Toddler: An Embryonic Signal That Promotes Cell Movement via Apelin Receptors

Andrea Pauli,* Megan L. Norris, Eivind Valen, Guo-Liang Chew, James A. Gagnon, Steven Zimmerman, Andrew Mitchell, Jiao Ma, Julien Dubrulle, Deepak Reyon, Shengdar Q. Tsai, J. Keith Joung, Alan Saghatelian, Alexander F. Schier*

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Introduction: Embryogenesis is thought to be directed by a small number of signaling pathways with most if not all embryonic signals having been identified. However, the molecular control of some embryonic processes is still poorly understood. For example, it is unclear how cell migration is regulated during gastrulation, when mesodermal and endodermal germ layers form. The goal of our study was to identify and characterize previously unrecognized signals that regulate embryogenesis.

Methods: To identify uncharacterized signaling molecules, we mined zebrafish genomic data sets for previously non-annotated translated open reading frames (ORFs). One such ORF encoded a putative signaling protein that we call Toddler (also known as Apela/Elabela/Ende). We analyzed expression, production, and secretion of Toddler using RNA in situ hybridization, mass spectrometry, and Toddler–GFP fusion proteins, respectively. We used transcription activator-like effector (TALE) nucleases to generate frame-shift mutations in the *toddler* gene. To complement loss-of-function analyses with gain-of-function studies, Toddler was misexpressed through mRNA or peptide injection. We characterized phenotypes using marker gene expression analysis and in vivo imaging, using confocal and lightsheet microscopy. *Toddler* mutants were rescued through global or localized *toddler* production. The relationship between Toddler and APJ/Apelin receptors was studied through genetic interaction and receptor internalization experiments.

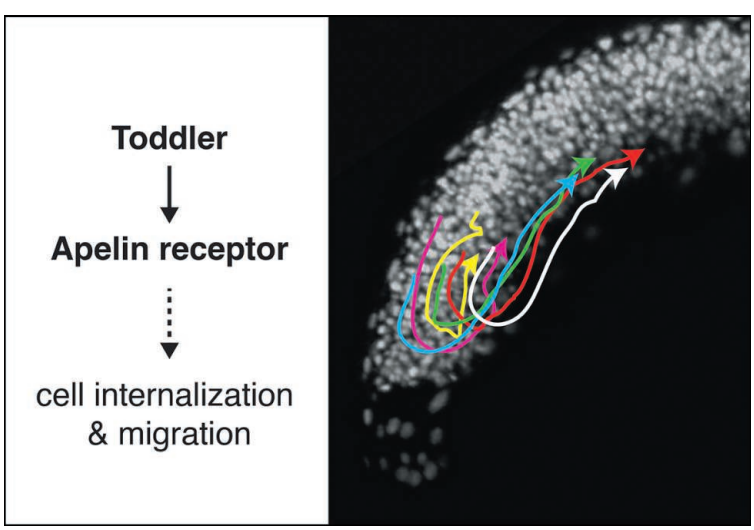
Results: We identified several hundred non-annotated candidate proteins, including more than 20 putative signaling proteins. We focused on the functional importance of the short, conserved, and secreted peptide Toddler. Loss or overproduction of Toddler reduced cell movements during zebrafish gastrulation; mesodermal and endodermal cells were slow to internalize and migrate. Both the local and ubiquitous expression of Toddler were able to rescue gastrulation movements in *toddler* mutants, suggesting that Toddler acts as a motogen, a signal that promotes cell migration. Toddler activates G-protein–coupled APJ/Apelin receptor signaling, as evidenced by Toddler-induced internalization of APJ/Apelin receptors and rescue of *toddler* mutants through expression of the known receptor agonist Apelin.

Discussion: These findings indicate that Toddler promotes cell movement during zebrafish gastrulation by activation of APJ/Apelin receptor signaling. Toddler does not seem to act as a chemo-attractant or -repellent, but rather as a global signal that promotes the movement of mesendodermal cells. Both loss and overproduction of Toddler reduce cell movement, revealing that Toddler levels need to be tightly regulated during gastrulation. The discovery of Toddler helps explain previous genetic studies that found a broader requirement for APJ/Apelin receptors than for Apelin. We propose that in these cases, Toddler—not Apelin—activates APJ/Apelin receptor signaling. Our genomics analysis identifying a large number of candidate proteins that function during embryogenesis suggests the existence of other previously uncharacterized embryonic signals. Applying similar genomic approaches to adult tissues might identify additional signals that regulate physiological and behavioral processes.

FIGURES IN THE FULL ARTICLE

SUPPLEMENTARY MATERIALS

Materials and Methods
Figs. S1 to S19
Full Reference List
Table S1
Data files S1 and S2



Toddler promotes gastrulation movements via Apelin receptor signaling. Toddler is an essential, short, conserved embryonic signal that promotes cell migration during zebrafish gastrulation. The internalization movement highlighted by the colored cell tracks requires Toddler signaling. Toddler signals via the G-protein–coupled APJ/Apelin receptor and may be one of several uncharacterized embryonic signals.

The list of author affiliations is available in the full article online.
*Corresponding author. E-mail: pauli@fas.harvard.edu (A.P.); schier@fas.harvard.edu (A.F.S.)

Toddler: An Embryonic Signal That Promotes Cell Movement via Apelin Receptors

Andrea Pauli,[1]* Megan L. Norris,[1]† Eivind Valen,[1]† Guo-Liang Chew,[1] James A. Gagnon,[1] Steven Zimmerman,[1] Andrew Mitchell,[2] Jiao Ma,[2] Julien Dubrulle,[1] Deepak Reyon,[3,4] Shengdar Q. Tsai,[3,4] J. Keith Joung,[3,4,5] Alan Saghatelian,[2] Alexander F. Schier[1,5,6,7,8]*

It has been assumed that most, if not all, signals regulating early development have been identified. Contrary to this expectation, we identified 28 candidate signaling proteins expressed during zebrafish embryogenesis, including Toddler, a short, conserved, and secreted peptide. Both absence and overproduction of Toddler reduce the movement of mesendodermal cells during zebrafish gastrulation. Local and ubiquitous production of Toddler promote cell movement, suggesting that Toddler is neither an attractant nor a repellent but acts globally as a motogen. Toddler drives internalization of G protein–coupled APJ/Apelin receptors, and activation of APJ/Apelin signaling rescues *toddler* mutants. These results indicate that Toddler is an activator of APJ/Apelin receptor signaling, promotes gastrulation movements, and might be the first in a series of uncharacterized developmental signals.

Many of the inductive events during early development are directed by a small number of signaling pathways whose agonists have been known for more than a decade (*1*). Therefore, it has been assumed that most, if not all, embryonic signals have been identified. However, the molecular control of some embryonic processes is still poorly understood. For example, it is largely unclear how cell migration is regulated during gastrulation or how cells coalesce into discrete tissues during organogenesis (*2*–*5*), suggesting that some of the involved signals are yet to be identified. Moreover, recent genomic studies have suggested that translation of short open reading frames (ORFs) and the generation of small peptides are much more pervasive than previously assumed (*6*, *7*). To search for new candidate signaling molecules, we used the Translated ORF Classifier (TOC) (*7*) to examine zebrafish transcript annotations and ribosome profiling data sets (*7*–*9*) for non-annotated translated ORFs (Fig. 1A) (materials and methods in the supplementary materials). This analysis identified 700 novel protein-coding transcripts (399 loci) (supplementary data files S1 and S2), of which 81% (562 transcripts in 325 loci) shared nucleotide sequence alignments with other vertebrates (table S1). Notably, this approach identified 28 candidate signaling proteins (40 transcript isoforms) characterized by the presence of putative signal sequences and lack of predicted transmembrane domains (table S1). Ribosome profiling and phylogenetic analysis suggest that these RNAs can generate secreted peptides with lengths ranging from 32 to 556 amino acids (Fig. 1A, fig. S1, and table S1). Although these genes have not been identified previously or are annotated in the zebrafish Ensembl database as noncoding RNAs, the majority (24 of 28) appear to be conserved in other vertebrates (fig. S1 and table S1).

Toddler Encodes a Short, Conserved, and Secreted Peptide

To test the functional potential of these candidate signals, we focused on a gene that we named *toddler* on the basis of the phenotype described below (Fig. 1B). *Toddler* (*tdl*) mRNA is expressed ubiquitously during late blastula and gastrula stages and becomes restricted to the lateral mesoderm, endoderm, and anterior and posterior notochord after gastrulation (Fig. 1C). *Toddler* is annotated as a noncoding RNA in zebrafish (*ENSDARG00000094729*), mouse [*Gm10664*; also called *Ende* (*10*)], and human (*LOC100506013*) (fig. S2) and is present in two lncRNA catalogs (*9*, *11*); however, it contains a 58–amino acid ORF with a predicted signal sequence and high conservation in vertebrates, including human (Fig. 1D and fig. S3). Sequence comparisons with the highly conserved C-terminal portion did not identify homology to any other known proteins, raising the possibility that this gene encodes an uncharacterized embryonic signal.

Six lines of evidence indicate that *toddler* is translated and encodes a secreted peptide. First, phylogenetic comparisons of synonymous versus nonsynonymous codon changes reveal strong amino acid preservation in the *toddler* ORF (PhyloCSF score of 98 (*8*); see Fig. 1, B and D, and table S1). Second, previous ribosome profiling data in mouse (*6*) and zebrafish (*7*) indicate that the *toddler* ORF is protected by actively translating ribosomes in vivo (Fig. 1B). Third, mass spectrometric analysis of nontrypsinated protein extracts from embryos expressing *toddler* mRNA detected the 11–amino acid C-terminal Toddler peptide fragment that is predicted to be a convertase cleavage product (Fig. 1D and fig. S4). Fourth, enhanced green fluorescent protein (eGFP) fusion proteins containing the wild-type signal sequence of Toddler are found extracellularly, whereas signal peptide cleavage site mutants are retained in the cell (Fig. 1E). Fifth, as described below, extracellular injection of in vitro–synthesized Toddler peptide (C-terminal 21 amino acids) elicits the same gain-of-function phenotypes as excess of *toddler* mRNA. Sixth, wild-type but not frameshifted *toddler* mRNA rescues *toddler* mutants (see below), providing direct evidence that it is the peptide product rather than the RNA that is functional in vivo. Together, these findings identify Toddler as a short, conserved, and secreted peptide.

Toddler Is Essential for Embryogenesis

To disrupt *toddler* function, we generated mutants by TALEN-mediated mutagenesis (fig. S5 and materials and methods) (*12*, *13*). Seven *toddler* alleles were recovered, each of which introduces a frameshift immediately after the signal peptide sequence (fig. S5, B and C). The vast majority of homozygous *toddler* mutants die between 5 and 7 days of development and display small or absent hearts, posterior accumulation of blood cells, malformed pharyngeal endoderm, and abnormal left-right positioning and formation of the liver (Fig. 2, A and B, and fig. S6). Penetrance and expressivity of *toddler* mutants vary, including occasional escapers that live to adulthood and rare instances of *toddler* mutants that display more severe defects in endoderm and mesoderm formation (fig. S7). Notably, the lethality of *toddler* mutants (survival, 0 of 25 animals) was rescued by injection of low amounts (2 pg) of wild-type (survival, 23 of 30 animals) but not frameshifted (survival, 0 of 32 animals) *toddler* mRNA (Fig. 2, A, C, and D). Embryonically rescued *toddler* mutants survived to adulthood and were fertile in the absence of any later source of Toddler peptide, indicating that zebrafish Toddler is only essential during early embryogenesis.

Toddler Is Required for Normal Gastrulation Movements

To determine when Toddler function is required during early embryogenesis, we used a heat shock–inducible transgene. Induction of *toddler* expression during late blastula and early gastrula stages,

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA. [2]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, USA. [3]Molecular Pathology Unit, Center for Computational and Integrative Biology, and Center for Cancer Research, Massachusetts General Hospital, Charlestown, MA 02129, USA. [4]Department of Pathology, Harvard Medical School, Boston, MA 02115, USA. [5]The Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142, USA. [6]FAS Center for Systems Biology, Harvard University, Cambridge, MA 02138, USA. [7]Center for Brain Science, Harvard University, Cambridge, MA 02138, USA. [8]Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA.

*Corresponding author. E-mail: pauli@fas.harvard.edu (A.P.); schier@fas.harvard.edu (A.F.S.)

†These authors contributed equally to this work.

but not at later times, rescued *toddler* mutants (fig. S8 and materials and methods).

The early requirement for Toddler, together with its expression peak during gastrulation (Fig. 1C), suggested that the later phenotypes originate from earlier defects. We therefore analyzed morphology and gene expression during blastula and gastrula stages and discovered that *toddler* mutant mesendodermal progenitors did not move properly toward the animal pole during gastrulation. Although ventral and lateral mesendodermal cells in wild-type embryos internalized at the margin and moved toward the animal pole (Fig. 2, C and E), these cells were closely packed and confined to a band near the margin in *toddler* mutant embryos (Fig. 2, C and D, and fig. S9). These defects were apparent by analysis of endodermal (*sox17*) and mesodermal (*fibronectin1/fn1*, *spadetail/tbx16*, *fascin*, *draculin/drl*) markers (Fig. 2C and fig. S9). In contrast, ectodermal (*sox3*), dorsal mesodermal (*goosecoid/gsc*, *hgg1*), and tail mesodermal (*ntla*) markers were largely unchanged in their expression domains (fig. S10). In addition to the ventrolateral movement defects, *toddler* mutants contained ~20% fewer endodermal cells at mid-gastrulation (Fig. 2, C and D, and fig. S9A). The initial expression of mesendodermal markers appeared unaffected (fig. S10B), suggesting that mesendodermal cells are specified normally in *toddler* mutant embryos but proliferate less. Notably, the *toddler* gastrulation phenotypes could be rescued by injecting low levels (2 pg) of *toddler* mRNA at the one-cell stage (Fig. 2, C and D, and fig. S9, A and C). These results reveal an important role for Toddler in the movement of ventral and lateral mesendodermal cells during gastrulation.

Toddler Promotes Endodermal and Mesodermal Cell Migration

To determine how Toddler affects the movement of cells during gastrulation, we performed live cell imaging and followed cell trajectories in wild-type and

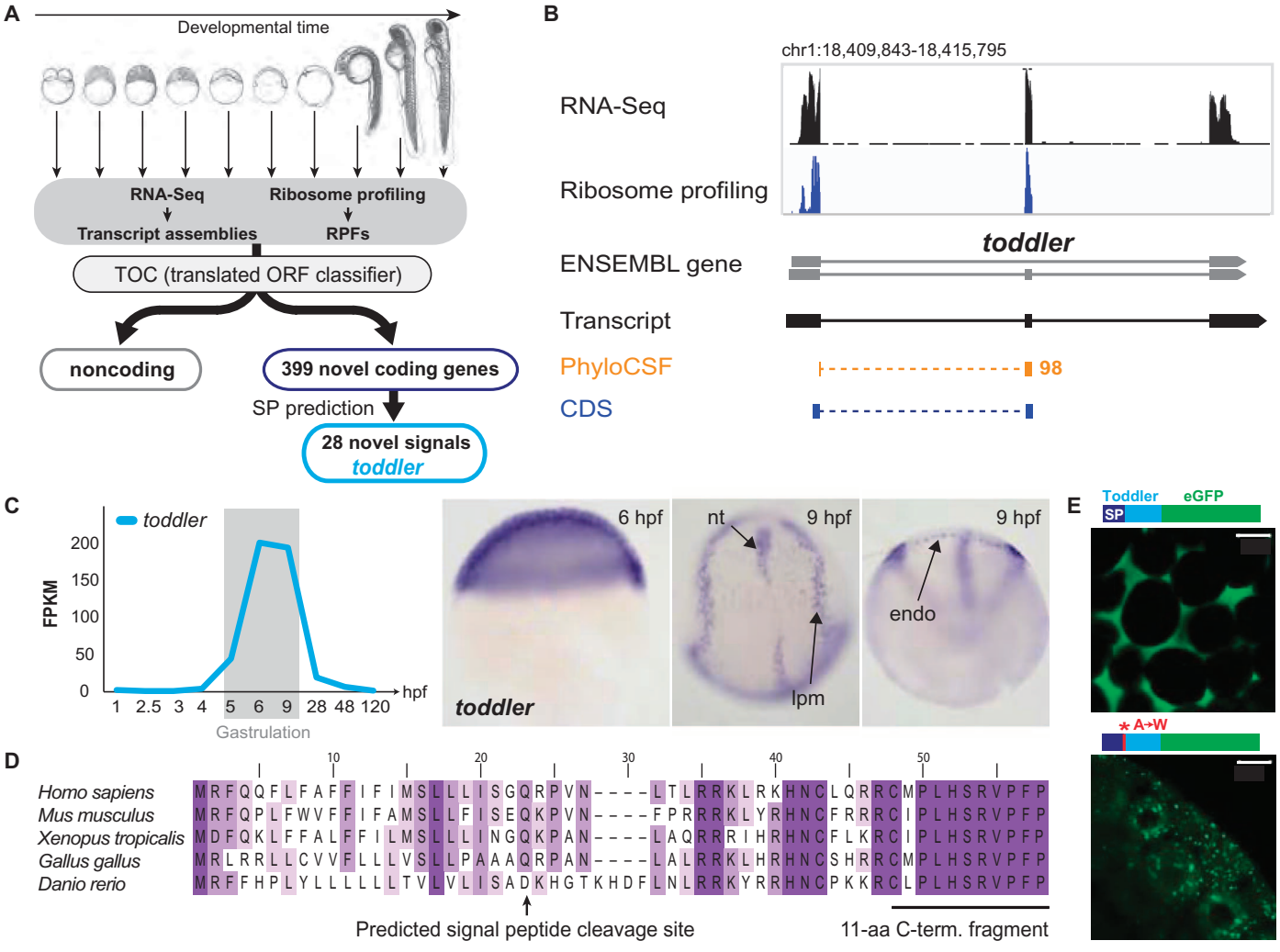


Fig. 1. Identification of the novel embryonic signal Toddler. (**A**) Overview of the individual steps used to identify novel coding and noncoding transcripts. SP, signal peptide; RPFs, ribosome protected fragments. (**B**) Genomic features of *toddler*. Coverage tracks for RNA-Seq (black) and ribosome profiling (blue), and tracks outlining the highest scoring regions in PhyloCSF (orange). Note that both PhyloCSF (*8*) and ribosome profiling (*7*) predict *toddler* to be protein-coding. (**C**) Expression analysis of *toddler* transcripts during embryogenesis. *toddler* transcripts peak during gastrulation [RNA-Seq data (*8*)]. FPKM, fragments per kilobase of transcript per million mapped reads. RNA in situ hybridization reveals ubiquitous expression of *toddler* transcripts at the beginning of gastrulation [6 hours postfertilization (hpf)]; expression becomes restricted to mesendodermal cells toward the end of gastrulation (9 hpf). nt, notochord; lpm, lateral plate mesoderm; endo, endoderm. (**D**) Toddler is conserved in vertebrates. ClustalW2 multiple protein sequence alignment of Toddler peptide sequences from five vertebrates. Darker shading indicates higher percentage identity of the amino acid. The predicted signal peptide cleavage site and the highly conserved C-terminal 11–amino acid (aa) peptide fragment that was detected by mass spectrometry are indicated. (**E**) Toddler signal sequence drives secretion. Injection of mRNAs encoding C-terminal Toddler-eGFP fusion proteins reveals that the wild-type Toddler signal sequence drives secretion (extracellular localization of eGFP), whereas mutation of A→W in the signal peptide cleavage site causes Toddler-eGFP to remain intracellularly (top, wild-type Toddler ORF; bottom, A→W mutant Toddler ORF). Fusion protein diagrams are not drawn to scale. Scale bars, 20 μm.

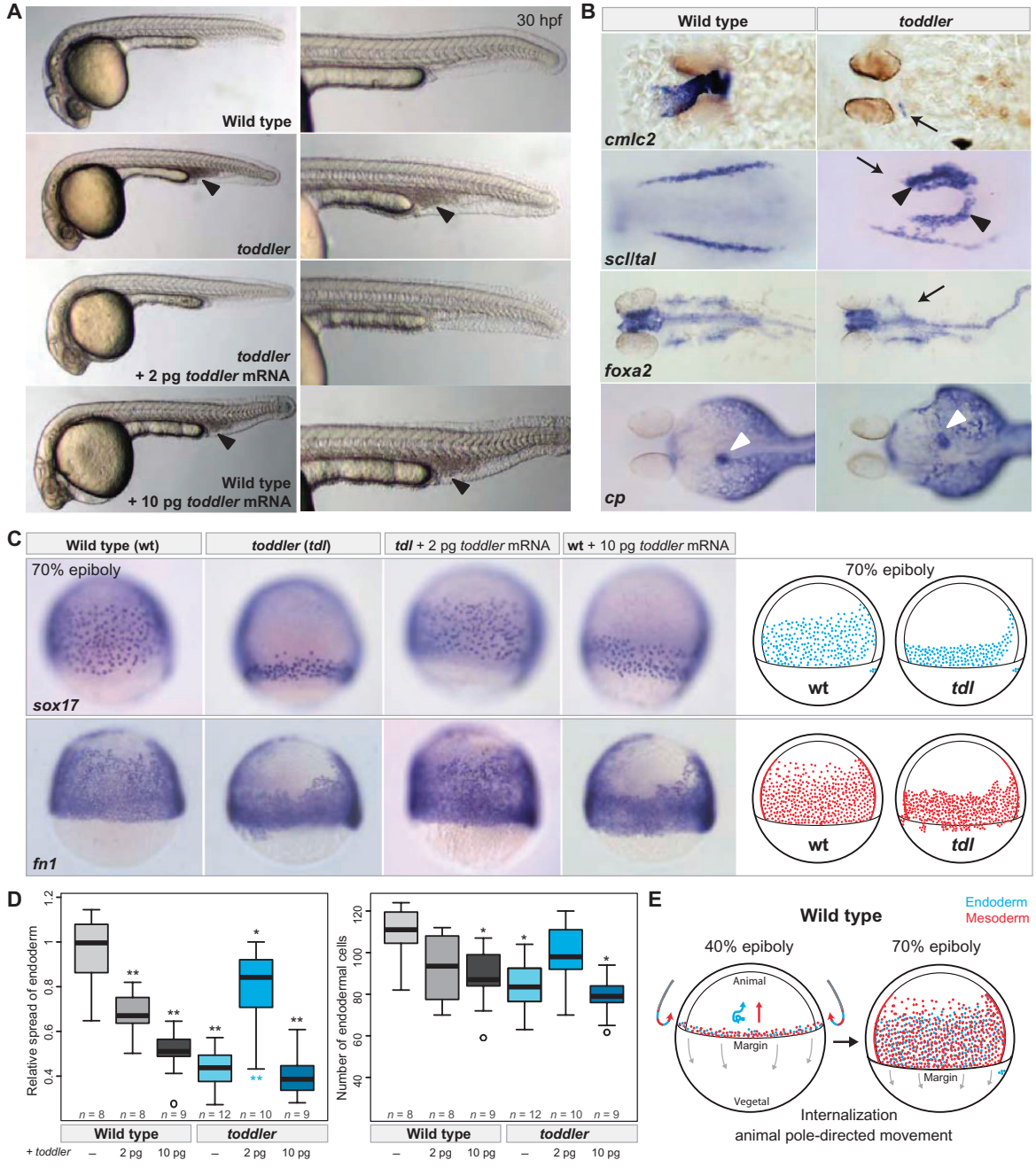


Fig. 2. Toddler is essential for embryogenesis. (**A**) Morphological analysis of *toddler* mutants. TALEN-induced *toddler* null mutants (see fig. S5) lack a functional heart, have no blood circulation, and accumulate blood posteriorly (black arrowheads). Defects in *toddler* mutant embryos are rescued by low doses (2 pg) of *toddler* mRNA. Injection of higher doses of *toddler* mRNA (≥10 pg) causes phenotypes in wild-type embryos reminiscent of *toddler* loss-of-function mutants. Shown are lateral views of embryos of the indicated genotypes at 30 hpf. (**B**) Marker gene analysis in wild-type and *toddler* mutant embryos at 36 hpf (*cmlc2*), at the 8 to 10 somite stage (*scl/tal*), at 30 hpf (*foxa2*), and at 3 days postfertilization [*ceruloplasmin* (*cp*)]. Black arrows indicate lack of or reduced staining in *toddler* mutant embryos; black arrowheads indicate ectopic expression; white arrowheads point to the liver in wild-type (>70% on left side) and *toddler* mutant embryos (expression: 45% right, 15% medial, 40% none/nonspecific). (**C**) Toddler is required for movement of ventrolateral endoderm and mesoderm toward the animal pole. Both absence of Toddler (*toddler*) and overexpression of *toddler* mRNA (wild-type embryos + 10 pg of *toddler* mRNA) reduce the movement of endodermal (*sox17*) and mesodermal [*fibronectin 1* (*fn1*)] cells toward the animal pole, as detected by in situ hybridization. All in situ images are lateral views of embryos at 70% epiboly (dorsal to the right). Illustrations of the observed endodermal (blue) and mesodermal (red) phenotypes in wild-type (wt) and *toddler* mutant (*tdl*) embryos are shown on the right. (**D**) Quantification of the endodermal defects at 70% epiboly. Left, relative spread of lateral endoderm along the animal-vegetal axis (that is, height of lateral band of *sox17*-expressing cells divided by the wild-type mean); right, number of endodermal cells within a lateral, fixed-size area. Gray, wild-type genomic background; cyan, *toddler* mutant genomic background. *P* values for pairwise comparisons with wild-type (black, top) or *toddler* mutant (cyan, bottom) were calculated on the basis of a standard Welch's *t* test (*$P < 0.01$; **$P < 0.00001$). (**E**) Illustration of early gastrulation movements in wild-type zebrafish embryos. Mesodermal (red) and endodermal (blue) cells are induced and internalized at the margin (40% epiboly stage). Whereas internalized cells migrate toward the animal pole in either a directional (mesoderm) or random walk–like pattern (endoderm) (*3*, *15*), epiboly movements are directed toward the vegetal pole (gray arrows). At 70% epiboly, mesodermal and endodermal cells have moved animally and cover most of the lateral side of the embryo.

toddler mutant embryos (movies S1 to S6). *Toddler* mutant endodermal cells [*sox17::GFP* (*14*)] displayed reduced movement toward the animal pole (Fig. 3A, fig. S11, and movies S1 and S2), migrated more slowly, and showed reduced net (start-to-end) displacement compared to wild-type cells (Fig. 3B and fig. S11). During early gastrulation, *toddler* mutant endodermal cells exhibited the characteristic random walk–

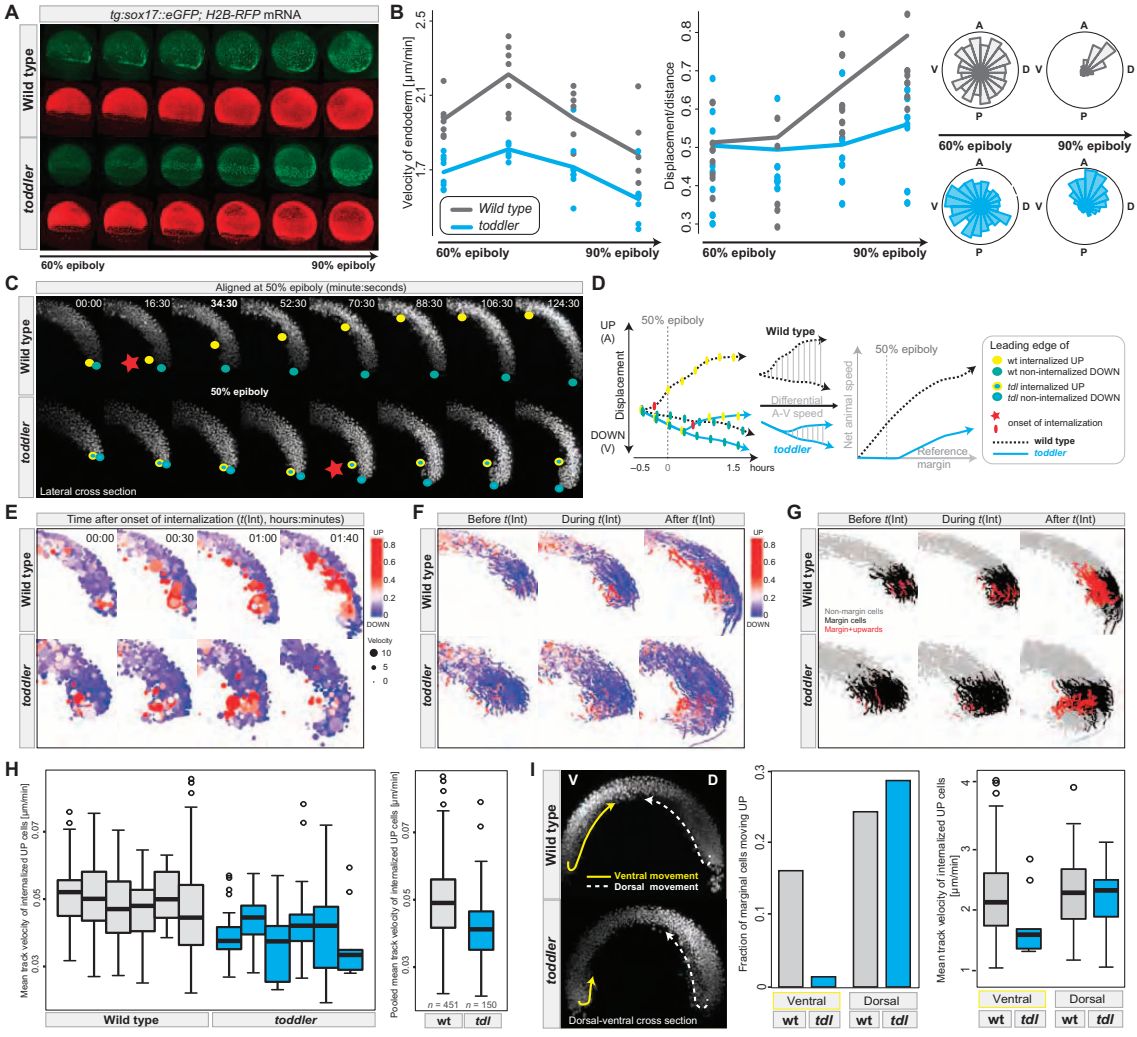


Fig. 3. Abnormal gastrulation movements in *toddler* mutants. (**A** and **B**) Analysis of endodermal cell migration in *sox17::eGFP* transgenic wild-type and *toddler* mutant embryos by confocal microscopy. Green, endodermal cells (marked by *sox17::eGFP*); red, nuclei [*human histone2B-RFP* (*H2B-RFP*) mRNA injection]. (A) Still images of maximum intensity projections of a time-lapse movie from 60 to 90% epiboly (movies S1 and S2). (B) Quantification of the average (median) velocity of endodermal cells (left), displacement versus distance travelled (middle), and directionality (roseplots; right) in wild-type (gray) and *toddler* mutant (cyan) embryos. Each dot represents the average speed (or the ratio between displacement versus distance travelled) of all endodermal cells tracked within a single embryo during a 45-min time interval with respect to its previous position [speed = actual distance (micrometers)/time (min)]. Shown are the data for four consecutive 45-min time windows. Roseplots display the random movement of endodermal cells during early gastrulation and the more directional migration at later stages [animal (A), posterior (P), dorsal (D), ventral (V)]. (**C** to **I**) Analysis of early gastrulation movements in *H2B-RFP* mRNA injected wild-type and *toddler* mutant embryos by light-sheet microscopy (single-plane illumination microscopy). (C to H) Internalization and animal pole–directed movement of lateral mesendodermal cells are reduced in *toddler* mutants. Analyses are shown for lateral cross sections of a time-lapse movie (movie S4) of a wild-type–*toddler* mutant embryo pair, imaged in parallel at 90-s intervals within a single experiment. (C) Still images of maximum intensity projections of 40-µm lateral cross sections (20 z-slices) during the time of internalization (time in minutes:seconds). Movies were aligned at 50% epiboly (48:00). Leading edges of internalizing mesendodermal cells (yellow dots) and vegetally moving cells (green dots) highlight the opposing paths of cells during gastrulation. Red stars mark the onset of cell internalization. (D) Comparison of animally and vegetally directed migratory paths of the wild-type and mutant embryo pair shown in (C). Frame-to-frame displacements (plotted on the left) were used to derive the net animal pole–directed cell movement. *Toddler* mutants (cyan) show delayed onset of internalization and reduced step-to-step and net animal pole–directed movement. (E to G) Cell tracking and digital analysis of gastrulation movements. (E) Position, speed (dot size), and directionality [color-coded from blue (vegetal movement) to red (animal movement)] of tracked cells during and after the time of internalization [*t*(Int)]. Movies were aligned to the onset of internalization [*t*(Int) = 00:00; time in hours:minutes]. (F and G) Cell tracks before ($t < -5$ min), during (-5 min $< t <$ 1 hour), and after ($t > 1$ hour) internalization in wild-type and *toddler* mutant embryos. In (F), tracks were color-coded on the basis of the total number of animal pole–directed (red) or vegetal pole–directed (blue) movements, normalized to the total number of frames per track. In (G), tracks were color-coded on the basis of their relative position and directionality with respect to the margin at the time of internalization (margin cells: cells located within 100 µm above the margin at the onset of internalization). Gray, nonmargin cells; black, margin cells; red, internalizing and upward-moving margin cells. (H) Quantification of the mean velocity of internalizing, animal pole–directed movement in wild-type and toddler mutant embryos. Mean track velocities were obtained from cell-tracking data derived from lateral cross sections of six wild-type (gray) and six *toddler* mutant (cyan) embryos, imaged in parallel in three independent experiments. Pooled wild-type and *toddler* mutant mean track velocities are plotted on the right (n = number of cell tracks). (I) *Toddler* mutant embryos are defective in ventrolateral but not dorsal internalization. (Left) Still image of maximum intensity projections of 40-µm dorsal-ventral cross sections (20 z-slices) of a wild-type–*toddler* mutant embryo pair 110 min after the onset of internalization. Arrows highlight the paths that the leading internalizing cells took on dorsal (D, dashed white line) and ventral (V, solid yellow line) sides of the embryo. Ventral movement toward the animal pole is severely reduced in the *toddler* mutant embryo, whereas dorsal internalization occurs normally. (Right) Quantification of the fraction and speed of internalizing marginal cells based on their positioning in the embryo (dorsal versus ventral) and genotype [wild type (gray) versus *toddler* mutant (cyan)] (see also movie S6).

like migration pattern observed in wild-type embryos (*3*, *15*), but they migrated in a less directional fashion than their wild-type counterparts during later gastrulation (movie S1 and Fig. 3B).

To analyze the earliest steps of mesendoderm movement, we followed the paths of H2B-RFP–labeled nuclei by light-sheet microscopy in wild-type and *toddler* mutant embryos (movie S3 and fig. S12). Analysis of 10 wild-type and 11 *toddler* mutant embryos confirmed that the movement of ventrolateral but not dorsal internalizing cells toward the animal pole was impaired in *toddler* mutants (Fig. 3, C to I, figs. S12 to S14, and movies S3 to S6). Internalization of ventrolateral cells at the margin was delayed (Fig. 3, C and D, fig. S13A, and movies S4 and S5) and reduced (Fig. 3, E to G and I, fig. S13, and movies S3 to S6). Although internalization in wild-type embryos started about 30 min before embryos reached 50% epiboly, it often commenced only after the 50% epiboly stage in *toddler* mutants (Fig. 3, C and D, fig. S13A, and movies S4 and S5). Ventrolateral internalized cells moved more slowly (Fig. 3, H and I) and often piled up at the margin (Fig. 3, C and E, figs. S13 to S15, and movies S3 to S6). In addition, epiboly movements were often delayed in *toddler* mutants, particularly during the time of internalization (fig. S13, A and B). In rare cases, we observed an almost complete absence of animal pole–directed ventrolateral cell movements; in these embryos, ventral and lateral marginal cells instead moved vegetally (movies S3, S5, and S6), likely contributing to the ectopic accumulation of posteriorly located blood cells at later stages (Fig. 2, A and B). These results identify Toddler as a key signal that promotes the internalization and animal pole–directed movement of mesendodermal cells during gastrulation.

Overexpression of Toddler Phenocopies *Toddler* Mutants

In contrast to inducers of specific cell fates, many signals involved in cell migration or tissue morphogenesis share loss- and gain-of-function phenotypes. For example, both reduction and increase in Wnt/planar cell polarity signaling disrupt convergence and extension movements during gastrulation (*2*–*5*). To determine whether Toddler might share this feature, we carried out overexpression analyses. Injection of *toddler* mRNA at levels only five times higher (≥10 pg) than needed for rescue caused phenotypes in wild-type embryos that resembled *toddler* loss-of-function mutants, including gastrulation and heart defects (Fig. 2, A, C, and D, and fig. S9, A and C). Similar phenotypes were observed upon extracellular injection of an in vitro–synthesized Toddler peptide fragment (C-terminal 21 amino acids; fig. S16). These observations reveal that proper levels of Toddler are required for normal mesendodermal movement and provide further evidence of an important role for Toddler in cell migration.

Toddler Functions as a Motogen

Most genes encoding signals that attract or repel cells are expressed in specific domains (*16*), and ubiquitous production of such signals interferes with guided cell migration. In contrast, we find that *toddler* RNA is expressed ubiquitously (Fig. 1C and fig. S17A) and that ubiquitous expression of *toddler* mRNA upon injection at the one-cell stage promotes the normal movement of mesendodermal cells in *toddler* mutants (Fig. 2, C and D). To further test the role of Toddler in cell migration, we locally expressed Toddler in the vegetal or animal regions of *toddler* mutants. In both scenarios, localized Toddler production was able to promote the migration of mesendodermal cells and rescue *toddler* mutants (Fig. 4). Although more complex scenarios are formally possible [for example, local processing (*17*) and self-generated gradient formation (*18*, *19*)], these results suggest that Toddler does not attract cells to or repel cells from specific sites. Instead, Toddler appears to act as a motogen (*20*–*22*)—a general promoter of mesendodermal cell migration.

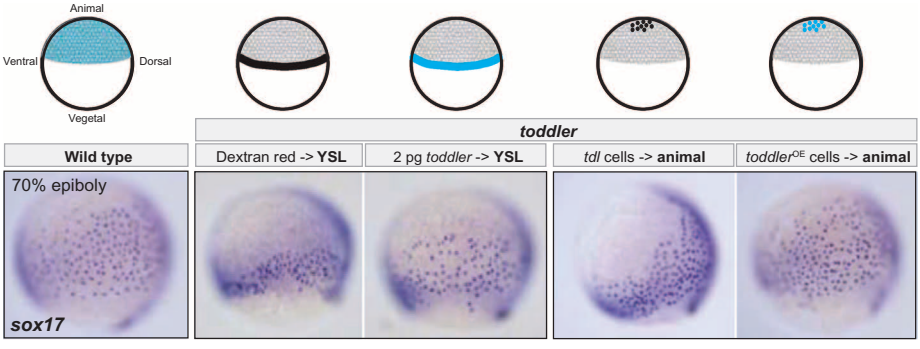


Fig. 4. Toddler functions as a motogen. Ubiquitous or localized expression of Toddler promotes animal pole–directed endodermal cell migration in *toddler* mutant embryos. Toddler was expressed either vegetally from the yolk syncytial layer (YSL) (injection of *toddler* mRNA into the YSL) or animally from a *toddler*-overexpressing (OE) clone of cells transplanted into the animal pole. Dextran red injections into the YSL and transplantation of uninjected *toddler* mutant cells served as controls. Different treatments are illustrated on top; *toddler* expression domains are highlighted in cyan. All *sox17* in situ hybridization images are lateral views of embryos at 70% epiboly (dorsal to the right).

Toddler Acts via APJ/Apelin Receptors

To identify candidate receptors for Toddler, we compared the *toddler* phenotype to previously described receptor mutant phenotypes. On the basis of the small size of Toddler peptide and the involvement of G protein signaling in gastrulation movements, we focused on G protein–coupled receptors (GPCRs) as candidate Toddler receptors (*14*, *23*–*30*). Four observations raised the possibility that the G protein–coupled APJ/Apelin receptor might mediate Toddler signaling. First, loss of APJ/Apelin receptor signaling results in small hearts and affects lateral mesoderm migration in zebrafish (*24*–*26*), phenotypes reminiscent of some aspects of the *toddler* mutant phenotype. However, in contrast to the broad roles of Toddler in mesendoderm migration, APJ/Apelin receptor signaling had been specifically implicated in cardiovascular development (*24*–*26*, *31*–*36*). Second, overexpression of Apelin, the only known ligand for the APJ/Apelin receptor (*35*–*38*), interferes with gastrulation movements in zebrafish (*24*–*26*). Third, the expression levels of Apelin receptors and Toddler peak during gastrulation (Fig. 5A), and Apelin receptors are expressed in mesendodermal cells [fig. S16A and (*24*, *25*, *39*)], the cell types affected in *toddler* mutants. Fourth, we found that Apelin is expressed only at the end of gastrulation [Fig. 5A and (*24*)], after the *toddler* and *APJ/apelin receptor* phenotypes (*24*, *25*, *40*) are apparent. These observations, together with the milder phenotypes observed in the absence of Apelin as compared to loss of APJ/Apelin receptors (*24*–*26*, *34*, *36*, *41*–*46*), raised the hypothesis that Toddler might be the bona fide activator of APJ/Apelin receptor signaling during gastrulation. We tested three predictions of this model.

First, we determined whether the absence of Apelin receptor function phenocopies *toddler* mutants. We reexamined *aplnra* and *aplnrb* double morphants (*24*–*26*) and found phenotypes that were highly similar to *toddler* mutants, including reduced movement of ventrolateral mesendoderm during gastrulation (Fig. 5, B and C). We also confirmed and extended previous analyses of the effects of Apelin overexpression (*24*–*26*) and found defects very similar to those caused by Toddler overexpression (Fig. 5, B and C). In addition, we observed that coexpression of Toddler and Apelin receptor at levels that individually did not cause major defects resulted in abnormal gastrulation movements reminiscent of Toddler and Apelin (*24*–*26*) overexpression phenotypes (Fig. 5D). These results reveal shared morphogenetic activities of the Apelin receptor and Toddler signaling pathways.

Second, we tested the epistatic relationship between Toddler and Apelin receptor signaling. The similarity of gain- and loss-of-function phenotypes precluded standard tests such as overexpression of Toddler in Apelin receptor mutants. Instead, we tested whether activation of Apelin receptor signaling can bypass the requirement

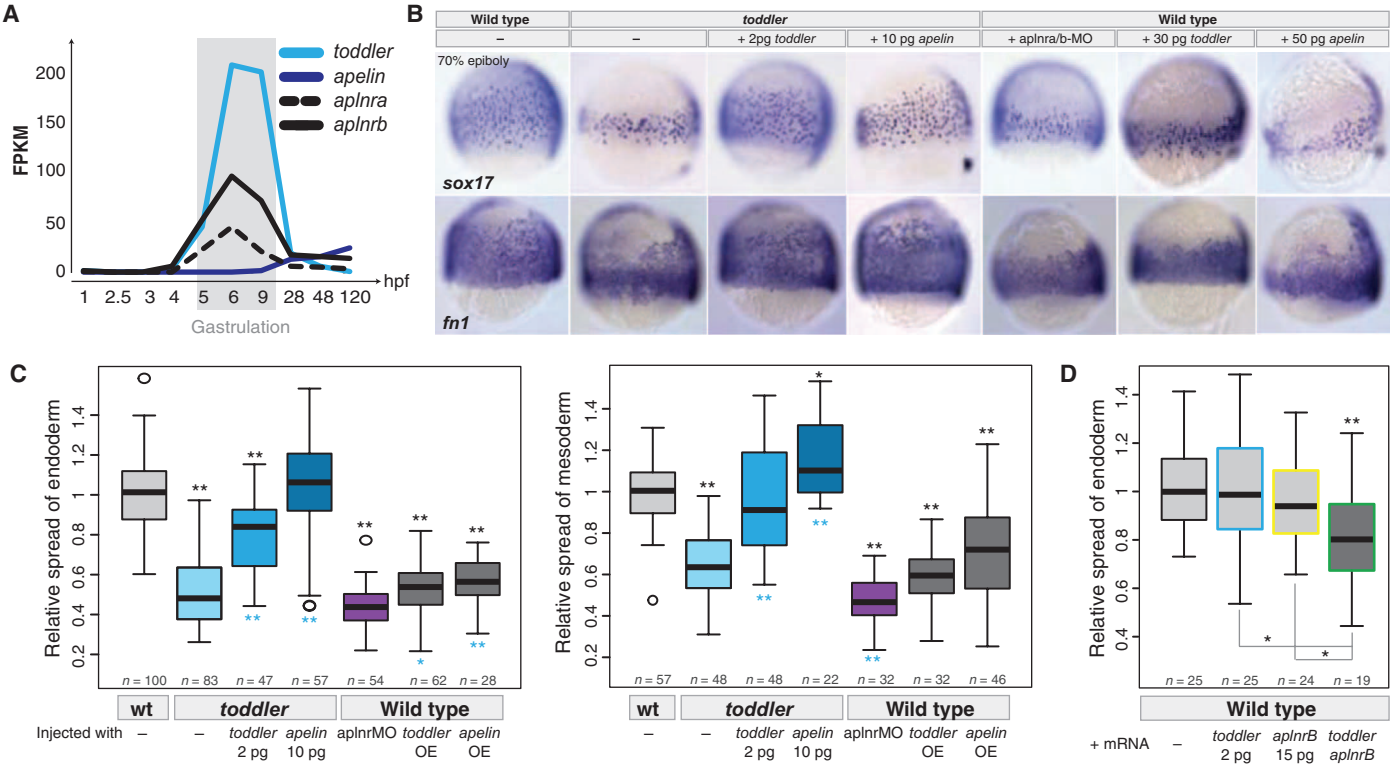


Fig. 5. Toddler acts via Apelin receptors. (**A**) RNA-Seq–based expression levels of *toddler*, *apelin*, and *apelin receptors* (*aplnra* and *aplnrb*) during embryogenesis. (**B**) Genetic evidence for Toddler signaling via the Apelin receptor. Endodermal (*sox17*) and mesodermal [*fibronectin 1* (*fn1*)] cell distributions were analyzed by in situ hybridization at 70% epiboly. Apelin receptor knockdown [aplnra/b morpholino (MO) injection] phenocopies *toddler* mutants, and Apelin production can rescue *toddler* mutants. Overexpression of Apelin causes phenotypes resembling *toddler* mRNA overexpression. (**C**) Quantification of the relative lateral spread of endoderm (left) and mesoderm (right). Quantifications are from multiple experiments (n = number of embryos per category). P values for pairwise comparisons with wild type (black, top) or *toddler* mutant (cyan, bottom) were calculated on the basis of a standard Welch's t test (*$P < 0.01$; **$P < 0.00001$). (**D**) Synergistic effect of Toddler and Apelin receptor b on endodermal cell migration. Injection of *toddler* or *aplnrb* mRNA at low concentrations (2 and 15 pg, respectively) did not cause significant defects in animal pole–directed movement of endodermal cells (different batch of *toddler* mRNA than used in Fig. 2D). However, coinjection of both mRNAs reduced the extent of endoderm movement. Shown are the combined data of two independent experiments. P values for pairwise comparisons with wild type (top) or individual mRNA injections (bottom) were calculated on the basis of a standard Welch's t test (*$P < 0.01$; **$P < 0.00001$).

for Toddler. *Apelin* mRNA injection into *toddler* mutant embryos restored normal mesendoderm migration (Fig. 5, B and C), cardiac development, and survival to adulthood. These results suggest that Toddler and Apelin activate the same signaling pathway.

Third, we tested whether Toddler can drive the internalization of Apelin receptors (Fig. 6), a hallmark of activated GPCR signaling (*47*–*50*). We misexpressed *toddler* mRNA with eGFP-tagged Apelin receptor a or b and observed strong internalization of the receptors from the plasma membrane (Fig. 6B). This effect was specific because other signaling proteins (chemokines Sdf1a/Cxcl12a or Sdf1b/Cxcl12b) did not alter the distribution of membrane-bound Apelin receptors, nor did Toddler alter the distribution of other chemokine receptors (Cxcr4a-eGFP, Cxcr4b-eGFP, and Cxcr7b-eGFP) (Fig. 6B and fig. S18). Moreover, Toddler produced from a local clone of cells was sufficient to cause Aplnrb-eGFP internalization at a distance from the source, suggesting that secreted Toddler peptide can act on neighboring cells (Fig. 6C). This conclusion was further strengthened by the observation that extracellular injection of in vitro–synthesized C-terminal Toddler or Apelin peptides induced efficient internalization of Aplnr-eGFP (Fig. 6D). These results indicate that Toddler activates Apelin receptors.

Discussion

Our study indicates that Toddler is an activator of APJ/Apelin receptor signaling, promotes gastrulation movements (see summary in Fig. 6E), and may be the first in a series of previously unknown developmental signals. While this study was under review, Toddler (named ELABELA) was independently reported to signal via APJ/Apelin receptors during endoderm differentiation and heart formation (*51*). The HUGO Gene Nomenclature Committee (HGNC) has recently designated the name Apela (apelin receptor early endogenous ligand) as the standardized symbol for Toddler/ELABELA/Ende. Our results lead to four major conclusions.

First, Toddler is a previously unrecognized signal that promotes cell movement during gastrulation. The rescue of *toddler* mutants by ubiquitous Toddler expression suggests that Toddler acts neither as a chemoattractant nor as a chemorepellent, but rather as a nondirectional signal to promote the internalization and movement of ventrolateral mesendodermal cells. Dorsal mesendoderm movement is largely unaffected in *toddler* mutants, consistent with the absence of Apelin receptor expression in this region and the role of other pathways in dorsal gastrulation movements (*3*). Both loss and overproduction of Toddler reduce cell movement, revealing that Toddler levels need to be tightly regulated to allow for normal gastrulation. It remains to be determined whether Toddler promotes motility by regulating cell shape, cellular protrusions, cell-substrate interactions, and cell-cell adhesion or through other means.

Second, Toddler-Apelin receptor signaling provides a long-sought link between mesendoderm induction and migration. Nodal signaling not only induces mesendoderm formation (*52*) but also activates the expression of Apelin receptors [fig. S17B and (*39*)]. Thus, Nodal-mediated induction of Apelin receptor expression might render cells competent to respond to Toddler and to become more motile (Fig. 6E). In this scenario, the activation of Apelin receptor expression in

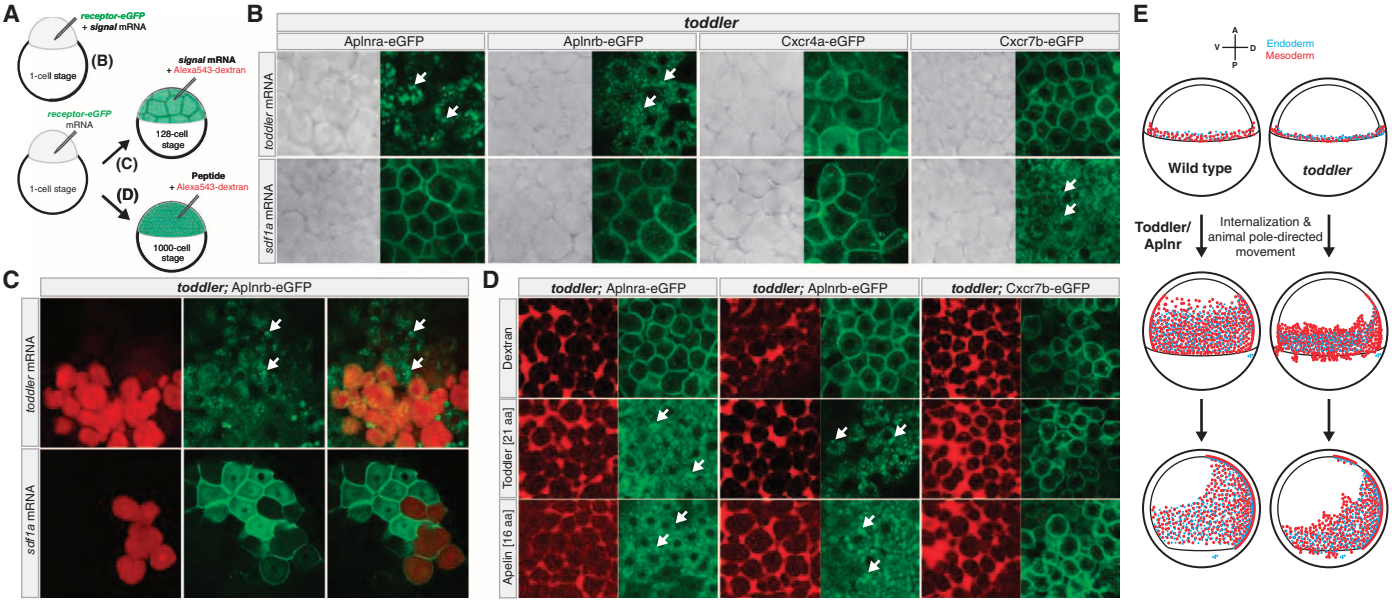


Fig. 6. Toddler drives internalization of Apelin receptors. (**A**) Schematic illustration of different treatments used to test for Toddler-mediated Apelin receptor internalization. (**B**) Test for signal-mediated internalization of eGFP-tagged receptors in zebrafish by coinjection of *signal* and *receptor-eGFP* mRNA into one-cell stage *toddler* mutant embryos. Receptor internalization was monitored by confocal microscopy. White arrows point to fluorescent foci of internalized receptors. In the absence of signal peptide overexpression, ectopically expressed receptors localize to the plasma membrane in pregastrulation *toddler* mutant embryos [see control Alexa543-dextran injections in (D)]. (**C**) Generation of a local source of Toddler or Sdf1a by injection of *toddler* or *sdf1a* mRNA (together with Alexa543-dextran as tracer) into a single cell at the 128-cell stage. Local expression of Toddler is sufficient to cause Aplnrb-eGFP internalization in cells that do not express *toddler* mRNA (non-red cells). (**D**) Extracellular injection of in vitro–synthesized C-terminal Toddler or Apelin peptide fragments is sufficient to drive internalization of Apelin receptors. (**E**) Model of the role of Toddler-Apelin receptor signaling in mesendodermal cell migration during zebrafish gastrulation. Left, wild type; right, *toddler*; top, 40% epiboly (mesendoderm specification and internalization); middle, 70% epiboly (animal pole–directed cell movement); bottom, 90% epiboly (dorsal convergence).

cells located at the margin at the end of the blastula stage would restrict the motogenic effects of Toddler and prevent ectopic and premature cell motility.

Third, Toddler is a novel agonist of APJ/Apelin receptor signaling, as evidenced by Toddler-induced internalization of Apelin receptors and rescue of *toddler* mutants by production of the known receptor agonist Apelin. Additionally, a fusion protein of alkaline phosphatase and Toddler binds to cells expressing Apelin receptors (*51*). Previous studies have implicated APJ/Apelin receptor signaling in a variety of biological processes, including the regulation of cardiovascular development and physiology, the control of fluid homeostasis, or even as a co-receptor for HIV infection (*53*, *54*). Although Apelin has previously been the only known agonist of the APJ/Apelin receptor, genetic studies have found discrepancies between the roles of Apelin and its receptor in mouse (*34*, *36*, *41*, *45*, *55*) and zebrafish (*24*–*26*). For example, Apelin knockout mice are viable and fertile (*45*, *46*, *56*), whereas APJ/Apelin receptor mutant mice are born at sub-Mendelian ratios (*34*). Our studies suggest that both Toddler and Apelin can activate APJ/Apelin receptors and indicate that it is endogenous Toddler—not Apelin—that activates APJ/Apelin receptor signaling during zebrafish gastrulation. Analogously to the promise of Apelin in biomedical applications (*53*, *54*), Toddler and its derivatives may take the place of Apelin in therapeutic contexts. Indeed, Toddler may also activate mammalian APJ/Apelin receptors because misexpression of zebrafish, mouse, and human Toddler induces similar overexpression phenotypes in zebrafish (fig. S19).

Fourth, our RNA-Seq and ribosome profiling data indicate that Toddler might just be one of several poorly characterized developmental signals that may have been missed in mutagenesis screens because of their small size. Applying similar genomic approaches to adult tissues might identify additional previously unknown signals that regulate physiological and behavioral processes.

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Supplementary Materials

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Science

Toddler: An Embryonic Signal That Promotes Cell Movement via Apelin Receptors

Andrea Pauli, Megan L. Norris, Eivind Valen, Guo-Liang Chew, James A. Gagnon, Steven Zimmerman, Andrew Mitchell, Jiao Ma, Julien Dubrulle, Deepak Reyon, Shengdar Q. Tsai, J. Keith Joung, Alan Saghatelian and Alexander F. Schier

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Toddler Welcome

It has been assumed that most, if not all, major signals that control vertebrate embryogenesis have been identified. Using genomics, **Pauli *et al.*** (10.1126/science.1248636, published online 9 January) have now identified several new candidate signals expressed during early zebrafish development. One of these signals, Toddler, is a short, conserved, and secreted peptide that promotes the movement of cells during zebrafish gastrulation. Toddler signals through G protein–coupled receptors to drive internalization of the Apelin receptor, and activation of Apelin signaling can rescue *toddler* mutants.

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