Developmental Cell *15*

Supplemental Data

The Endoderm of the Mouse Embryo Arises by Dynamic Widespread Intercalation of Embryonic and Extraembryonic Lineages

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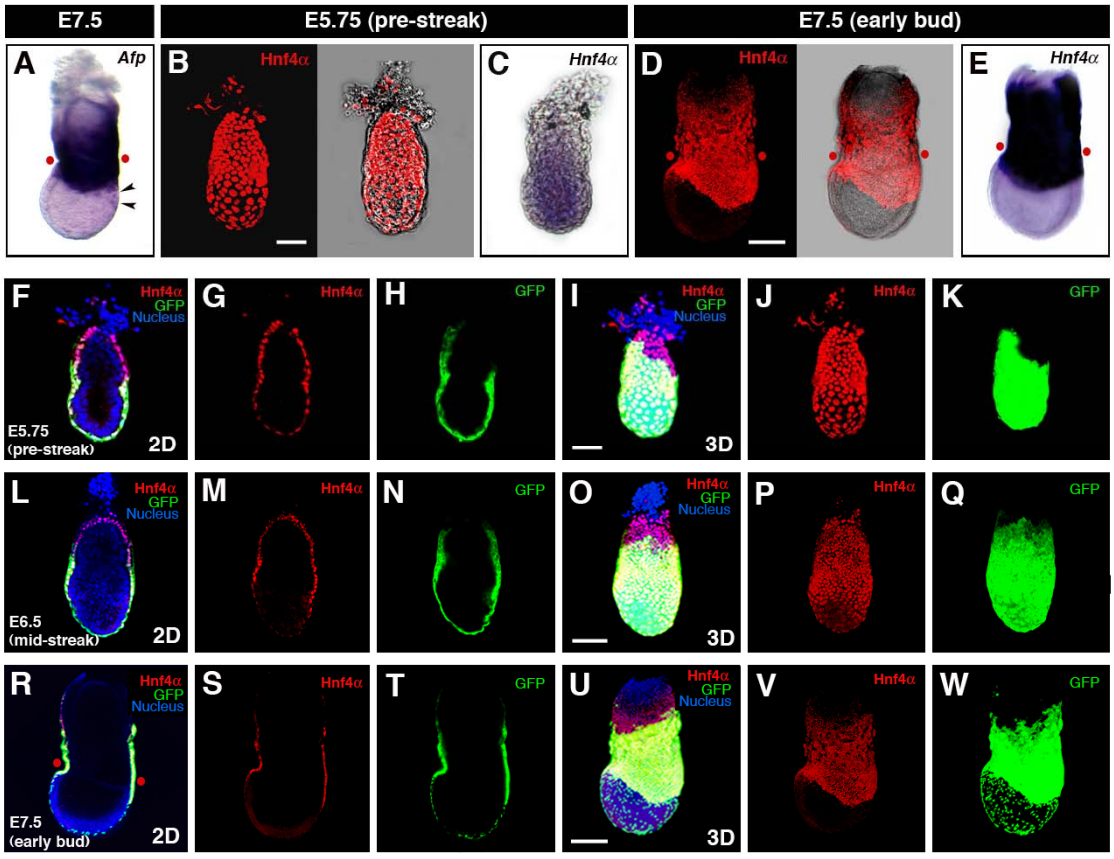
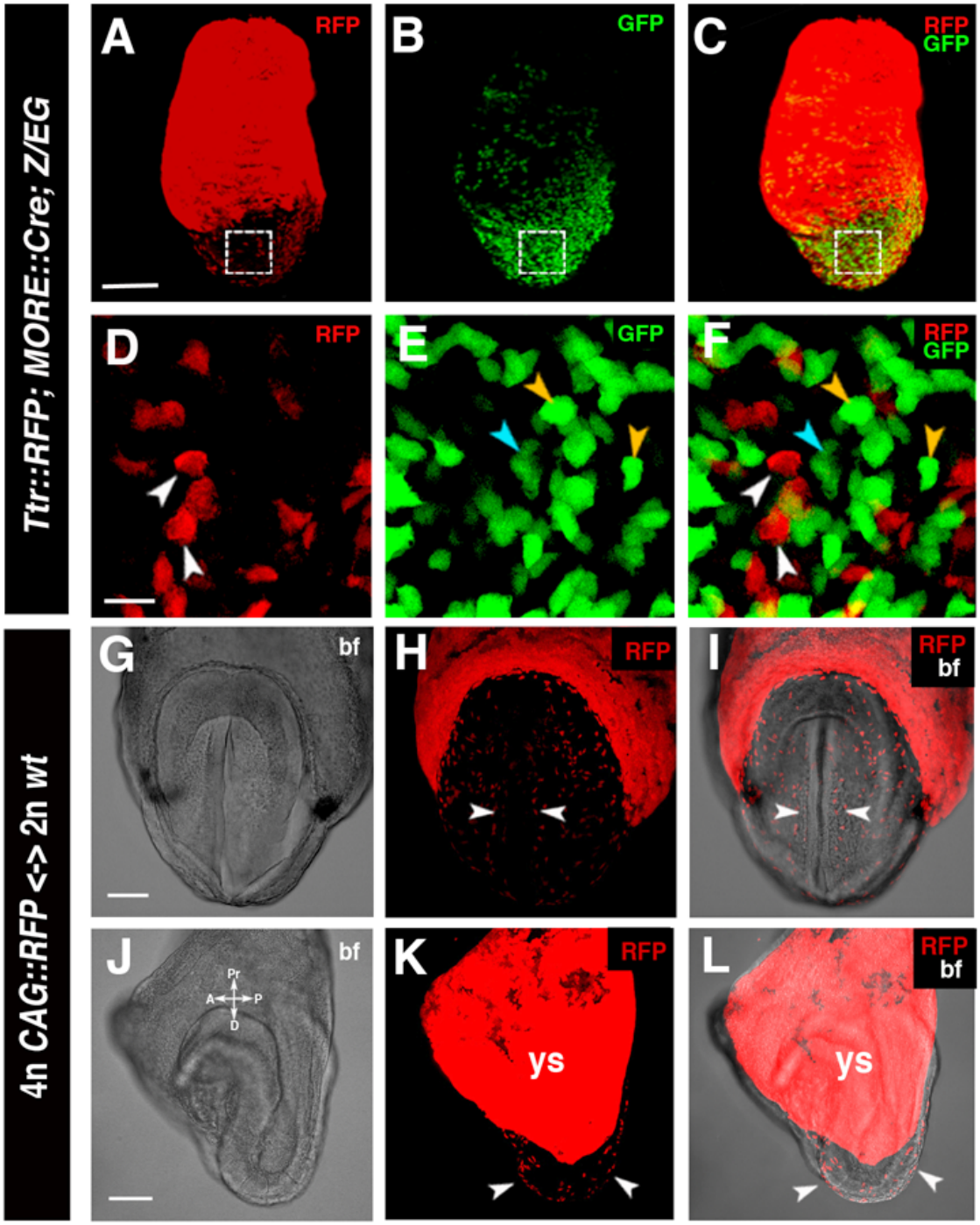


Kwon et al., Figure S1

Figure S1. Rapid Downregulation of Visceral Endoderm Markers in Visceral Endoderm-Derived Cells Associated with the Epiblast

Localization of *Afp* mRNA within the visceral endoderm overlying the extraembryonic ectoderm as well as speckled mRNA expression within the visceral endoderm overlying the epiblast in an early bud stage (EB) embryo (black arrowheads, A). Data in panels B and D are depict 3D reconstructions of confocal z-stacks acquired from wholemount embryos: darkfield fluorescence channel (left panel) and fluorescence channel overlayed on brightfield image (right panel). At pre-streak stages (E5.75) Hnf4α is present throughout the proximo-distal length of the embryo in cells of the visceral endoderm (B and C). Localization of protein (red fluorescence, B), and expression of mRNA (blue chromogenic staining, C) in stage-matched embryos. By the early bud stage (E7.5) Hnf4α marks a more proximal location within the embryo, where it is present within the visceral endoderm overlying the extraembryonic ectoderm (D and E). Localization of protein (red fluorescence, D), and expression of mRNA (blue chromogenic staining, E) in stage-matched embryos. Pre-streak stage (F-K), mid-streak stage (L-Q) and early bud stage (R-W) *Afp::GFP*$^{Tg/+}$ embryos visualized for GFP (green), and counter-stained for Hnf4α protein (red) and Hoechst (labeling nuclei - blue). Single saggital (2D) optical sections with three-channel merge (F, L and R), and single channels depicting visceral

endoderm-specific nuclear Hnf4α staining (G, M, and S) as well as visceral endoderm-localized GFP (H, N and T) in *Afp::GFP*$^{Tg/+}$ embryos. 3D reconstructions of z-stack taken through the lateral half of each embryo (acquired from the surface through to the mid-axial level) with three-channel merge (I, O and U). 3D reconstructions depicting single channel nuclear-localized Hnf4α (J, P and V) and GFP localization (K, Q and W) in *Afp::GFP*$^{Tg/+}$ embryos. 2D, 2-dimensions = single xy slice from a z-stack; 3D, 3-dimensions = 3D reconstruction of a z-stack. Red dots correspond to the position of the amnion, the morphological landmark of the boundary between the extraembryonic and embryonic part of the conceptus. Scale bars = 50 μm in B and I; 100 μm in O; 200 μm in D and U.



Kwon et al., Figure S2

Figure S2. Scattered Cells on the Surface of the Embryo Overlying the Epiblast Are Not of Epiblast Origin

3D reconstructions of *z*-stacks taken through a headfold stage (E7.5) *MORE2::Cre*$^{Tg/+}$; *Z/EG*$^{Tg/+}$; *Ttr::RFP*$^{Tg/+}$ embryo (A-C). High magnification views of boxed region depict individual RFP +ve cells derived from the visceral endoderm in close apposition to, but not overlapping with epiblast-derived GFP^{+} cells (D-F). RFP^{+} cells located on the surface of the embryo (white arrowheads); subpopulation of GFP^{+} cells located on the surface of the embryo (orange arrowheads); subpopulation of GFP^{+} cells that are not superficially located (blue arrowheads).

(G-L) LHF and ESom stage 4n *CAG::RFP*$^{Tg/+}$ <-> 2n R1 ES cell chimeras. Brightfield image (G and J), 3D reconstruction of z-stack of mages acquired in red fluorescent channel (H and K), and overlay (I and L). Dispersed population of 4n RFP^{+} cells organized around the anterior midline (white arrowheads; H and I) and overlying the epiblast (white arrowheads; K and L). Scale bars = 200 µm in A and J; 100 µm in G; 20 µm in D.

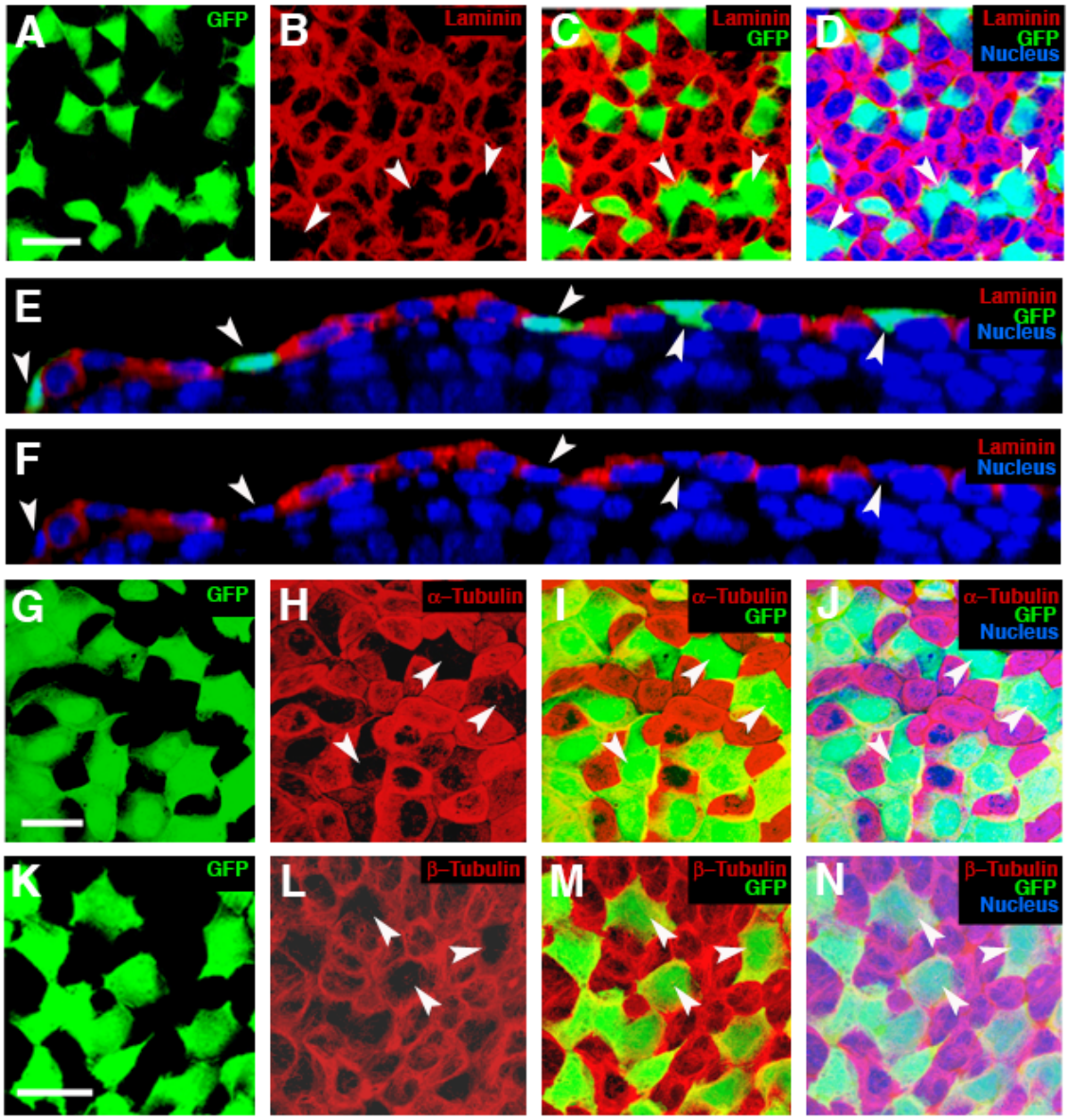


Figure S3. Differences in the Extracellular Matrix, Cytoskeletal and Signaling Protein Distinguish Visceral Endoderm-Derived Cells from Neighboring Epiblast-Derived Cells Occupying the Outer Layer of the Embryo

3D reconstructions of high magnification z-stacks taken through the region of the epiblast of early bud (E7.5) stage *Afp::GFP*$^{Tg/+}$ embryos visualized for GFP (green), counterstained for Hoechst (nuclei – blue) and laminin (A–D and E–F are orthogonal sections), α-tubulin (G-J) and β-tubulin (K-N). Data are depicted as single and merged channels. White arrowheads highlight visceral endoderm-derived cells exhibiting reduced levels of protein as compared to their neighbors. Scale bars = 50 μm.

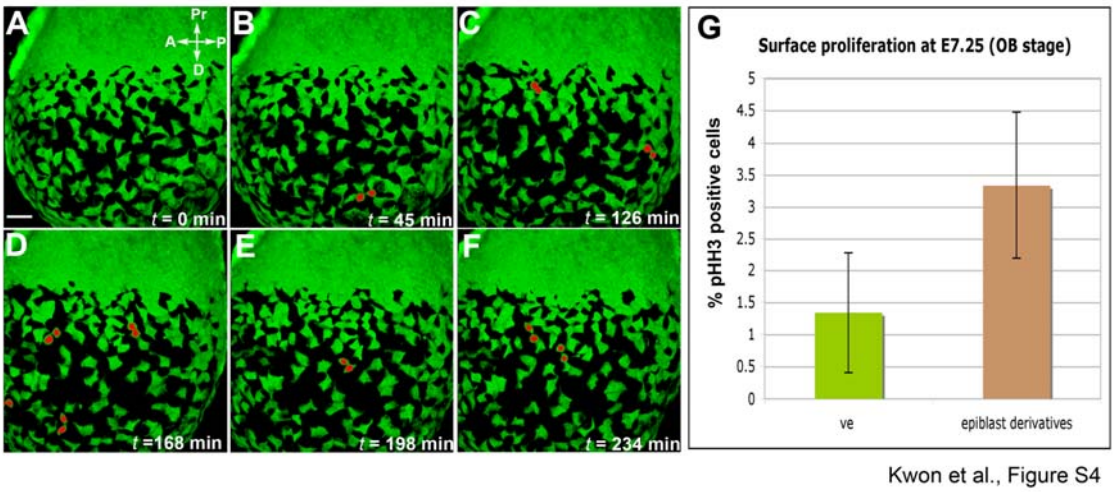


Figure S4. Scattered Visceral Endoderm Derived Cells Overlying the Epiblast Continue to Proliferate

Still images from 3D time-lapse movie (Movie S10) of *Afp::GFP*$^{Tg/+}$ embryo with dividing GFP^{+} cells overlying the epiblast labeled in red (A-F). Scale bar = 50 μm. Quantitation of mitotic cells by anti-pHH3 stain (data not shown) overlying the epiblast at E7.5 (OB stage) shows a two-fold proliferation of GFP-negative cells (G). N=7.

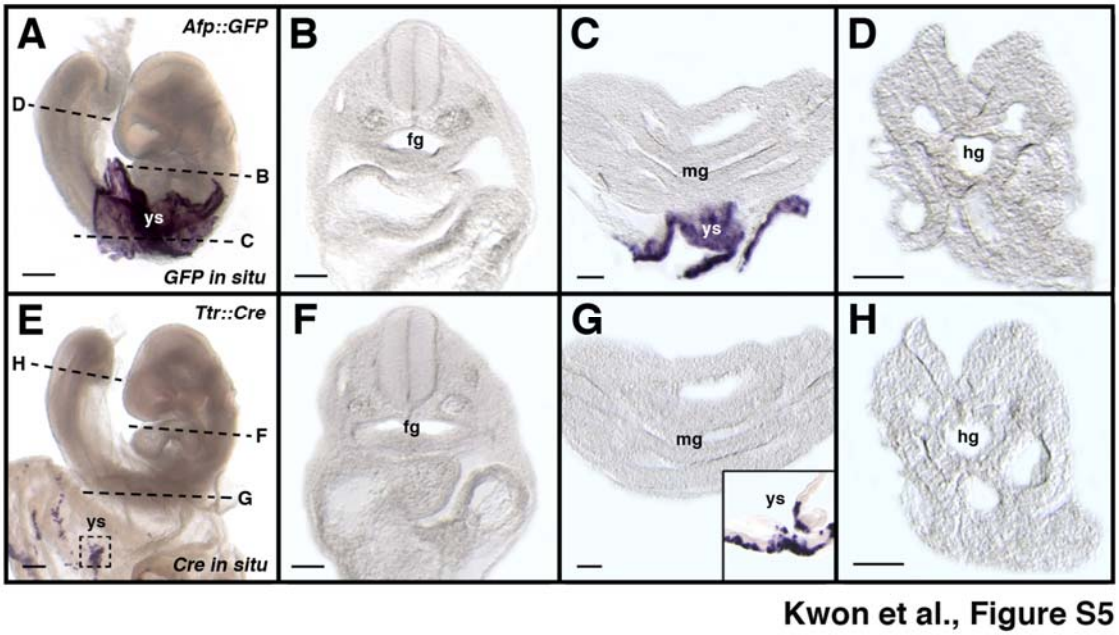


Figure S5. Reporter Expression in the Embryonic Gut Does Not Correspond to De Novo Activation of Transgenes

(A) Wholemount view of a 16 somite stage (E9.0) *Afp::GFP*$^{Tg/+}$ embryo processed for *GFP* mRNA *in situ* hybridization.

(B-D) Transverse sections at different rostrocaudal levels (dashed lines) though the embryo in A. *GFP* transcripts were detected in the yolk sac (blue chromogenic stain, C), but not in the fore- (B), mid- (C) and hindgut (D) regions.

(E) Wholemount view of a 16 somite stage (E9.0) *Ttr::Cre*$^{Tg/+}$ embryo processed for *Cre* mRNA *in situ* hybridization.
(F-H) Transverse sections at different rostrocaudal levels (indicated by dashed lines) though the embryo in E. *Cre* transcripts were detected in the yolk sac (blue chromogenic stain in inset, G), but not in the fore- (F), mid- (G) and hindgut (H) regions. ys, yolk sac; fg, foregut; mg, midgut; hg, hindgut. Scale bars = 200 µm in A and E; 100 µm in B-D and F-H.

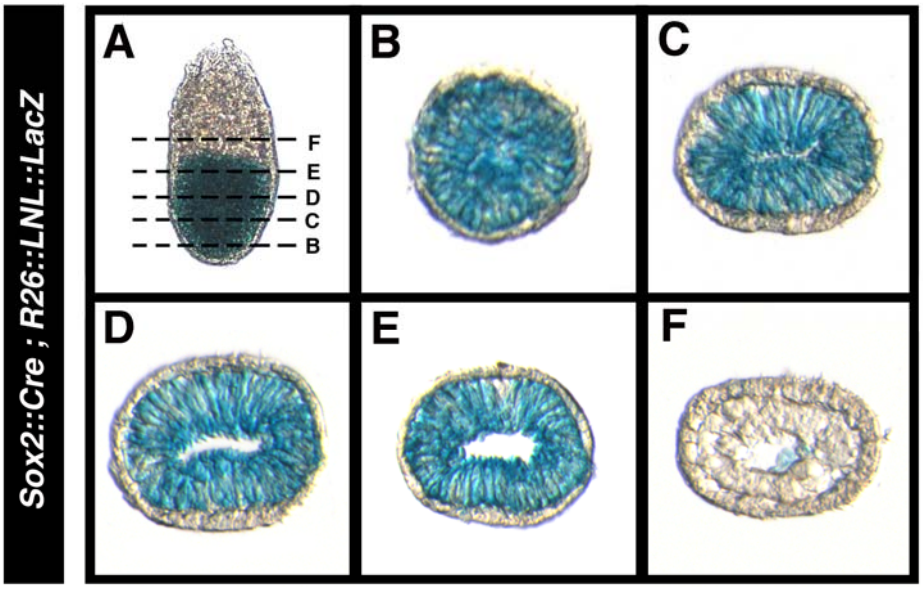


Figure S6. The Visceral Endoderm Is Not Colonized by Direct Egression of Epiblast Cells in the PS-ES Embryos
Wholemount view of an early streak stage (E6.0) *Sox2::Cre*$^{Tg/+}$ *; R26::LNL::LacZ*$^{+/-}$ embryo stained for LacZ (A). Transverse sections taken at sequential rostrocaudal levels through the embryo (indicated by dashed lines in A), showing LacZ^{+} cells within the epiblast and LacZ^{-} cells comprising the visceral endoderm (B-F).

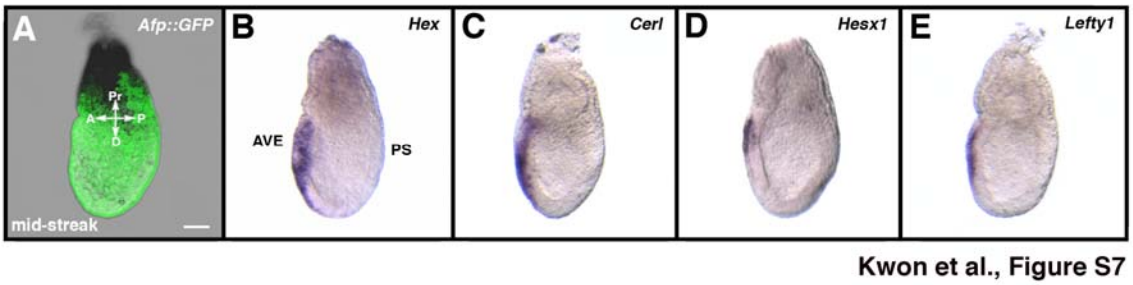


Figure S7. Migration of the AVE Precedes Dispersal of the Visceral Endoderm Overlying the Epiblast
3D reconstruction of z-stacks taken through a mid-streak (E6.5) stage *Afp::GFP*$^{Tg/+}$ embryo visualized for GFP (green), and overlayed on brightfield (A). *In situ* hybridizations on stage-matched embryos with *Hex* (B), *Cerl* (C), *Hesx1* (D) and *Lefty1* (E), markers of the anterior visceral endoderm (AVE), which has already migrated anteriorly at this stage. Scale bar = 50 µm.