Published in final edited form as:

Curr Opin Genet Dev. 2013 August ; 23(4): 461–469. doi:10.1016/j.gde.2013.04.001.

Genetic Dissection of Ventral Folding Morphogenesis in Mouse: Embryonic Visceral Endoderm-supplied BMP2 Positions Head and Heart

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Abstract

Ventral folding morphogenesis, a vital morphogenetic process in amniotes, mediates gut endoderm internalization, linear heart tube formation, ventral body wall closure and encasement of the fetus in extraembryonic membranes. Aberrant ventral folding morphogenesis underlies a number of birth defects, such as gastroschisis and ectopia cordis in human and misplacement of head and heart in mouse. Recent cell lineage-specific mouse mutant analyses identified the Bone Morphogenetic Protein (BMP) pathway and Anterior Visceral Endoderm (AVE) as key regulators of anterior ventral folding morphogenesis. Loss of BMP2 expression solely from embryonic visceral endoderm (EmVE) and the AVE blocks formation of foregut invagination, and simultaneously, aberrantly positions the heart anterior/dorsal to the head, suggesting a mechanistic link between foregut and head/heart morphogenesis.

Introduction

Malformations of the ventral body wall represent a prevalent but understudied class of human birth defects, that includes gastroschisis (development of intestines outside the abdominal wall) and ectopia cordis (heart formation outside the thoracic cavity) [1–2]. These defects likely arise from irregularities in ventral folding morphogenesis (VFM), a fundamental developmental process that orchestrates internalization of gut endoderm, closure of the ventral body wall, formation of a linear heart tube (Figure 1), and encasement of the fetus in extraembryonic membranes (Figure 2). The human pathology underlying these disorders remains poorly defined, due partly to the absence of standardized nomenclature and of methodology for classifying body wall-associated birth defects. The lack of suitable animal models has also impeded studies of the genetic pathways and cellular mechanisms guiding VFM [1–3].

In mouse, VFM begins in the anterior region of the Early Head Fold (EHF) embryo, around Embryonic Day (E) 7.75–8.0, following the generation of the ectoderm, mesoderm, and endoderm lineages during gastrulation. Through concurrent rostral-caudal and ventral-to-lateral tissue movements, VFM achieves inversion of the germ layers in the anterior region of the embryo, formation of the primitive heart tube, and placement of the head dorsal/

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anterior to the heart (Figure 1B). By the 8–10 somite stage, both the hindgut and foregut pockets have formed; the midgut region remains on the external surface, and the ventral region caudal to the heart, continues to reside outside the amnion and yolk sac (Figure 2A) [4]. Between E8.5 (6–8 somites) and E9.0 – E9.5 (14–16 somites), while completing endoderm internalization and formation of the primitive gut tube, the mouse conceptus undergoes turning/axial rotation (Figure 2) [4]. Vital to viability and further development, turning achieves enclosure of the entire embryo within the extraembryonic membranes (Figure 2D).

In contrast to the hollow cylindrical shape of the mouse epiblast, the human epiblast resembles a flattened disc at the onset of VFM during the fourth week of gestation [5]. This variant topology accounts for a key difference in VFM between human and mouse; human embryos need not undergo turning to complete internalization of gut endoderm and enclosure of the fetus in the amnion. Nonetheless, lineage tracing studies -and histological examination of serially sectioned early somite mouse embryos- suggest that mouse and human embryos employ comparable cell and tissue movements to achieve foregut invagination, formation of the primitive heart tube, and placement of the headfolds rostral to the heart during early VFM (Figure 1B) [4,6–8].

The morphogenetic events mediating ventral body wall closure and gut tube formation are complete early in the fifth week of human embryonic development [1], months before body wall malformations are assessed in newborns. Thus distinguishing the etiology of this class of birth defects from secondary complications presents a difficult challenge. Moreover, disruption of the initial steps of VFM, at the 0-to10 somite stage, likely results in early lethality and spontaneous abortion; a factor contributing to the current dearth of knowledge about the pathways and cellular mechanisms mediating these events in mammalian embryos.

Here we review recent findings from lineage-specific mouse mutant analyses that uncovered temporally and spatially distinct roles for BMP2 signaling in VFM. These studies revealed that the onset and early steps of anterior VFM (Figure 1B) require EmVE-expressed BMP2; later (post 8–10 somite stage) and more posterior steps of VFM (Figure 2) require BMP2 expressed by epiblast derivatives. We discuss the implications of these findings for post-gastrulation roles of the AVE and for the origin cardia bifida. We also highlight promising leads for further study to elucidate the mechanisms through which EmVE-BMP2 signals are transduced into morphogenetic movements that extensively rearrange tissue layers during anterior VFM.

Potential cell source of signal initiating VFM: tissue layer organization at site of foregut invagination

VFM initiates at the early somite stage with the formation of foregut invagination, which manifests as the anterior intestinal portal (AIP), an arc of thickened endoderm spanning the anterior midline. Thus signals initiating VFM must emanate from one of the tissue layers in the vicinity of the prospective AIP during the allantoic bud-to-pre-somite/EHF stages. As illustrated in Figure 1A & Bi, cardiac crescent progenitor cells reside just anterior to the emergent head folds at the rostral end of the pre-somite/EHF embryo, where amniotic ectoderm transitions into embryonic ectoderm. Two cell populations comprise the surface layer overlying the heart and neuroectoderm and both will contribute to the invaginating foregut: (1) prechordal plate (PrCP) and (2) gut endoderm located on either side of the anterior midline.

The PrCP lies at the anterior end of the midline, just caudal to the cardiogenic region and just rostral to the anterior head process (AHP, Figure 1A). It is a bilaminar structure containing ectoderm directly juxtaposed to endoderm/axial mesendoderm derivatives of the

early gastrula organizer (EGO; anterior region of the early primitive streak, PS) [9]. The ectoderm layer overlying the PrCP contains neuroepithelial precursors to the forebrain. Molecularly defined by the expression of *Gsc* and the absence of *Brachyury* [10–11], the PrCP marks the position of the forthcoming foregut invagination. Lineage tracing of PrCP cells labeled with Dil/DiO at the pre-somite/EHF stage demonstrated that derivatives of the PrCP populate the oral cavity, ventral midline and dorsal foregut endoderm, and ventral cranial mesenchyme [12].

Two distinct sources of cells give rise to gut endoderm on the anterior surface of the pre-somite/EHF embryo: PS derived definitive endoderm (DE) and EmVE (reviewed in [13]). Single cell fate-mapping studies originally defined epiblast cells ingressing through the anterior PS as the primary source of endoderm generated during gastrulation [14–16]. The EmVE contribution to gut endoderm remained unknown for nearly two decades, until the Hadjantonakis group applied live imaging to track the movements of fluorescently labeled VE cells. These experiments revealed that during the Early-to-Late Allantoic Bud (EB-LB) stages, epiblast/streak-derived DE cells intercalate multifocally into the VE layer, rapidly dispersing the EmVE cells [17**]. Relevant to consideration of potential sources and targets of signals initiating foregut invagination, EmVE derivatives persist within the gut tube until at least the 20 somite stage [17**].

BMP2 expression in EmVE/AVE and epiblast lineages regulates temporally distinct steps of VFM

The initial characterization of the *Bmp2* knockout (KO) noted phenotypic defects suggestive of impairment of one or more aspects of VFM; notably the heart developed abnormally outside the amnion [18]. This study ascribed the observed malformations to loss of expression from the only tissues known at that time to transcribe *Bmp2*: extraembryonic mesoderm of the amnion, yolk sac, & allantois and cardiac progenitors. All four tissues activate *Bmp2* transcription during the neural plate (no bud, EB, LB)-to-EHF stages, just before or coincident with formation of foregut invagination. Subsequent studies provided evidence for even earlier expression of *Bmp2* in the VE [19–20] and documented a spatially dynamic pattern of *Bmp2* transcription that is restricted to EmVE during pre-streak and gastrulation stages (Figure 3A) [21–22; 23**]. NODAL activates *Bmp2* expression throughout EmVE at E5.25–E5.5, during the specification and anterior migration of the distal visceral endoderm (DVE). Upon the onset of gastrulation at ~E6.25, early streak (ES) embryos express *Bmp2* predominantly in the proximal EmVE, with the highest levels occurring anteriorly. As demonstrated by two-color *in situ* hybridization to *Bmp2* and either *Cer1* (an AVE marker) or *Brachyury* (a posterior PS marker) *Bmp2* expression becomes confined to a patch of cells in the proximal-most anterior VE of the mid-streak embryo [23**]. *Bmp2* expression persists in anterior endoderm at the neural plate/pre-somite/EHF stages. Relevant to the *Bmp2* KO phenotype, the EmVE provides the primary source of BMP2 until the neural plate/EHF stage [23**].

Lineage-specific mutant analyses recently revealed that execution of VFM requires BMP2 expression in both EmVE and epiblast derivatives [23**]. KO embryos lacking BMP2 expression in all cell types display one or more of four distinct defects at early somite stages (E8.5–E9.5): open proamniotic canal (OPC); heart-outside the amnion; disorganized anterior (DAT); and unturned [23**] (Figure 3Biii–iv). VE-CKO (conditional KO) embryos, which lack a functional *Bmp2* gene in VE while maintaining BMP2 expression in epiblast derivatives, arrest before turning at the 8–10 somite stage; they display the DAT phenotype in which the heart forms anteriorly/dorsally to the head rather than in its normal posterior/ventral location (Figure 3Bv). However, *Bmp2* VE-CKO embryos always contain the misplaced heart within a closed amnion; indicating the absence of OPC and heart-outside the amnion defects [23**]. Conversely, EPI-CKO embryos, lacking a functional *Bmp2* gene in

all epiblast derived lineages while maintaining BMP2 expression in EmVE, never present the DAT phenotype; they fail to turn and exhibit OPC and heart-outside the amnion defects (Figure 3Bvi). In addition to amnion defects, *Bmp2* EPI-CKO embryos recovered at E9.5 display a new phenotype, not found in the global *Bmp2* KO, termed posterior-specific delay. *Bmp2* EPI-CKO embryos with this phenotype develop head structures comparable to those in stage matched WT littermates, but form a posterior region resembling that in the global KO (Figure 3Bvii) [23**]. *Bmp2* EPI-CKO embryos also properly position the primitive heart tube, but reflecting requisite BMP2 functions in cardiac mesoderm, the heart tube fails to undergo looping.

The mispositioned heart and head in VE-CKO embryos suggests that loss of BMP2 expression from EmVE disrupts the initial steps of VFM, which invert the anterior germ layers and place the heart posterior and ventral to the headfolds (Figure 1B). The posterior-specific delay phenotype of EPI-CKO embryos indicates that expression of BMP2 in the EmVE is sufficient for the normal execution of anterior VFM up to the 7–10 somite stage. The inability of the EPI-CKO mutant to turn, complete germ layer inversion, and incorporate the embryo in the extraembryonic membranes suggests that BMP2 expression in epiblast lineages contributes not only to amnion formation but also to subsequent steps in VFM between 8-to-16 somite stages.

EmVE-expressed BMP2 links foregut invagination with morphogenesis of the head and heart

Histological sectioning and scanning EM of early somite stage *Bmp2* VE-CKO and KO embryos identified three traits that invariably coincide in mutants with the DAT phenotype: (i) absence of foregut invagination, (ii) appearance of ectopic neural folds and (iii) an elongated, flattened anterior axis (Figure 4A–F) [23**]. This constellation of defects indicates that loss of EmVE-expressed BMP2 impairs two fundamental and concurrent aspects of early VFM: formation of foregut invagination and positioning of head and heart. These three defining features of DAT suggest that BMP2 signaling mechanistically connects rostral-caudal folding of endoderm with that of neuroectoderm to coordinate the morphogenetic movements mediating formation of foregut invagination and repositioning of head and heart. How EmVE-expressed BMP2 orchestrates the early steps of VFM remains largely unknown; yet different lines of investigation provide propitious clues and identify important areas for further study.

Type I Signaling Receptor—DAT likely results from loss of EmVE-BMP2 activation of BMPR1A (ALK3) on epiblast-derived cell types [23**]. KO embryos lacking *Bmpr1a*, which is expressed ubiquitously at pre-streak and early gastrulation stages [24–25], arrest prior to gastrulation with defects most comparable to those observed in the *Bmp4* KO [26]. However, mutants with a mosaic –but epiblast specific– loss of *Bmpr1a* display the three characteristic traits of the DAT phenotype [27]. Studies on *Smad5*, a receptor-regulated transcription factor in the BMP pathway [28], additionally support an EmVE-BMP2 → epiblast-BMPR1A pathway for activating anterior VFM [23**]. Epiblast cells –but not extraembryonic ectoderm and VE cells– transcribe *Smad5* and late headfold-to-early somite stage *Smad5*^{−/−} mutants lack foregut invagination and contain ectopic neural folds [29].

BMP2 Activation of VFM - Developmental Timing—Since the VE-specific Cre transgene (*Tr:Cre*) used by Madabhushi and Lacy [23**] inactivated *Bmp2* by E5.5, their analysis of VE-CKO embryos could not determine whether the DAT phenotype reflects a requirement for *Bmp2* expression throughout the EmVE at E5.5 and/or in the proximal, anterior region of the EmVE from E6.5 to the EHF stage (Figure 3A). Notably, the anterior location of BMP2-expressing EmVE cells at E6.5–E7.5 corresponds to the site where the

cardiac crescent and PrCP will reside in the EHF embryo. The close juxtaposition of the anterior BMP2-expressing EmVE to the very tissues that will be repositioned by rostral-caudal and lateral-to-ventral tissue folding (cardiac progenitors, the AIP, and neuroectoderm) make it a compelling candidate for the source of signals activating VFM.

BMP2 Activation of VFM – a Proposed Post-gastrulation AVE Function—

During the ES-MS stages, the *Bmp2*-expressing EmVE cells comprise a subregion of the AVE, a well defined organizing center that establishes and patterns the A-P axis during the PS-ES stages [30–31]. Although the AVE is generally portrayed as consisting of functionally equivalent cells, Takaoka et al. [32**] recently reported temporal and spatial shifts in the EmVE populations contributing to the AVE, suggesting functional heterogeneity [32**]. Genetic fate mapping experiments found, contrary to prevailing models, that distal EmVE cells (DVE) expressing *Lefty1* at E5.25–E5.5 do not give rise to the *Hhex* expressing AVE at E6.5. Instead, the descendants of E5.25–E5.5 *Lefty1*+ DVE cells occupy an anterior/lateral region of the EmVE, distinct from and non-overlapping with the AVE, which forms from distal EmVE cells first expressing *Lefty1* and *Cer1* only after E5.5 [32**]. Noting that the ES domain of *Bmp2* expression resides within an area projected to contain all progeny of E5.25 *Lefty1*+ DVE cells, Madabhushi and Lacy [23**] proposed that activation of VFM represents a late, but integral function of the AVE, potentially one performed by direct descendants of the *Lefty1*+DVE cells at E5.25. Such a role, coinciding with the confinement of *Bmp2* expression to the proximal AVE in ES-EHF embryos, is also consistent with the suggestion that signals from more distal AVE cells regulate the anterior expression of *Bmp2* [33].

Before the discovery that embryonic gut endoderm forms through an intercalation of epiblast-derived derived DE cells into EmVE layer [17**], the prevailing model for endoderm morphogenesis posited that a steady production of nascent endoderm cells by the anterior PS propelled a contiguous layer of DE cells proximally, until the EmVE, including the AVE, was displaced into the extraembryonic region by E7.5, before foregut invagination [13,15]. Since relocation to an extraembryonic position would separate the AVE/EmVE from the embryo proper, a post-gastrulation role directing tissue patterning and morphogenesis has not been considered for the AVE. In contrast to lateral EmVE cells, which become incorporated into gut endoderm, the ultimate fate of anterior EmVE/AVE cells remains unknown. Genetic labeling experiments, using GFP perdurance as a short term lineage tracer, detected EmVE derivatives reside on either side of anterior midline tissue that will form notochord [17**,34]. However these studies did not examine whether anterior EmVE cells contribute to PrCP [11–12]. Lineage and marker-expression analyses assessing whether BMP2-expressing anterior EmVE cells become incorporated into PrCP and the foregut tube should prove quite informative regarding the mechanism by which BMP2 regulates foregut invagination and the onset of VFM.

BMP2 Activation of VFM – Potential Target Tissues—Elucidation of the mechanisms coordinating foregut invagination with repositioning of head and heart will require experimentally identifying the subpopulation of epiblast-derived cells that responds to the EmVE-BMP2 signal. As depicted in Figures 1A and 3A, anterior EmVE cells expressing BMP2 reside in close proximity to multiple tissues, including PrCP endoderm, neuroectoderm, and cardiac mesoderm. Thus determining whether EmVE-expressed BMP2 signals to one or more distinct cell layers will be crucial for understanding its role in VFM.

BMP2 Activation of VFM – Coordination of Morphogenetic Cell Behaviors—An examination of markers for specification of AVE, cardiac mesoderm, neuroectoderm, and foregut endoderm found a comparable distribution of transcripts in *Bmp2* VE-CKO and WT embryos, indicating that the absence of EmVE-BMP2 did not detectably perturb cell

specification and lineage patterning [23**]. This observation suggested, instead, that appropriately specified cells in tissues normally responsive to EmVE-BMP2 fail to participate in necessary morphogenetic cell behaviors. Madabhushi and Lacy [23**] proposed that EmVE-BMP2 acts to initiate and coordinate cell behaviors required to achieve the extensive and simultaneous tissue rearrangements inherent to anterior VFM. Directly pertinent to this proposed function of EmVE-BMP2 are recent findings in cell culture models demonstrating an interaction between cell shape/cytoskeletal tension and levels of BMP2-induced signaling [35**–36*]. These studies highlight interplay between the transduction of biochemical signals and the transmission of mechanical forces as an intriguing and credible paradigm for future investigations into how EmVE-BMP2 signaling is transduced into the morphogenetic movements that achieve formation of the foregut tube simultaneously with repositioning of cardiac mesoderm and neuroectoderm during anterior VFM.

Anterior VFM: a revised model for cardia bifida

Current models for cardia bifida propose that a single morphogenetic process coordinates the movement of endoderm and lateral mesoderm to drive the formation of foregut invagination and the linear heart tube. The finding that defective foregut development invariably accompanies cardia bifida in several mouse knockouts, including *Gata4* [37*–38*], *Hgs/Hrs* [39*], *Furin* [40, 42*], and *Flrt3* [41*] led to the hypothesis that the generation of a single linear heart tube depends on the formation of foregut invagination. The presence of a medial heart tube in *Bmp2* VE-CKO embryos disputes this view [23**]. The absence of EmVE-BMP2 blocks both rostral-to-caudal and ventral-to-lateral folding of anterior endoderm, resulting in the lack of the foregut pocket and a closed gut tube. Yet a single heart tube still forms, indicating that the lateral-to-ventral movement of anterior splanchnic mesoderm proceeds independently of rostral-caudal folding of anterior gut endoderm.

Figure 4G diagrams a revised model for the origin of cardia bifida that incorporates distinct differences found in the anterior VFM defects of the *Bmp2* VE-CKO compared to those of *Gata4*^{−/−}, *Hrs*^{−/−}, *Furin*^{−/−}, and *Flrt3*^{−/−} embryos. This model proposes that a single morphogenetic process achieves both formation of foregut invagination and correct rostral-to-caudal positioning of head and heart and that this process is separate from that producing the medial heart tube. Consistent with this view, in both the *Gata4* and *Hrs* mutants, which completely lack foregut invagination, the bilateral heart primordia reside anterior to the headfolds [37*, 38*, 39*]. In contrast, the foregut pocket does form in the *Furin* and *Flrt3* mutants, but fails to close ventrally [41*–42*]. As would be predicted by a single morphogenetic process driving foregut invagination and rostral-caudal positioning of head and heart, the bilateral heart primordia appropriately reside posterior to the headfolds. The complete absence of foregut invagination in the *Bmp2* VE-CKO still leaves open the possibility that lateral-to-ventral movements to close the gut tube may be mechanistically connected to those that join the bilateral heart fields.

Acknowledgments

We thank Branislav Brkic for his work on the graphic art included in the figures and Ellen Freed for insightful comments on the manuscript and valuable discussions. Our research receives support from NIH/NICHD 1 R01 HD072499, NIH/NCI 5 P30 CA008748, and NYSTEM C026879

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Figure 1. Ventral folding morphogenesis encompasses multiple concurrent tissue rearrangements: EHF, 0-to-8 somites

(A) An oblique frontal view of an EHF mouse embryo; planes indicate positions of sagittal and transverse sections shown in B. Before the onset of VFM, ectoderm (green) lines the amniotic cavity and encompasses the inner-most layer of the distal (embryonic) portion of the conceptus; endoderm (yellow, blue) forms the surface layer and overlies the mesoderm (pink). The heart primordia (red), which develop bilaterally from splanchnic mesoderm, reside just anterior to the nascent ectodermal headfolds [6]. The anterior midline consists of three structures [9,11]: the node, which will generate trunk and tail notochord; anterior head process (AHP), axial mesoderm -derived from the early/mid gastrula organizer- that underlies the prospective midbrain and rostral hindbrain; and Prechordal plate (PrCP), axial mesendoderm -derived from the early gastrula organizer- underlies the prospective forebrain. (B) The tissue folding propelling VFM occurs along two axes, rostral-caudal (depicted in sagittal sections i–iii) and ventral-to-lateral (depicted in transverse sections, iv–vi), during the EHF (i, iv), 2 somites (ii, v), and 7 somites (iii, vi) stages. The horizontal lines (labeled iv, v, vi) in respectively, panels i, ii, and iii indicate the level of the transverse section in the corresponding lower panel. Rostral-caudal folding repositions endoderm cells along the midline as it transforms the sheet of anterior gut endoderm from a convex external layer into a concave internal foregut pocket protruding rostrally [7–8]. Concurrent rostral-caudal folding of neuroectoderm repositions the headfolds to reside anterior and dorsal to cardiac mesoderm [4,6,39]. Lateral-to-ventral folding of the bilateral heart primordia positions cardiac progenitors at the ventral line, where they merge to form the primitive

heart tube. Concomitantly, lateral regions of gut endoderm and of the body wall (apposed somatic mesoderm and surface ectoderm layers) move ventrally toward the midline and fuse to close, respectively, the foregut tube and the body wall. The visceral yolk sac (extraembryonic visceral endoderm –yellow plus extraembryonic mesoderm –pink dashed line) undergoes ventral-to-lateral tissue movements to envelope the anterior region (head and heart) of the early-somite embryo. Arrows indicate direction of rostral-caudal (ii, iii) and lateral-to-ventral (iv) folding. Abbreviations: AHP- anterior head process; amn- amnion; fg, foregut pocket; hf-head folds; PrCP-prechordal plate; ys-visceral yolk sac.

Figure 2. Ventral folding morphogenesis encompasses multiple concurrent tissue rearrangements: 8-to-20 somites

(A) 8–10 somite stage embryo at the onset of turning with hindgut and foregut pockets (dashed blue lines), external midgut region (solid blue line), and the ventral region, caudal to the heart, outside the amnion and yolk sac. Lateral edges of the amnion and yolk sac are joined to the embryo at the umbilical ring (boundary between the body wall and future attachment site of the umbilical cord, an allantois derivative). The amniotic ectoderm (green line) exists as an extension of the embryonic ectoderm, whilst the amniotic mesoderm lies in direct contact with the mesodermal layer of the visceral yolk sac (red dashed lines). (B–D) Turning/axial rotation inverts the lordotic U-shaped trunk region, placing the embryo in the fetal position with the head looking toward, rather than away from, the tail region; (compare A and D). Upon completion of turning, with embryo now encased within the extraembryonic membranes (amnion and visceral yolk sac), the ectoderm and mesoderm layers of the ventral body wall are continuous with the ectoderm and mesoderm layers of the amnion, which now covers the surface of the allantois/umbilical cord (D) [43]. Turning places the umbilical cord in the midline, at the meeting point of the original anterior and posterior connection sites between the amnion and embryo (* in D). al- allantois; amn- amnion; ys- visceral yolk sac.

Figure 3. BMP2 expression in EmVE/AVE and epiblast lineages regulates distinct steps in VFM (A) Schematic diagrams of *Bmp2* expression in EmVE during the pre-streak (E5.5), early-streak (E6.5); mid-streak (E6.5) and early bud (E7.5) stages. Diagrams illustrate lateral views or transverse sections at the indicated levels; anterior is to the left. Results of two-color *in situ* hybridizations to *Bmp2* (purple) and *Cer1* (orange) or *Brachyury/T* (blue) are illustrated. ant midl- anterior midline; AVE- anterior visceral endoderm; EB-early bud; ES-early streak; MS-mid- streak; meso-mesoderm; n-node; PS- primitive streak. (B) Schematic representations of lineage specific *Bmp2* knockout phenotypes. (i and ii) Wild type embryos show proper arrangement of the head, heart, amnion (thin gray line) and allantois (hatched gray lines) before (8–10 somites) and after turning (15–20 somites). The heart lays posterior/ventral to the head (8–10 somites) and has undergone looping (15–20 somites); both head and heart sit inside the amnion. *Bmp2* KO embryos display one or more of four defects: (iii) disorganized anterior phenotype (DAT); (iv) open proamniotic canal (OPC), heart outside the amnion (ht-out), DAT and unturned. (v) *Bmp2* VE-CKO displays DAT phenotype; (vi) *Bmp2* EPI-CKO displays ht-out and OPC phenotype (8–10 somites) plus (vii) posterior specific delay (15–20 somites) in which the heart fails to loop and the embryo remains unturned.

Figure 4. Disorganized anterior phenotype: formation of foregut invagination and linear heart tube are independent morphogenetic events

(A–F) *Bmp2* KO mutants with the disorganized anterior phenotype display ectopic neural folds and absence of foregut invagination. Frontal views of (A) WT and (B) KO embryos at the 4 somite stage. The two symmetric head folds (red asterisks) and a single neural groove (red ^) present in WT are absent in the KO. (C–E) Transverse sections of WT at the regions indicated in A; (D–F) transverse sections of KO at the regions indicated in B; anterior/ventral is downward. (C) Proximal WT section showing symmetrical head folds and foregut. (E) Distal WT section showing two head folds, medial hinge point (red ^) and surface ectoderm (red >). (D) Proximal KO section showing uneven head folds (red asterisks) and absence of the foregut pocket (red arrow). (F) Distal KO section showing additional neural folds (red asterisks), ectopic hinge points (red ^), and surface ectoderm (red >); (reproduced with permission from Madabhushi and Lacy [23**]). (G) Model for origin of cardia bifida. EHF (0 somite) embryo before onset of VFM is shown on top; the heart progenitors (red) reside just anterior to the nascent ectodermal headfolds (green). Arrows point to four different scenarios at the 8–10 somite stage: 1) wild type - rostral-caudal folding positions the heart posterior to the head folds and generates the foregut invagination; lateral-to-ventral folding fuses the bilateral heart primordia and closes the gut tube at the ventral midline (closed yellow-blue triangle); 2) *Bmp2* VE-CKO - absence of rostral-caudal folding results in absence of foregut invagination and retention of heart progenitors anterior to the headfolds; the presence of a single heart field indicates that lateral-to-ventral folding occurred to fuse the bilateral heart primordia; 3) *Gata4* KO or *Hrs* KO - similar to the *Bmp2* VE-CKO absence of rostral-caudal folding results in absence of foregut invagination and retention of heart progenitors anterior to the headfolds; but lateral-to-ventral folding also does not occur, resulting in lack of fusion of the anteriorly mispositioned bilateral heart primordia; and 4) *Furin* KO or *Flrt3* KO - rostral-caudal folding occurs and positions the heart primordia posterior to the head folds and generates the foregut invagination but absence of lateral-to-ventral folding results in lack of fusion of the properly posteriorly positioned bilateral heart primordia and failure to close the foregut tube at the ventral midline (open yellow-blue triangle). The diagrams are based on Madabhushi and Lacy [23**] and on targeted mutations in the transcription factor *Gata4* [37*–38*], the vesicular

trafficking protein *Hgs/Hrs* (HGF-regulated tyrosine kinase substrate) [39*], *Furin*, a proprotein convertase [40, 42*], and *Flrt3* (fibronectin leucine rich transmembrane protein 3) [41*].