The T-box transcription factor Eomesodermin is essential for AVE induction in the mouse embryo

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Reciprocal inductive interactions between the embryonic and extraembryonic tissues establish the anterior–posterior (AP) axis of the early mouse embryo. The anterior visceral endoderm (AVE) signaling center emerges at the distal tip of the embryo at embryonic day 5.5 and translocates to the prospective anterior side of the embryo. The process of AVE induction and migration are poorly understood. Here we demonstrate that the T-box gene Eomesodermin (Eomes) plays an essential role in AVE recruitment, in part by directly activating the homeobox transcription factor Lhx1. Thus, Eomes function in the visceral endoderm (VE) initiates an instructive transcriptional program controlling AP identity.

Results and Discussion
We performed immunohistochemistry to determine the onset of Eomes expression. Eomes was undetectable in the primitive endoderm of the late blastocyst (E4.5) but present in the EmVE at E5.5, as assessed by colocalization with a PdgfraH2B-GFP reporter (Plusa et al. 2008) and Hnf4α immunoreactivity (Fig. 1A–A3,B–B3; Kwon et al. 2008). Thus, Eomes is not expressed when DVE cells are initially specified in the late blastocyst but rather is induced throughout the EmVE by E5.5, coincident with AVE re-recruitment as well as in specification of the definitive endoderm and cardiac mesoderm (Arnold et al. 2008; Costello et al. 2011). Intriguingly, immunohistochemistry and studies of GFP reporter mice have revealed Eomes expression in the embryonic VE (EmVE) at embryonic day 5.5 (E5.5), prompting our efforts to investigate its role in this tissue (Kwon and Hadjantonakis 2007; Arnold et al. 2009).

Formation of the anterior–posterior [AP] axis of the mouse embryo relies on precisely coordinated reciprocal signaling activities between the pluripotent epiblast and its neighboring tissues, the extraembryonic ectoderm (ExE) and visceral endoderm (VE). AP axis formation is initiated at late blastocyst stages with the specification of the distal VE [DVE], a subpopulation of primitive endoderm cells, which, a day later, around E5.5, reside at the distal tip of the post-implantation egg cylinder (Takaoka et al. 2011). Nodal signals from the epiblast lead to recruitment of additional cells—the so-called anterior visceral endoderm (AVE)—that, together with the initial DVE pioneer population, migrate toward the embryonic/extraembryonic boundary, thereby triggering reorgani-

zation of cells within the VE epithelium (Arnold and Robertson 2009; Rossant and Tam 2009; Nowotschin and Hadjantonakis 2010). This Nodal signaling-dependent, unilateral movement of cells converts the pre-existing proximodistal (PD) axis of the egg cylinder to an AP axis (Norris et al. 2002). Cells of the AVE express secreted Nodal, Bmp, and Wnt antagonists, thereby restricting signaling to the posterior epiblast and confining the site of nascent mesoderm induction to the primitive streak.

Eomesodermin (Eomes, also referred to as Tbr2), a member of the T-box family of transcription factors, is dynamically expressed in both the embryonic and extraembryonic tissues of the early embryo. Eomes mutants exhibit defects in the trophoderm and arrest at implantation (Russ et al. 2000), obscuring its role at later stages of development. Chimeric analysis, together with epiblast-specific ablation, has uncovered essential functions for Eomes in epithelial-to-mesenchymal transition (EMT) and mesoderm delamination as well as in specification of the definitive endoderm and cardiac mesoderm (Arnold et al. 2008; Costello et al. 2011). Intriguingly, immunohistochemistry and studies of GFP reporter mice have revealed Eomes expression in the embryonic VE (EmVE) at embryonic day 5.5 (E5.5), prompting our efforts to investigate its role in this tissue (Kwon and Hadjantonakis 2007; Arnold et al. 2009).

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The investigate the dynamics of AVE specification, we used wild-type littermates (Fig. 2A–D,A1–D1). To furthered at the distal tip of the embryo, as compared with significantly reduced levels or loss of expression, localized at the embryonic/extraembryonic junction (Fig. 2G,G1), as compared with wild-type embryos at E5.5, has been reported previously (Srinivas et al. 2004). In contrast, as compared with wild-type embryos at E5.5, EomesVE/−; Hex-GFPTg/C0 embryos exhibited low levels of GFP fluorescence in a reduced number of distal cells (Fig. 2F,F1). Moreover, fluorescence was undetectable at later stages (Fig. 2H,H1), suggesting that loss of Eomes results in failure to correctly maintain the DVE and disrupts recruitment of further Hex-expressing cells that normally give rise to the migratory AVE population. Failure to position the AVE results in aberrant mesoderm induction, with nascent mesoderm markers T/Brachyury, Fgf8, and Mixl1 (Fig. 2I–K) observed throughout the proximal epiblast. Coincident with a perturbation of PD polarity, Otx2 was also expressed more broadly in the distal epiblast and overlying VE (Fig. 2L,L1). In wild-type embryos, Nanog expression is restricted to the proximal posterior epiblast (Fig. 2M), whereas in contrast, here we observed Nanog expressed throughout the entire epiblast in EomesVE/− mutants (Fig. 2M1). However, expression of the epiblast marker Oct4 was unperturbed in EomesVE/− mutants (data not shown).

Both Wnt and Nodal signaling play essential roles in AP patterning. Next, we investigated the activity of the TOPGAL. Wnt signaling reporter (DasGupta and Fuchs 1999) in EomesVE/− mutants. Reporter expression levels were unaffected, but expression was restricted proximally due to the failure to correctly position the

**Figure 1.** Eomes is activated in the EmVE, and genetic ablation in the VE results in morphogenesis defects. ([A–A3]) E4.5 Pdgfrafluous-GFP embryo depicting nuclear-localized Eomes (red) in trophoderm (TE) and absence in primitive endoderm (PE). ([B–B3]) Colocalization of Eomes (red) and Hnf4α (green) in the EmVE (white arrowhead). ([C–C3]) Localization of Eomes and Hnf4α in E5.5 embryo with VE-specific Eomes inactivation (EomesVE/−). Note the lack of colocalization of Eomes and Hnf4α protein (C2, orange arrowhead). ([D,D1]) E5.75 EomesVE/− mutant embryos (D1) exhibit increased thickening of the VE at the distal tip (D1, black arrowhead) compared with wild-type [WT; D]. EomesVE/− at E6.5, the AVE has migrated anteriorly in wild type [E], but remains thickened distally in EomesVE/− mutants [E1, black arrowhead]. At E7.25 ([F,F1]), EomesVE/− mutants display aberrant morphology ([F1] compared with wild type [F]), becoming exacerbated at E7.5 [G1]. ([G,G1]) Note constriction at embryonic/extraembryonic junction (ExEM) (red arrowheads). ([2D]) Single optical section, (3D) projection of z-stack; (Em) embryonic; (Epi) epiblast; (ExE) extraembryonic ectoderm.

hybridization for Hesx1, Hex, Cer1I1, and Dkk1 revealed significantly reduced levels or loss of expression, localized at the distal tip of the embryo, as compared with wild-type littermates (Fig. 2A–D,A1–D1). To further investigate the dynamics of AVE specification, we used the Hex-GFP reporter strain [Rodriguez et al. 2001]. Time-lapse imaging of Hex-GFP embryos revealed GFP expression in a small group of DVE cells at the distal tip of the egg cylinder by E5.5 (Fig. 2E,E1) and an increase in fluorescence levels of the reporter over time, concomitant with the directional migration of a cohort of GFP-positive DVE/AVE cells toward the embryonic/extraembryonic junction (Fig. 2G,G1), as has been reported previously (Srinivas et al. 2004). In contrast, as compared with wild-type embryos at E5.5, EomesVE/−; Hex-GFPTg/+ embryos exhibited low levels of GFP fluorescence in a reduced number of distal cells (Fig. 2F,F1).

**Figure 2.** Eomes is required for the maintenance of the DVE and specification and migration of the AVE. ([A–D1]) Whole-mount in situ hybridization of E6.5 embryos of the indicated genotypes using AVE/DVE-specific markers Hesx1, Hex, Cer1I1, and Dkk1. ([E–H]) Aberrant DVE specification (E5.5; E) and migration (E6.5; G) in EomesVE/− mutants [F,H] versus wild type [WT] [E,G]. ([Green] Hex-GFP, [red] F-actin, [blue] nuclei. ([I–L]) Whole-mount in situ hybridization of E6.5 embryos using posterior (T) and intermediate streak (Fgf8, Mixl1) markers ([I–K]) and the anterior marker Otx2 ([L–M]) in wild type and EomesVE/− mutants ([I–L,M,MI]). ([I,M]) Expression of Nanog in E6.0 wild-type and EomesVE/− mutant embryos.
primitive streak [data not shown]. In contrast, Nodal expression was markedly up-regulated throughout the epiblast [Fig. 3A,A1]. Additionally, expression of Lefty2, a direct target and antagonist of Nodal, as well the Nodal coreceptor CRIPTO became radialized in EomesΔVE−/− mutants [Fig. 3B–C1]. In contrast, expression of CRIPTO, another Nodal coreceptor, was down-regulated in the VE of EomesΔVE−/− mutants [Fig. 3D1]. Cryptic expression is normally restricted to the proximal EmVE at prestreak stages [Fig. 3D]. Loss of Cryptic expression strengthens the argument for defective specification of the EmVE in EomesΔVE−/− mutants.

To test whether reducing Nodal levels could rescue some of the observed defects, we generated EomesΔVE−/−; NodalLacZ/+ embryos. EomesΔVE−/−;NodalLacZ/+ embryos were recovered at Mendelian ratios at midstreak (E6.5) and early bud (E7.5) stages [Supplemental Fig. 2A–F]. As judged by gross morphology, NodalLacZ/+ reporter expression, and marker gene analysis, at E6.5, EomesΔVE−/−;NodalLacZ/+ embryos exhibited an intermediate phenotype between wild-type embryos and EomesΔVE−/− mutants [Fig. 3E–M]. The VE thickening observed in the EomesΔVE−/− mutants was absent in EomesΔVE−/−;NodalLacZ/+ embryos. EomesΔVE−/−;NodalLacZ/+ embryos express Cerl1 and Lefty1, albeit at very reduced levels [Fig. 3P,M]. Consequently, the primitive streak became localized posteriorly as judged by NodalLacZ/+ (Supplemental Fig. 2A–C) and T/Bachyury expression [Supplemental Fig. 2D–F]. However, at E7.5, EomesΔVE−/−;NodalLacZ/+ embryos exhibited a constriction at the embryonic/extraembryonic boundary [Supplemental Fig. 2C,F]. Hence, increased Nodal signaling from the epiblast appeared to exacerbate the EomesΔVE−/− mutant phenotype. Restraining Nodal signaling in EomesΔVE−/− embryos was sufficient for initiation of DVE migration, as DVE formation is not defective in EomesΔVE−/− mutants [Fig. 2F]. However, VE specification and migration was still impaired in EomesΔVE−/−;NodalLacZ/+ embryos.

Conditional deletion of Eomes from the early EmVE prevents the correct specification and maintenance of the VE. The simplest scenario is that Eomes acts upstream to activate the hierarchy of transcription factors governing VE formation. Nodal signaling in the early epiblast is known to be responsible for induction of the VE via a Smad2-dependent pathway [Brennan et al. 2001]. The LIM domain factor Lhx1 and forkhead factor Foxa2 are both required for correct formation and function of the VE. Loss of either gene results in failure to establish a normal AP axis, likely due to impaired migration of the VE and consequent defects in primitive streak formation [Ang and Rossant 1994; Shawlot and Behringer 1995; DuFort et al. 1998; Kinder et al. 2001]. Interestingly, the Lhx1;Foxa2 double-mutant phenotype closely resembles that of Smad2 mutants. Markers of the DVE/AVE are not induced, and the entire epiblast adopts a mesodermal character [Waldrip et al. 1998; Perea-Gomez et al. 1999], suggesting that these transcription factors work in parallel or cooperatively to regulate the gene regulatory network [GRN] underlying VE specification. In wild-type E5.5 embryos, Eomes, Lhx1, and Foxa2 are expressed in the EmVE [Fig. 4A–A3,A–E3]. In Smad2−/− embryos, both Eomes and Lhx1 are lost, while Fox2 expression is still evident [Fig. 4B–B3,F–F3]. In contrast, in Lhx1 mutants, Eomes and Foxa2 expression is unaffected [Fig. 4C–C3,G–G3]. Foxa2 is not required for the VE to maintain Eomes or Lhx1 expression [Fig. 4D–D3,H–H3], whereas in EomesΔVE−/−;NodalLacZ/+ mutants, Lhx1 is absent in the EmVE [Fig. 4l–I3,J–J3]. Hence, activation of Lhx1 depends on the Smad2–Eomes pathway, whereas Nodal signaling levels are less critical for Foxa2 expression.

To test whether Lhx1 is a direct Eomes target, we exploited cell culture protocols that promote the formation of extraembryonic endoderm populations. First, we used forced expression of Gata6 to direct embryonic stem (ES) cells toward an extraembryonic endoderm fate [Shimosato et al. 2007]. A regulatable Gata6 expression vector was stably introduced into wild-type or Smad2-deficient ES cells [Tremblay et al. 2000], in which Nodal signaling is significantly impaired. Following induction of Gata6 via addition of dexamethasone, Eomes and Lhx1 transcript levels were assessed. Wild-type cell clones expressed Eomes and Lhx1 transcripts in response to Gata6 induction, while expression of both genes was severely reduced in Smad2-deficient clones [Supplemental Fig. 3A]. Thus, robust Eomes and Lhx1 induction depends on active Nodal–phospho-Smad2 signaling. Next, we examined XEN cell cultures [Kunath et al. 2005]. XEN cells express low or undetectable levels of Eomes and Lhx1 as assessed by RT–PCR [Fig. 5A] and expression microarrays [Supplemental Fig. 3B]. Stably trans-
implicated the role of the Lim domain transcription factor Lhx1 in activating expression of the T-box transcription factor Eomes in the EmVE population. Conditional loss of Eomes expression in the VE does not perturb DVE formation but rather blocks correct specification of the AVE. Our ChIP and epistasis experiments reveal that this is due to a requirement for Eomes in binding to and activating expression of the Lim domain transcription factor Lhx1. Amot, which regulates VE movement in the early embryo (Shimono and Behringer 2003), is regulated by Lhx1 (Shimono and Behringer 1999), and Lhx1 is also known to bind the promoter of the AVE marker Hesx1 (Chou et al. 2006). Thus, Eomes acts upstream of Lhx1 in the GRN underlying AVE specification and migration [Fig. 5J]. In contrast, Foxa2 expression is Nodal-independent (Brennan et al. 2001), as loss of either Smad2, Eomes, or Lhx1 prevents expression of Foxa2 in the VE. Hence, both Foxa2 and Lhx1 are independently required for proper AVE function. In addition to Nodal–Smad2, a second signaling pathway must function in AVE formation. Identification of upstream regulators of Foxa2 will require additional experiments.

Signaling cues from the epiblast and ExE temporally and spatially intersect to precisely pattern the growing VE and restrict induction of the AVE to the most distal population within the EmVE. At E5.5, the EmVE uniformly expresses Eomes, Lhx1, and Foxa2, the three transcription factors identified as having critical roles in AVE formation. Anterior patterning of the early post-implantation embryo requires a continuum of opposing Nodal/Bmp signaling activities. Nodal and Bmp signals from the early epiblast and ExE, respectively, are required to distinguish the EmVE versus the extraembryonic VE [ExVE] (Mesnard et al. 2006; Yamamoto et al. 2009). As the embryo expands, the most distal VE cells become positioned increasingly farther away from the ExE, the source of BMP ligands, while chromatin immunoprecipitation (ChIP).

Both endogenous Eomes and EomesER [4 d post-differentiation] were specifically enriched at the T-site. Eomes occupancy of this site correlates with RNA polymerase II [PolII] binding at the Lhx1 TSS, consistent with induction of Lhx1 transcription [Fig. 5D,E]. Since Eomes is the only T-box transcription factor known to be expressed in the EmVE (Arnold et al. 2009), these data provide compelling evidence that Lhx1 is a direct target of Eomes during AVE specification. To explore whether Eomes acts to not only induce but also maintain Lhx1 expression during migration of the AVE, we examined expression in embryos. Double-labeling experiments revealed colocalization of both transcription factors in the AVE of E6.5 of wild-type embryos [Supplemental Fig. 4].

The present study provides new insights into DVE/AVE functions in promoting anterior fates in the early post-implantation embryo. We identify a critical role for the Nodal–Smad2 pathway in activating expression of the T-box transcription factor Eomes in the EmVE population. Conditional loss of Eomes expression in the VE does not perturb DVE formation but rather blocks correct specification of the AVE. Our ChIP and epistasis experiments reveal that this is due to a requirement for Eomes in binding to and activating expression of the Lim domain transcription factor Lhx1. Amot, which regulates VE movement in the early embryo (Shimono and Behringer 2003), is regulated by Lhx1 (Shimono and Behringer 1999), and Lhx1 is also known to bind the promoter of the AVE marker Hesx1 (Chou et al. 2006). Thus, Eomes acts upstream of Lhx1 in the GRN underlying AVE specification and migration [Fig. 5J]. In contrast, Foxa2 expression is Nodal-independent (Brennan et al. 2001), as loss of either Smad2, Eomes, or Lhx1 prevents expression of Foxa2 in the VE. Hence, both Foxa2 and Lhx1 are independently required for proper AVE function. In addition to Nodal–Smad2, a second signaling pathway must function in AVE formation. Identification of upstream regulators of Foxa2 will require additional experiments.

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Role of Eomes in AVE induction

Materials and methods

Mouse strains, embryo collection, and staining

Mouse strains used were as follows: Eomesflox/flox [Mao et al. 2008], Ttr-Cre (Kwon and Hadjantonakis 2009), PdgfraH2B-GFP [Hamilton et al. 2003], Hex-GFP [Rodriguez et al. 2001], Nodal−/− [Collignon et al. 1996], Lhx1 (Shawlot and Behringer 1995), Foxa2 (Ang and Rossant 1994), and Smad2−/− [Waldrip et al. 1998]. EomesVE-deleted embryos [referred to as Eomes−/−] were generated by crossing Eomes−/−;Ttr-Cre+/+ males with Eomesflox/flox females. For time-lapse imaging, embryos were cultured in 50% rat serum/50% DMEM-F12, 5% CO2, at 37°C.

Materials and methods

Cell culture, ChIP, and RNA analysis

P19CL6 and P19EoER cells were cultured as previously described (Costello et al. 2011). ES cells were differentiated in medium containing 1% FBS, 5% mouse serum, 5% KGM, 0.5 μg/mL puromycin to selective for pCAG-EomesER-IRESPuro (Niwa et al. 2005)–transduced P19CL6 and P19EoER cells. Activin A (R&D Systems) was added at a concentration of 50 μg/mL. Whole-mount in situ hybridization and LacZ staining were performed as previously described (Kunath et al. 2005). pCAG-EomesER-IRESPuro (Niwa et al. 2005)–transduced P19CL6 and P19EoER cells were cultured as previously described (Costello et al. 2011). ES cells were cultured as previously described (Costello et al. 2011). Cells were electroporated with pCAG-Gata6GR-IRESPuro (Shimosato et al. 2007) and selected in puromycin. Addition of 100 mM dexamethasone (Sigma) resulted in the activation of Gata6GR and conversion of cells to a XEN-like phenotype (Shimosato et al. 2007).
RNA was isolated and analyzed as previously described (Costello et al. 2011). Primer sequences are provided in Supplemental Table S3. Wide-field images were acquired with a Zeiss AxioCam camera on a Leica M165FC. Laser-scanning confocal data were acquired on a Zeiss LSM510META or Olympus FV1000. Raw data were processed using Zeiss AIM/ZEN and Bitplane Imaris software.

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