viewer (McGowan et al., 2013). CTCF-motifs were identified using the FIMO function of MEME Suite (Bailey et al., 2009; Grant et al., 2011).

**NG Capture-C and analysis**

NG Capture-C was performed as described previously (Davies et al., 2016) on single cell suspensions of ESC, DE or erythrocytes. Samples were indexed for multiplexing and co-capture of enhancers or promoters using biotinylated 120-mers (Sigma, IDT) designed with the CapSeq webtool (http://apps.molbiol.ox.ac.uk/CaptureC/cgi-bin/CapSeq,cgi) (Hughes et al., 2014) and pooled to a final concentration of 2.9 nM (Table S4). Captured material was pooled and sequenced using the Illumina NextSeq platform with 150 bp paired-end reads (300 cycle kit, Illumina). Reads were mapped using Capture-C scripts (https://github.com/teilenius/captureC/releases), analysed as previously described (Hay et al., 2016), and additionally with FourCseq (Klein et al., 2015) and DESeq2 (Love et al., 2014).
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Competing interests
The authors declare no competing or financial interests.

Author contributions
C.S.S., I.C.E.K.B. and E.J.R. designed the project; C.S.S., D.J.D., M.E.G. and E.J.R. performed the experiments; C.S.S., D.J.D., J.T., D.R.H., J.R.H. E.K.B. and E.J.R. analysed and interpreted the data; C.S.S., D.J.D., E.K.B. and E.J.R. wrote the paper.

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Data availability
ChIP-seq, ATAC-seq and NG Capture-C data have been deposited in NCBI GEO (GSE94250). Accession numbers of published ChIP-seq data sets used in this study are listed in Table S5.

Supplementary information
Supplementary information available online at http://dev.biologists.org/lookup doi:10.1242/dev.147322 supplemental

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