Supplemental Materials & Methods

Fly strains and genetics

The \textit{HLH54F}\textgreater H2A-mCherry reporter (“HC3”) was described in a previous study (Kadam et al., 2012). The \textit{HLH54}\textgreater GAP-Venus (“GV2”) reporter was created in the course of this study (see below). Wild type refers to yw or yw; GV2 or yw; HC3 fly stocks, as indicated. 5053>GAL4 (#2702), UAS-\textit{p35} (#5072), \textit{wun2TRiP} (#32423), and \textit{w^1118}; Df(2R)Exel7098/CyO (#7864) fly stocks were obtained from the Bloomington \textit{Drosophila} Stock Center (BDSC). Additional RNAi lines were obtained from the Vienna \textit{Drosophila} Resource Center (VDRC): \textit{wun2} (#103452), \textit{wun} (#6446), and \textit{wun} (#51090). The following stocks were obtained from published sources: \textit{wunen^GL}/CyO and \textit{wunen^{CE} vg cn prl}/SM3 (Zhang et al., 1996), \textit{faf}-LacZ; FRT \textit{tud^B45}/CyO, hs-hid \textit{faf}-LacZ; FRT \textit{tud^B42}/CyO, hs-hid (Arkov et al., 2006) and \textit{tud} Df(PurP133)/SM1 (Tearle and Nusslein-Volhard, 1987; Wang and Lehmann, 1991), \textit{Trel^{AEF3}} and \textit{Trel^{AEPS}} (Dahanukar et al., 2001; Ueno et al., 2001), \textit{osk^{301}/TM3} and \textit{osk^{CE4}/TM3} (Lehmann and Nusslein-Volhard, 1986), G447>GAL4 (Georgias et al., 1997), \textit{nos} >GAL4.VP16 (Van Doren et al., 1998), \textit{vasa} >GFP (Nakamura et al., 2001), \textit{gcl^A}/CyO (Robertson et al., 1999), and \textit{HLH54^A598}/CyO (Ismat et al., 2010).

To each mutant stock and Gal4 driver stock, either GV2 or HC3 was introduced so that the CVM cells could be followed with anti-GFP (Venus) or anti-RFP (mCherry) staining, respectively. To identify mutant embryos from heterozygotes, the balancer chromosomes were switched to \textit{Gla}/CyO, \textit{twi-GAL4, UAS-GFP} (BDSC#6662) or \textit{Sb, Ser/TM3, twi-GAL4, UAS-GFP} (BDSC#6663).

Females containing \textit{wun wun2} germline clones were made as previously described (Renault et al., 2010). In brief, hsFLP12, \textit{yw; Sco/CyO} (BDSC#1929) virgins were crossed to FRTG13 \textit{ovo^D}/CyO (BDSC#2125) males. Males from this cross of the genotype hsFLP, \textit{yw}; FRTG13 \textit{ovo^D}/CyO were crossed to virgin females of the genotype \textit{w}; FRT42B \textit{wun^{49} wun2^EP2650\textit{ex34}/CyO f\textit{tz}+lacZ} (Renault et al., 2010). Embryos collected from this pairing were heat shocked for 2 hours at 37°C on day 3 and day 5 after egg lay. Germline clone virgin females were collected and crossed to \textit{wun^GL}, \textit{GV2/CyO, twi-GAL4, UAS-2xEGFP} males.
Ectopic expression of \textit{wun2} in \textit{wun} \textit{wun2} zygotic mutants was accomplished by crossing UAS-\textit{wun2}-myc, \textit{wun}^{CE}/CyO, \textit{ftz}>\textit{LacZ}; HC3 to \textit{wun}^{GL}, \textit{en}>GAL4/ CyO, \textit{ftz}>\textit{LacZ} (Mukherjee et al., 2013). The HC3 reporter was crossed into the UAS-\textit{wun2}-myc, \textit{wun}^{CE}/CyO line using standard genetic crosses.

\textit{gcl}^{Δ}/Df(2R)Exel7098 maternal mutants were generated by crossing the balanced stock to \textit{w}^{1118}; Df(2R)Exel7098, and identifying the hemizygotes in the progeny. Similarly, \textit{tud}^{B45} and \textit{tud} Df(Pu^{P133}) balanced stocks were crossed to generate \textit{tud}/Df hemizygotes. \textit{osk}^{301}/\textit{osk}^{CE4} transheterozygotes were generated by an analogous scheme. For maternal \textit{osk} mutants, the females were raised and kept at 18°C for the experiment, as \textit{osk}^{301} (also known as \textit{osk}^{8}) is temperature sensitive with loss of PGCs and normal segmentation at 18°C, but abdominal segmentation phenotypes at 25°C (Ephrussi et al., 1991; Hay et al., 1990; Kim-Ha et al., 1991). These maternal mutants were then crossed to the GV2 reporter to generate embryos for fixed analysis, or the HC3 reporter for live analysis.

\textit{Molecular cloning of CVM membrane marker and generation of transgenic lines}

The GV2 reporter was constructed by modifying the p-attB vector (Bischof et al., 2007). An ~1.6kb enhancer from the \textit{HLH54F} locus was placed 5’ of the \textit{even-skipped} minimal promoter as described previously (Kadam et al., 2012) followed by a sequence coding the dual palmitoylation sequence (MLCCMRRRTKQVEKNDEDQKI) from the growth associated protein 43 (GAP43) (McCabe and Berthiaume, 1999) fused in frame to Venus YFP. Site-directed transgenesis was used to create transgenic lines on the second chromosome using flies carrying the 51C attP landing site (BDSC#24482).

\textit{Fixation, in situ hybridization, and antibody staining}

Embryos were fixed and stained using standard protocols (Frasch, 1995; Jiang et al., 1991; Kosman et al., 2004). The following antibodies were used: rabbit anti-Green Fluorescent Protein (1:1000, Life Technologies A11122, lot 1753594), goat anti-GFP (1:5000, Rockland 600-101-215, lot 25297), rabbit anti-RFP (1:1000, MBL PM005, lot 044), mouse anti-Fas3 (1:200, DSHB 7G10), rat anti-Vasa (1:200, DSHB), rabbit anti-Vasa (1:1000, Santa Cruz D-260), mouse anti-Myc (1:200, Santa Cruz SC-40, lot
G2708), guinea pig anti-Hb9 (Broihier and Skeath, 2002). All fluorescent secondary antibodies used were from Life Technologies (1:400). DAB staining was performed using standard procedures and the VECTASTAIN Elite ABC kit (Vector Laboratories) followed by detection with DAB Peroxidase Substrate Kit (Vector Laboratories). Fluorescent images were obtained with a LSM5 Pascal confocal microscope (Carl Zeiss).

For cross-sections, stained embryos were embedded in acetone-araldite (Electron Microscopy Science) and blocks were allowed to harden at 65°C overnight. Sections at 8 µm were obtained by using a microtome (LKB Bromna 2218 Historange) and mounted in 1:1 acetone:araldite solution.

Larval guts were dissected from 3rd instar larvae in ice cold Phosphate Buffered Saline (PBS) and then fixed immediately for 20 minutes in 4% formaldehyde in PBS at room temperature. The guts were washed with PBST (PBS plus 0.1% Tween-80) and then permeabilized with 1% Triton-X 100 in PBS for 10 minutes. After washing again in PBST, Phalloidin staining was performed according to manufacturer’s instructions using ActinRed 555 ReadyProbes reagent (Molecular Probes R37112). Guts were briefly washed in PBST and mounted with Vectashield.

**Live imaging and image processing**

In short, embryos were collected for 30 minutes and aged for ~5.0-5.5 hours in a humidified 24°C incubator to reach stage 10. These staged embryos were hand dechorionated and placed on a coverslip coated with dried heptane-glue mixture. ddH2O was added on the embryos, which were subsequently imaged under a 40x water lens using a Zeiss LSM5 Pascal confocal microscope. Time-lapse imaging was set to scan every 3 minutes and there were 6-10 z-slices at each time point. The scan speed and pixel averaging was adjusted to complete scanning about one minute at each time point in order to minimize phototoxicity. We confirmed that wild type embryos hatch after live imaging using this setting. Mutant embryos were identified after embryo mounting but before the imaging session using either CyO, twi>Gal4, UAS-2xEGFP, or TM3, twi>Gal4, UAS-2xEGFP, Sb Ser balancers based on the lack of GFP expression in the trunk mesoderm at stage 10.
The time-course of images collected by LSM software was handled using ImageJ, where linear adjustments such as brightness/contrast and levels were made. Movie files and temporal-color coded projections were generated using ImageJ after adding the appropriate time stamps. Other confocal images shown are z-projections of confocal slices (maximum pixel) and generated using the LSM software. Imaris 7.2.1 (Bitplane) was used to create 3D projections models used for Tre1 analysis.

CVM reporter lines used
To further understand the relative movements of CVM cells, we created a CVM-specific membrane reporter to visualize these cells’ migration and morphology as no antibodies specific to this cell type are available. For a previous study, we used an enhancer sequence, shown to support expression of the *hlh54f* gene within CVM cells, to drive expression of a histone H2A fusion to the mCherry fluorescent protein (*HLH54F*>H2A-mCherry on the 3rd chromosome) (Kadam et al., 2012); this reporter of CVM cells is referred to as “HC3”. This construct supports expression predominantly within CVM cells, that exhibit directed anterior movement, but also within another cell type (possibly malphigian tubule precursor cells) that do not migrate in a directed fashion but remain located at the posterior (Kadam et al., 2012). Similarly, here we created another CVM-reporter; one tailored to monitor cell shape through expression of a membrane-tethered protein (i.e. *HLH54F*>Gap-Venus on the 2nd chromosome) referred to as “GV2”.
SUPPLEMENTARY INFORMATION

Figure S1. CVM migration defects in later stage zygotic and maternal/zygotic wun wun2 mutant embryos

(A-F) Lateral view of embryos fluorescently stained with α-GFP (GV2 reporter to identify CVM) and α-Vasa (PGCs).

(A-C) Stage 12 embryos showing (A) wild-type migration of CVM and PGCs compared to (B) a M+Z- wun wun2 mutant where PGCs are mislocalized, clustering largely near the pmg, while the CVM is similarly disorganized in this region, and (C) a M-Z- wun wun2 mutant where all PGCs have died by this stage and CVM cells show a loss of organization, but continue to migrate towards the anterior.

(D-F) Stage 13 embryos showing wild-type (D) CVM spreading along the gut with defined anterior to posterior polarity, and localization of PGCs to the presumptive gonadal mesoderm. (E) M+Z- wun wun2 showing disorganization and crossing over of
the CVM, as well as fewer PGCs making it to the presumptive gonadal mesoderm. (F) M-Z- wun wun2 embryos lack PGCs and show lack of organization of CVM. (G,H) Lateral view of stage 13 embryo stained for RFP (CVM nuclei, green), GFP (CVM cell bodies, red) and FasIII (TVM, cyan). Wild-type embryos show a close association of CVM cells to the TVM (G,G’) while in the M+Z- wun wun2 mutant many of the CVM cells are detached from the TVM (H,H’).
**Figure S2. Comparison of CVM migration defects with and without ectopic expression of wun2 in the en domain**

(A-G) Fluorescent antibody staining of embryos for CVM (HC3, α-RFP) and PGCs (α-Vasa). In D-G α-Myc antibody was used in addition to identify ectopic expression of UAS-wun2-myc. Embryos in A-G are oriented laterally.

(A,D) CVM migration appears similar at stage 12 with or without ectopic Wun2 expression.

(B,E) The CVM completes its migration to the anterior of the embryo in stage 13 with (E) or without (B) the ectopic stripes of Wun2 expression, although there are fewer PGCs and fewer CVM cells at stage 13 when Wun2 is ectopically expressed (compare B to E).

(C,F) At later stages in wun wun2 zygotic mutations with ectopic expression of Wun2 in the en domain, the CVM phenotypes are similar to the wun wun2 zygotic phenotypes.

(G,G’G’’) Single Z slices from stage 11 embryo. Aberrantly migrating CVM can be seen near PGCs, but in areas free of PGCs the CVM migrates in a straight course around the posterior of the embryo.
Figure S3. Knockdown of wun2 in either the pmg or CVM does not affect migration of CVM cells

(A) Graphic depiction of expression regions for the GAL4 drivers used in this study at stage 10 and stage 11. G447>GAL4 and 5053>GAL4 are expressed in the CVM. nos>GAL4.VP16 is expressed in the PGCs, and 48Y>GAL4 is expressed in the pmg.

(B-I) Fluorescent antibody staining of CVM (HC3, α-RFP) and PGCs (α-Vasa) at stage 11 early, dorsal view (B,D,F,H), and stage 11 late, dorsal view (C,E,G,I). UAS-wun2TRiP driven by GAL4 in the CVM (D-G) or the pmg (B,C) showed no change from wild-type migration patterns. Likewise, p35 alone driven by nos>GAL4.VP16 (H,I) does not affect CVM migration.
Figure S4. Knockdown of *wun2* in the germ cells causes cell death which can be rescued by co-expression of p35

PGC numbers were counted in fixed whole mount embryos of stage 13-15 stained using Vasa antibody and detected by DAB staining. Number of embryos counted for each experimental class is indicated next to the label within parentheses, error bars represent the standard deviation. A two-tailed t-test was used to compare each of the experimental classes to wild-type (i.e. *yw*). Experimental classes found to be significantly different from wild-type were marked with *** (P<0.0001)
Figure S5. *tre1* zygotic expression does not rescue the maternal PGC and CVM mutant phenotypes

(A-C) Lateral view of *tre1* M-Z+ embryos stained for CVM (GV2, GFP), PGCs (Vasa) and pmg (Hb9). In the maternal *tre1* mutant the PGCs are unable to migrate out of the pmg and the CVM can be seen entering the pmg at stage 11 (A,A’). At stage 13 the CVM fails to migrate all the way to the anterior of the forming midgut (B). Finally, at stage 15 the CVM fails to fully ensheath the midgut (C). Thirty-six of the forty embryos examined at stage 15 had a mutant phenotype similar to or more severe than the one pictured in C.
**Figure S6. Range of CVM defects seen in germ cell-less mutants**

(A) The number of CVM cells entering the midline in the indicated genotypes. Although the means were just outside of significance (p = 0.528 by student’s t-test), the presence vs. absence of CVM cells in the midline was significant by Fisher’s exact test (p = 0.0202; yw = 21%, n=28 vs gclΔ/Df = 55%, n = 22).

(B) The synchrony of the front edge of CVM clusters in each embryo in stage 11 embryos was determined as in (I) for CVM in A5 to A3.5 (i.e. before they make a turn towards the anterior of the embryo). Synchrony was defined as the difference in abdominal segment reached by the two clusters in each embryo, and was indistinguishable between genotypes. Mean and standard deviation are shown. For yw, n = 28 embryos, and for gclΔ/Df , n = 22 embryos.

(C) The mean lengths of CVM clusters are the same in embryos derived from yw and gclΔ/Df females. Cluster length was determined by measuring the abdominal segment reached by the front of the cluster, to the last portion of the main cluster, defined as a contiguous group of CVM cells without significant gaps (>1-2 cells lengths) between adjacent cells, or other recognizable disruption in the cluster. Trailing CVM cells were not considered part of the main cluster. Segmental boundaries were determined by the
tracheal pits. The ventromedial grooves of the parasegments were used if the tracheal pits were not visible. Embryos were examined in two time intervals; mid-to-late stage 11, (front of the CVM had advanced between A5 and A3.5), and through late stage 11 (CVM in A3.5 to A1.5), but in the absence of germ band retraction. Exact determination of position within A3 (i.e. the “turn”) was not always possible, and a best estimation was made. Individual embryos values are shown on the graph, along with mean cluster length value (widest bar, with error bars showing standard deviation. n=56 CVM clusters (28 embryos) for yw at mid stage 11, n=44 clusters (22 embryos) for gcl^{5}/Df at mid stage 11, n =46 clusters (23 embryos) for yw at late stage 11 to stage 12, and n=38 clusters (19 embryos) for gcl^{5}/Df at mid stage 11 to stage 12.

(D-K) Embryos lacking germ cells from gcl^{5}/Df, tud^{B45}/Df, and osk^{301}/osk^{CE4} maternal mutants show various defects in CVM migration. Like those from gcl^{5}/Df maternal mutants, embryos from tud^{B45}/Df maternal mutants show abnormal consolidation of the CVM clusters in stage 11 (F), which occur in embryos from gcl^{5}/Df maternal mutants even when germ cells are present, albeit at a greatly reduced number (E, compare to Figure 5B). Other defects include: asynchrony at stage 11 (G), disorganized CVM at stage 13 (I, hashed line) and cell death outside of the main row of CVM in stage 13 (arrowheads, J,K). All embryos were from the indicated maternal mutants crossed to GV2 males, followed by immunostaining for GFP (D-K) and also Vasa (D-F, H-J).
Supplemental Movies

Movie 1. Co-migration of CVM cells and PGCs
Embryo expressing the HC3 reporter and vasa-GFP imaged live from the beginning of CVM migration, through germ band retraction. Time is displayed as hours: minutes. At the beginning, the migrating CVM cells have just begun to separate into two bilateral clusters and move forward while the germ cells are emerging from the pmg. By 1.5 hours, the CVM start to overtake the germ cells. At 50 minutes, a germ cell can be seen squeezing between CVM cells, as the germ cells are moving laterally. About half of the CVM cells have passed the “turn” when germ band retraction begins at ~3 hr. At this time the germ cells completely trail the CVM, and are present as two distinct bilateral groups that move toward the somatic gonadal precursors. Genotype of embryo is homozygous for the HC3, vasa-GFP reporter chromosome, and is in a wild-type background. Contrast was optimized with ImageJ to better visualize each cell population. First movie is two color showing CVM and PGCs; second movie is the same embryos showing CVM cell migration only for comparison.
Movie 2. CVM migration in a zygotic *wun wun2* mutant embryo is slow and disorganized

Two *wun wun2* zygotic mutant embryos (*wun^{GL}/wun^{CE}*) expressing the HC3 reporter imaged from the beginning of CVM migration, until the onset of germ band retraction. CVM cells migrate anteriorly, but show defective behavior including movement towards the midline. Overall, many cells in each cluster behave erratically, and seemingly “tumble” rather than displaying continuous anteriorly directed movement. The interval of acquisition is shorter than the other movies displayed, and the relative time scale was corrected to match all other movies shown.
Movie 3. CVM cell cluster movement in three wild-type embryos
Compilation of movies of three yw embryos crossed to males bearing the HC3 reporter to visualize CVM in the progeny. Clusters appear almost aerated with space between cells at times.
Movie 4. CVM cell cluster movement in gcl^Δ/Df mutant embryos is more compact

Compilation of movies of three embryos from maternal gcl^Δ/Df maternal mutants crossed to males bearing the HC3 reporter to visualize CVM in the progeny. Clusters appear more compact.