Developmental Cell
Short Article

ELABELA: A Hormone Essential for Heart Development Signals via the Apelin Receptor

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http://dx.doi.org/10.1016/j.devcel.2013.11.002

SUMMARY

We report here the discovery and characterization of a gene, *ELABELA* (*ELA*), encoding a conserved hormone of 32 amino acids. Present in human embryonic stem cells, ELA is expressed at the onset of zebrafish zygotic transcription and is ubiquitous in the naive ectodermal cells of the embryo. Using zinc-finger-nuclease-mediated gene inactivation in zebrafish, we created an allelic series of *ela* mutants. *ela* null embryos have impaired endoderm differentiation potential marked by reduced *gata5* and *sox17* expression. Loss of Ela causes embryos to develop with a rudimentary heart or no heart at all, surprisingly phenocopying the loss of the *apelin receptor* (*aplnr*), which we show serves as Ela's cognate G protein-coupled receptor. Our results reveal the existence of a peptide hormone, ELA, which, together with APLNR, forms an essential signaling axis for early cardiovascular development.

INTRODUCTION

Hormonal peptides are an important class of secreted signaling molecules. Endogenous peptides are most notable for their functions in innate defense as antimicrobial peptides (Cederlund et al., 2011), in immune regulation as chemokines (Bonecchi et al., 2009), and in modulation of behavior as neuropeptides (van den Pol, 2012). Deficiencies in hormonal peptides are the cause of several human diseases, the most prominent being the loss of insulin or insulin resistance in diabetes mellitus. Deficiency in the neuropeptide hypocretin causes narcolepsy (Nishino et al., 2000; Peyron et al., 2000), and anomalies in regulation of the appetite and satiety hormones leptin (Montague et al., 1997) and ghrelin are the underlying causes of congenital obesity and hyperphagia in Prader-Willi syndrome (Cummings et al., 2002).

Whereas many peptide hormones have been characterized and shown to play key roles in adult physiology, an involvement of these tiny signaling molecules during early development has not been established. During embryogenesis, six key signaling pathways (WNT, BMP/NODAL, FGF/IGF, NOTCH, HEDGEHOG, and HIPPO) are crucial for embryonic patterning. To our knowledge, no hormonal peptide has yet been implicated in the ability of naive blastomeres to differentiate into one of the three embryonic germ layers.

We have isolated a human gene, AK092578, which is currently annotated as a noncoding transcript. This gene is predicted to encode a 54 aa hormone with a signal peptide. We named this hormone, which is present in all vertebrate species, ELABELA (ELA). *ELA* expression has been reported to be rapidly downregulated during human embryonic stem cell (hESC) differentiation (Miura et al., 2004). To our knowledge, the second-earliest peptide hormone to be expressed during embryogenesis is apelin (APLN), the transcription of which begins during gastrulation in mice (D'Aniello et al., 2009). However, *Apln* knockout mice have no defects in early embryonic development (Kuba et al., 2007), which is inconsistent with the inactivation of its G protein-coupled receptor (GPCR) Aplnr (also known as Apj or Agtrl1), the loss of which leads to variable embryonic lethality due to growth retardation and cardiac malformations (Charo et al., 2009). In zebrafish, mutations in the *APLNR* homolog *aplnrb* impair the migration of cardiac progenitors from the lateral plate mesoderm into the heart field. Because *aplnra* and *aplnrb* are expressed in early precursors of the endodermal lineage, hours before the onset of *apln* expression, it has been postulated that APLNR transduces the signal of an earlier, yet to be discovered hormone (Charo et al., 2009; Scott et al., 2007).

Here, we demonstrate that ELA is a secreted peptide hormone. Using zinc-finger-nuclease (ZFN)-mediated gene inactivation, we created an allelic series of *ela* null zebrafish, and show that it is essential for endoderm differentiation and heart morphogenesis. We find that Ela, and not Apln, is the earliest ligand recognized by Aplnr, which mediates its effect on endoderm differentiation and subsequent cardiogenesis. Together, our results show the existence of an unannotated hormone, ELA, which is critical in vivo for heart morphogenesis that signals through the APLNR pathway.

RESULTS

ELA Is a Conserved Hormone

Human *ELA* consists of three exons on chromosome 4, which generates a transcript (AK092578) that is annotated as a noncoding RNA. However, *ELA* mRNA contains a conserved open reading frame (ORF) that encodes a predicted polypeptide of 54 aa. Phylogenetic analysis revealed that this polypeptide is

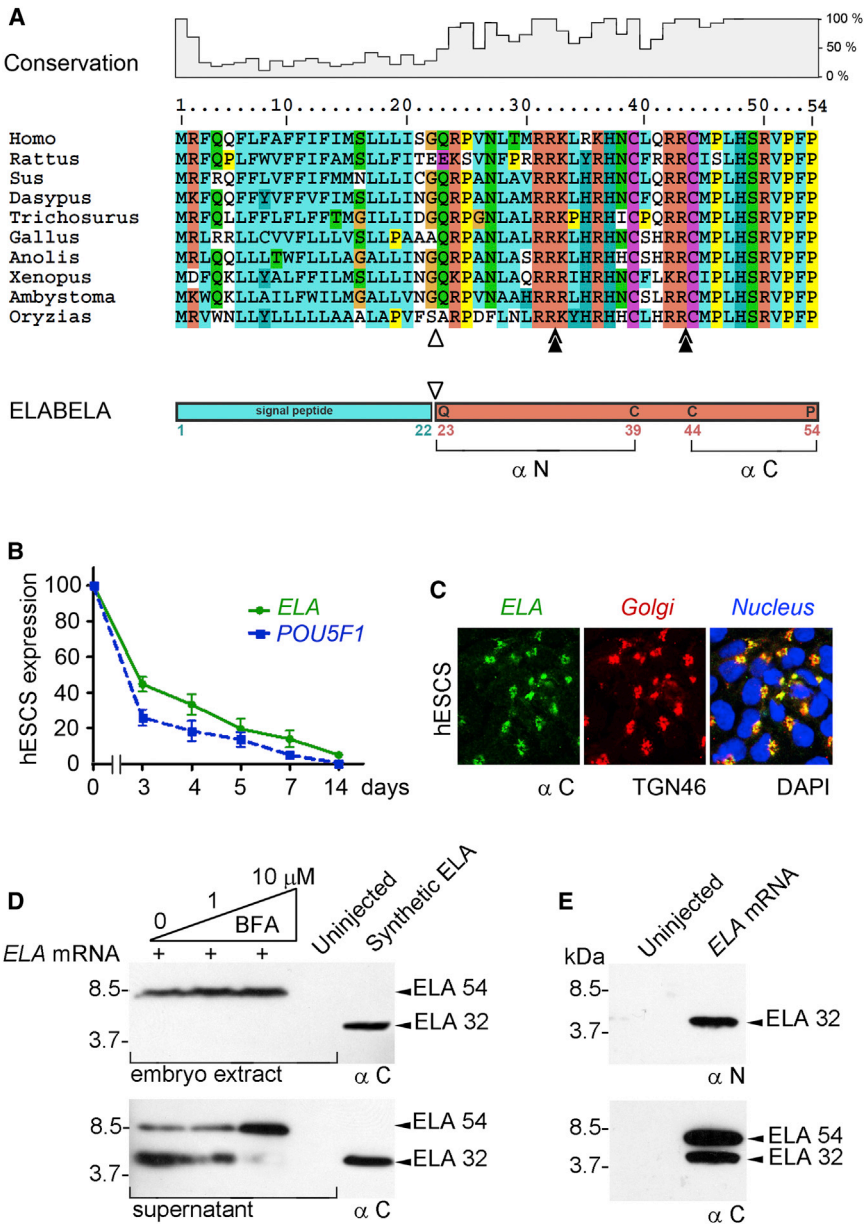

Figure 1. *ELA* Encodes a Conserved Hormone

(A) *ELA* encodes a conserved vertebrate protein of 54 aa consisting of a secretory signal and a mature 32 aa peptide. The carboxy terminus is invariant. White arrowhead: predicted signal peptide cleavage site between G22 and Q33. Black double arrowheads: possible furin cleavage sites after conserved di-arginines R31R32 and R42R43 motifs. The N- and C-terminal epitopes chosen for α N and α C antibody production are noted. The α C antibody is designed to recognize all ELA peptides regardless of species.

(B) *ELA*, like *POU5F1*, is rapidly silenced in hESCs during embryoid body differentiation (data are represented as mean ± SEM).

(C) By immunofluorescence in hESCs, endogenous ELA marked with the α C antibody colocalizes with the *trans*-Golgi network marked by TGN46.

(D) Western blot for ELA, which is translated, processed, secreted, and recognized by the α C antibody as a 4 kDa single band when overexpressed in *Xenopus laevis* embryos. BFA-mediated inhibition of secretion blocks ELA processing.

(E) The α C antibody recognizes both full-length ELA and processed ELA, whereas the α N antibody is specific to the mature processed ELA peptide.

a highly conserved protein with a predicted N-terminal signal sequence of 22 residues (Figure 1A). Along with a pair of conserved cysteines, the last 13 residues are nearly invariant in all vertebrate species. Based on this prediction, we raised antibodies against the N and C termini of the predicted mature ELA peptide (hereafter referred to as α N and α C antibodies; Figure 1A). *ELA* is most highly expressed in undifferentiated hESCs and is rapidly downregulated during differentiation (Figure 1B), as previously reported (Miura et al., 2004). In hESCs, the α C antibody detects ELA in the Golgi apparatus, as evidenced by colocalization with TGN46, a *trans*-Golgi marker (Figure 1C), suggesting that ELA is in the secretory pathway. To confirm that ELA is processed for secretion, human *ELA* ORF mRNA was microinjected into 4-cell-stage *Xenopus laevis* embryos. After 10 hr of secretion, we confirmed that *ELA* was translated, processed, and secreted by embryos (Figure 1D). In the supernatant, the processed ELA was the same size as a synthetically produced recombinant ELA consisting of the C-terminal 32 residues. Increasing amounts of Brefeldin A (BFA), an antibiotic that blocks the exit of secretory proteins from the endoplasmic reticulum, was able to block ELA processing and secretion (Figure 1D). Unlike the α C antibody, which recognizes both full-length and processed ELA, the α N antibody was specific to mature ELA, indicating that its epitope is revealed once the signal peptide is cleaved (Figure 1E). Taken together, these data confirm that *ELA* encodes a potentially soluble mature peptide with a molecular weight of <4 kDa.

Creating an Allelic Series of *ela* Mutants in Zebrafish

During zebrafish embryogenesis, *ela* is expressed from the mid-blastula transition (MBT) to 3 days postfertilization (dpf; Figures 2A and 2B). Without any measurable maternal contribution, *ela* is ubiquitous in dividing cells of the blastoderm before becoming restricted after gastrulation to axial structures, with most prominent expression in the neural tube (Figure 2A). *ela* is located on chromosome 1 and also consists of three exons (Figure 2C). To document the exact function of and requirement for *ela* in vivo, we designed and injected custom ZFNs to induce double-stranded breaks in exon 1 of *ela*, which codes for its signal peptide. Screening of the F1 generation allowed us to identify an

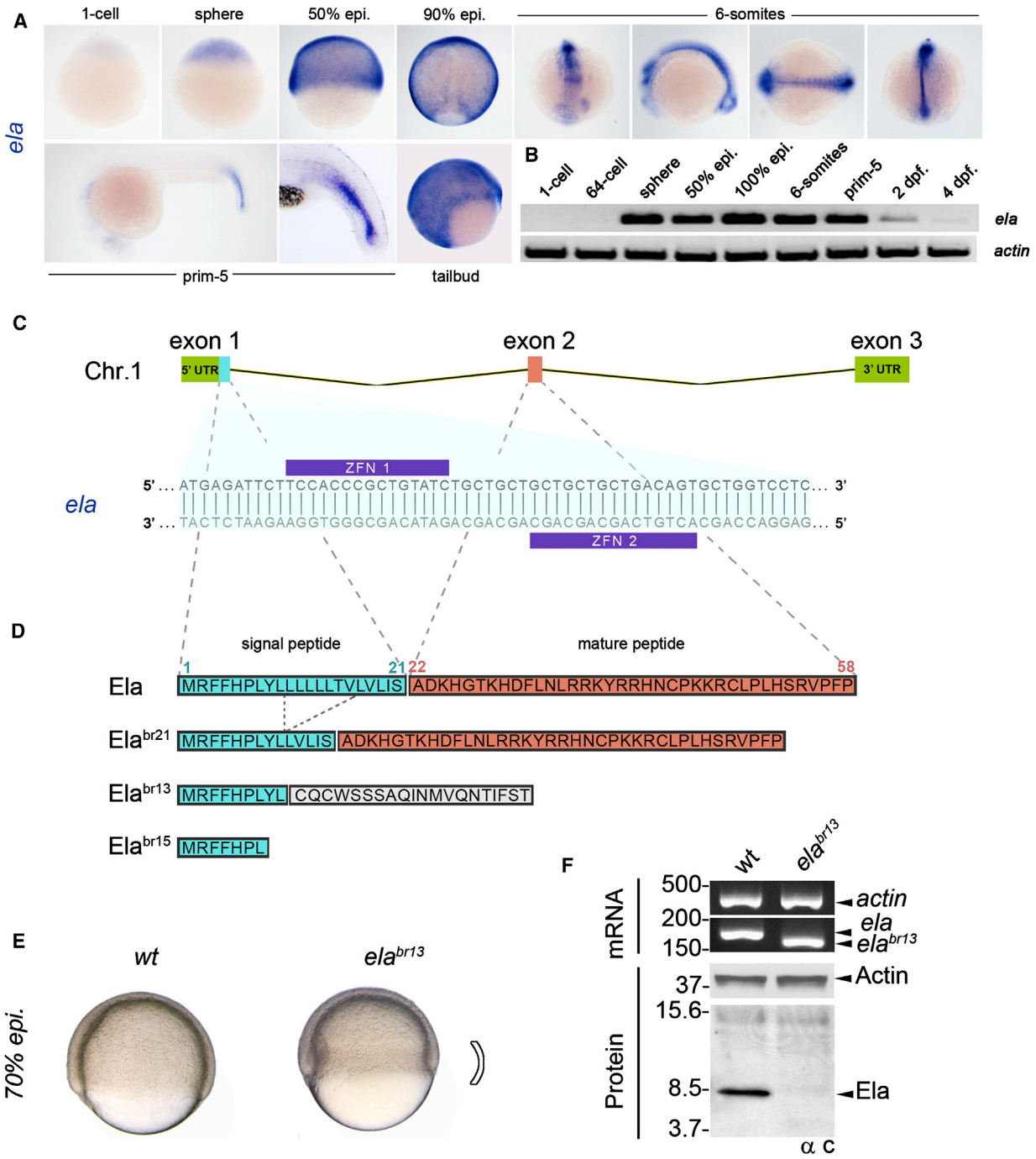

Figure 2. Generation of an Allelic Series of Mutant *ela* Zebrafish

(A) By WISH in zebrafish embryos, *ela* is found to be zygotically expressed and ubiquitous in the blastoderm. During gastrulation, its expression becomes axial and is strongest in the neural tube.

(B) RT-PCR analysis shows that, relative to *actin*, expression of *ela* is exclusively zygotic, peaking at 100% epiboly and becoming absent by 4 dpf.

(C) In zebrafish, *ela* consists of three exons located on chromosome 1. A custom pair of ZFNs targeting exon 1 of *ela* was used to create an allelic series of mutations within the signal peptide of Ela.

(D) Three distinct loss-of-function alleles were generated. The ela^{br21} allele results in a unique 7 aa in-frame deletion in Ela's signal peptide. Alleles ela^{br13} and ela^{br15} caused premature stop codons and disrupted the reading frame, resulting in no Ela mature peptide.

(E) Relative to WT embryos, homozygous null ela^{br13} embryos show defective epiboly movements and a constricted germ ring at the involuting margin at 70% epiboly.

(F) RT-PCR reveals that at 100% epiboly, endogenous *ela* and ela^{br13} mRNAs are of a distinct size. Western blotting using the α C antibody shows that endogenous Ela is recognized in WT embryos and is absent in null ela^{br13} siblings.

allelic series (Figure 2D) of heterozygous *ela* fish that were selected and backcrossed at least five times before phenotypic analysis was undertaken.

We analyzed three distinct *ela* alleles: ela^{br21}, ela^{br13}, and ela^{br15}. ela^{br21} is a 21 bp deletion that causes a unique 7 aa in-frame deletion within the signal peptide but leaves the mature peptide intact. ela^{br13} and ela^{br15} are frameshift alleles that are caused by deletion of 13 and 15 bp, respectively, and disrupt the entire mature Ela peptide (Figure 2D). *ela* null embryos developed normally up to 50% epiboly, after which migration anomalies were observed in the germ ring. We easily scored *ela* null embryos by eye using the "rough" and constricted appearance of the involuting marginal layer at the shield stage (Figure 2E). At 100% epiboly, when *ela* expression peaks (Figure 2B), we confirmed by RT-PCR that ela^{br13} mRNA was shorter than wild-type (WT) *ela* mRNA (Figure 2F), which is consistent with its biallelic genomic deletion. By western blotting using α C antibodies, we detected the endogenous Ela full-length protein in extracts of WT embryos, but not in ela^{br13} embryos (Figure 2F).

Loss of *ela* Causes Embryonic Lethality Due to Heart Dysgenesis

All *ela* homozygous mutant fish showed similar phenotypes and were present in the expected Mendelian recessive ratios (Figure 3A and Figures S1A–S1C available online), suggesting that the three alleles behave as loss-of-function mutants. *ela* heterozygous fish were phenotypically normal, whereas *ela* null fish presented with severe cardiac dysplasia ranging from a rudimentary heart to no heart (Figures 3A, 3B, and S1A–S1C). Loss or severe reduction of the embryonic heart marker *cmlc1* was seen in more than 95% of *ela* null embryos ($n > 370$) regardless of the allele analyzed (Figure 3C). Furthermore, *ela* null embryos had minimal blood circulation and we observed excess erythrocytes accumulating at the intermediate cell mass (ICM) (Figure 3A), as confirmed by *scl* upregulation in *ela* mutant embryos relative to heterozygous siblings (Figure 3D).

In addition, *ela* mutant larvae displayed variable posterior truncations and, at times, tailbud duplications. Posterior tissue defects ranged from loss of the ventral fin to complete tail and trunk truncations (Figure 3A). Unexpectedly, similar phenotypes were also observed upon *ela* mRNA overexpression (Figure S1D). Thousands of *ela* null embryos from all three genotypes were obtained and scored into three classes according to the severity of tail defects (Figures 3A and 3E). We observed that the relative proportions of each class varied greatly among heterozygous crosses (Figure 3E) and even among different clutches of embryos born to identical parents (data not shown), suggesting a notable phenotypic variance in tail development but not in heart morphogenesis. Remarkably, a very low percentage of *ela* null mutants developed to fertile adults (Figure 3F), ruling out any maternal *ela* effects and permitting homozygous crosses to yield 100% null clutches. These three *ela* loss-of-function alleles show that truncation of one-third of the Ela signal peptide results in phenotypes identical to those caused by frameshift mutations, indicating that Ela requires an intact signal peptide to be functional in vivo. Moreover, these recessive *ela* alleles suggest that reducing Ela levels by half is of no obvious consequence, whereas a total absence of Ela is incompatible with heart development, hematopoiesis, and, to a lesser extent, tail elongation.

ELA Is Required for Proper Endoderm Differentiation

In order to understand the embryological origin of the severe cardiac defects observed in *ela* mutant fish, we analyzed by whole-mount in situ hybridization (WISH) a series of markers for all three embryonic germ layers. Most prominently, *ela* mutant gastrulae displayed specific defects in the mesendodermal lineage (Figures 3G–3L). Mesoderm marked by *bra* exhibited impaired epiboly movements, presumably caused by convergent-extension anomalies. At 100% epiboly, *ela* mutant embryos were delayed and had an open blastopore with a shorter and thicker notochord relative to WT embryos (Figure 3G). The expression of *gata5*, which marks mesendoderm and promotes the development of cardiovascular progenitor cells (CPCs), was distinctly altered in *ela* embryos compared with WT embryos at 75% epiboly. Instead of marking a discrete axial population of cells in the heart-forming region, *ela* null embryos displayed a distinctive chevron shape around the organizer that was continuous with the marginal *gata5*-expressing cells (Figure 3H). The expression of *foxa2* was markedly reduced in its marginal, but not axial, component, indicating a diminished endoderm differentiation potential (Figure 3I) as confirmed by the reduced expression of *sox17* (Figure 3J), which marks the definitive endodermal precursors. *sox17* forerunner cells were unaltered by the absence of *ela*, indicating specificity vis-à-vis the endoderm lineage. The total number of $sox17^{+}$ cells at 75% epiboly were on average decreased by 30%–40% compared with their WT or *ela* heterozygous siblings (Figure 3K), similar to what was observed in homozygous ela^{br15} and ela^{br21} embryos (Figure 3L). We conclude that *ela* is essential in vivo for the proper differentiation of endodermal precursors that are known to be crucial for guiding the overlying cardiac progenitors to the heart-forming region.

Aplnr Is the Cognate Receptor for Ela during Heart Development

The *ela* mutant phenotype bears a striking resemblance to that of the apelin receptor (Aplnr), which has been implicated in heart development. The zebrafish mutant *grinch,* which carries a recessive W90L missense mutation in *aplnrb*, and *Aplnr* knockout mice both have defects in cardiac morphogenesis (Charo et al., 2009; Scott et al., 2007). Unexpectedly, however, loss of Apelin (Apln), the accepted ligand for Aplnr, does not recapitulate the phenotype of *grinch* in zebrafish or *Aplnr* null mice. We thus surmised that Ela might be the long-sought-after alternate and earlier ligand for Aplnr (Charo et al., 2009; Scott et al., 2007). Unlike most other hormones that have near-neutral isoelectric points, ELA and APLN are rich in basic residues and have isoelectric points above 12 (Figure 4A), suggesting they might share a common receptor. For Ela to be the earliest ligand of Aplnr, we contended that it should (1) be expressed concomitantly with *aplnr* before the onset of gastrulation, (2) be expressed in or adjacent to *aplnr*-expressing cells, (3) phenocopy *aplnr* mutants, and (4) bind to Aplnr on the surface of cells. Consistent with previous reports, we found that the onset of *aplnra* and *aplnrb* coincides with that of *ela* at MBT, whereas *apln* expression begins 5 hr later during gastrulation (Figure 4B). Cells expressing *aplnra* and *aplnrb* are in the hypoblast (Zeng et al., 2007), just beneath the overlying enveloping layer where *ela* is ubiquitously transcribed (Figure 2A). We found that the

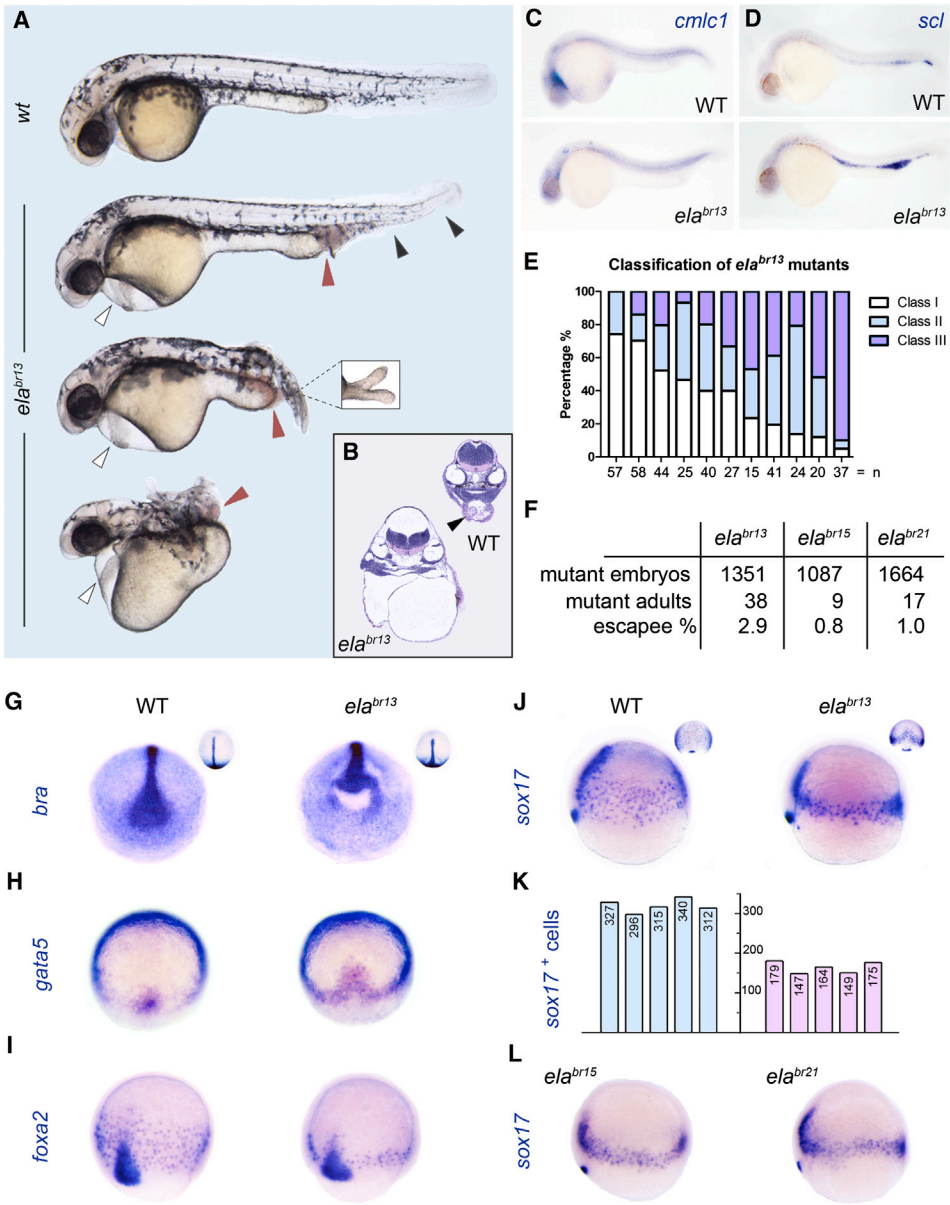

Figure 3. *ela* Knockout Zebrafish Have Severe Cardiovascular and Endoderm Defects

(A) Null *ela*^{br21} larvae display pericardial edema (white arrowheads) and accumulated erythrocytes (red arrowheads), and have no blood circulation. Variable posterior anomalies are observed, including loss of ventral fin (black arrowheads), tailbud duplications (inset), and extreme tail/trunk truncations (bottom embryo).

(B) Loss of *ela* causes severe cardiac anomalies ranging from mild heart dysplasia to total heart agenesis, as shown by hematoxylin and eosin staining on sections from the top and bottom embryos in (A).

(C) Null *ela*^{br13} fish have a severe reduction of *cmlc1* expression, which marks the developing heart.

(D) Null *ela*^{br13} fish display increased hematopoiesis as judged by the upregulation of *scl*, a marker of blood precursors.

(E) Classification of null *ela*^{br13} larvae with varying degrees of tail defects. Class I larvae are defined as having pericardial edema and tail blood clot. Class 2 larvae have ventral fin defects or tailbud duplications in addition to class 1 phenotypes. Class 3 larvae have all phenotypes of class 1 combined with mild to severe tail/trunk truncations.

(F) Percentages of *ela* mutant fertile adults that were obtained from heterozygous intercrosses using all three alleles.

(G) Relative to WT embryos, homozygous null *ela*^{br13} embryos show convergence-extension defects resulting in delayed blastopore closure and thickened notochord (insets) as indicated by altered *bra* expression.

(H) The loss of *ela* causes defective migration of mediolateral *gata5*-expressing cells, which mark mesendoderm cells that guide heart progenitors to the anterior lateral plate mesoderm.

(I) *ela* mutant embryos show reduced *foxa2* expression compared with WT embryos.

(J) *ela* mutant embryos show a more compact *sox17* expression pattern compared with WT embryos, whereas *sox17*+ forerunners cells are not affected.

(K) *ela* null embryos have approximately 40% less sox17+ cells than their heterozygous siblings at 75% epiboly.

(L) At 75% epiboly, *ela*^{br21} and *ela*^{br15} mutant embryos exhibit *sox17* expression defects similar to those observed for *ela*^{br13} (see also Figure S1).

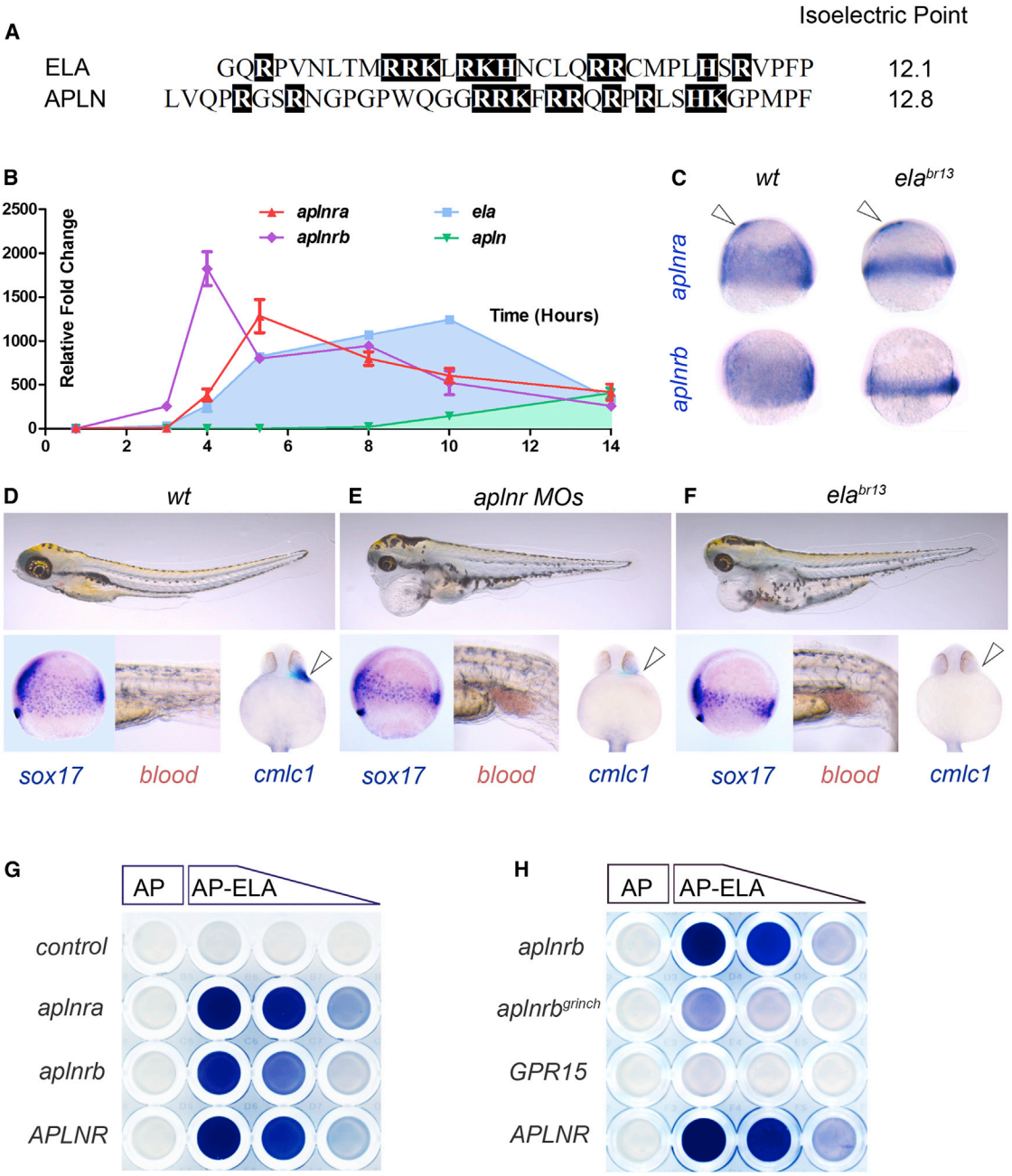

Figure 4. Ela's Cognate Receptor for Endoderm Differentiation Is Aplnr

(A) ELA and apelin are very basic hormones with isoelectric points exceeding 12.

(B) By qPCR, the onset of transcription of *aplnra* and *aplnrb* coincides with that of *ela* at the MBT; *apln* expression debuts 5 hr later during gastrulation (data are represented as mean ± SEM).

(C) Relative to control embryos, the expression of *aplnra* and *aplnrb* at 70% epiboly becomes stronger and confined to the most equatorial hypoblast in the *ela* mutant (white arrowheads: specific *aplnra* expression in the animal pole).

(D) WT embryo with a beating heart and blood circulation at 6 dpf, normal *sox17* expression in definitive endoderm at 75% epiboly, no erythrocyte accumulation in the ICM, and *cmlc1* expression in the heart-forming region at 30 hpf.

(E) *aplnr* morphants phenocopy *ela* mutant embryos, with no beating heart, loss of blood circulation at 6 dpf, reduced *sox17* expression in definitive endoderm at 75% epiboly, accumulation of erythrocytes in the ICM, and loss of *cmlc1* expression in the heart-forming region at 30 hpf.

(F) *ela* mutant embryos are nearly indistinguishable from *Aplnr* morphants and have no beating heart or blood circulation at 6 dpf, show reduced *sox17* expression in definitive endoderm at 75% epiboly, have accumulated erythrocytes in the ICM, and no *cmlc1* expression in the heart-forming region at 30 hpf.

(G) In 293T cells, overexpression of zebrafish *aplnra* or *aplnrb*, or human *APLNR* is sufficient to confer cell-surface binding to recombinant AP-ELA.

(H) In 293T cells, overexpression of zebrafish *aplnrb*, but not its mutant form (*grinch*) carrying the W90L missense mutation, or *GPR15* (an orphan GPCR closely related to APLNR), is enough to afford binding to recombinant AP-ELA.

expression of *aplnra* and *aplnrb* was also responsive to the loss of *ela*, displaying a more condensed pattern at the margin relative to WT embryos (Figure 4C). Using an array of markers, we found that *aplnr* morphants were phenotypically indistinguishable from *ela* null embryos. Both exhibited pericardial edema with markedly reduced *cmlc1* expression and accumulation of erythrocytes in the ICM at 30 hr postfertilization (hpf). By 6 days, all *ela* mutant and *aplnr* morphant embryos had cardiac dysplasia with little to no blood circulation (Figures 4D–4F). These in vivo data demonstrate that loss of *ela* phenocopies the loss of *aplnr*, arguing that they form a ligand-receptor pair in vivo. Lastly, overexpression of zebrafish *Aplnra and Aplnrb* in human embryonic kidney 293T (HEK293T) cells was sufficient to afford cell-surface binding to ELA conjugated to alkaline phosphatase (AP-ELA) (Figure 4G). In contrast, *Aplnrb*^{grinch} was unable to bind AP-ELA (Figure 4H). This interaction is conserved across species, since overexpression of human APLNR also confers binding to AP-ELA, but not GPR15 (Figure 4H), an orphan GPCR that is closely related to APLNR (Vassilatis et al., 2003). These results suggest that extracellular ELA can bind APLNR in a native cellular context. Taken together, our results support the notion that ELA, and not APLN, is the earliest agonist of APLNR, which works in tandem to direct endodermal differentiation for cardiac ontogeny.

DISCUSSION

We have uncovered a peptide hormone with potent embryonic signaling activity. Encoded by a transcript that is believed to be a long noncoding RNA, ELA is in fact the precursor for a small secreted peptide that is found in all vertebrate species. In vivo, ELA specifically affects the mesendodermal lineage, where its activity, transduced by Aplnr, brings about the migration and differentiation of the cardiac lineage.

Unannotated Hormones

We present evidence that uncharacterized peptide hormones exist in the human genome and have yet to be annotated as such. We surmise that more will surface as multiexonic transcripts are screened for the existence of phylogenetically conserved small ORFs. One such example is ELA, which up until now has been annotated as a long noncoding RNA. Several observations may explain, at least in part, why such tiny peptides are overlooked: (1) they are underrepresented in the proteome and escape mass-spectrometric detection, (2) ORF prediction algorithms are biased toward longer ORFs if phylogenetic conservation is not taken into consideration, and (3) despite evolutionary preservation, phylogenetic alignment may not be sufficient to uncover these hormones, since signal peptides are poorly conserved and only a portion of the mature peptide involved in signaling might be invariant, as in the case of ghrelin and bradykinin, which undergo further processing after secretion (Kojima et al., 1999; Sheikh and Kaplan, 1989). In this regard, we note the presence of two conserved di-arginine motifs in the ELA mature peptide (R31R32 and R42R43), which could serve as recognition and cleavage sites for furins (Hosaka et al., 1991). If ELA is further processed by furin-like proteases, a C-terminal 11-mer fragment that is evolutionarily invariant (Figure 1A) may be generated.

The Ela-Aplnr Axis in Cardiovascular Development

Scott et al. (2007) have shown that although *aplnr* is required prior to the onset of gastrulation for proper cardiac morphogenesis, its known ligand, *apln*, is not expressed until midgastrulation. Similarly, several groups have reported that knockouts of *Apln* and *Aplnr* in mice are not functionally allelic, as would be expected if a linear and exclusive ligand-receptor relationship linked Apln to Aplnr (Charo et al., 2009). This led some investigators to hypothesize that *Aplnr* may have ligand-independent functions, and recent reports have shown that Aplnr can respond to stretch (Scimia et al., 2012). An alternate and nonmutually exclusive explanation for this phenomenon invokes the existence of a second ligand for Aplnr. Our results suggest that Ela fulfills this role in early zebrafish embryogenesis together with Aplnr, to direct the migration and amplification of endodermal precursors for proper cardiac ontogeny.

At present, it is unclear whether Apln and Ela signal through Aplnr to elicit identical signaling cascades and therefore can partly compensate for one another. With respect to cardiovascular development, however, we note that *apln* zebrafish morphants and *apln* mouse knockouts do not display overt congenital cardiac anomalies (Kuba et al., 2007; Scott et al., 2007). The Ela-Aplnr axis thus appears to be exclusive for cardiac development, and Apln may be insufficient due either to divergent signaling downstream of Aplnr or simply to incompatible spatiotemporal patterns of expression.

How *ela* in the epibolyzing blastoderm affects the migration and differentiation of *aplnr*-expressing endodermal precursors in the hypoblast is not clear. Our analysis places *ela* upstream of *gata5*, one of the earliest markers of mesendodermal cells, which fails to coalesce at the midline in *ela* mutants. Mutation in *gata5* in *faust* zebrafish demonstrates that this transcription factor is required for precardiac mesoderm to migrate to the embryonic midline (Reiter et al., 1999). The myocardium lineage, which is one of the first paraxial cell populations to migrate into the anterior lateral plate mesoderm (ALPM), is very sensitive to changes in the endoderm lineage, which is specified by the Nodal pathway (Schier, 2003). Our loss-of-function alleles place signaling by Ela upstream of or parallel to the endodermal-mediated pathway for heart morphogenesis. Ela may be required for the correct proliferation of endoderm precursor cells, as judged by their decreased numbers in *ela* mutant fish. Alternatively, or concurrently, Ela may behave as a chemotactic stimulus that promotes the timely migration of endodermal cells toward the midline, which in turn guide the CPCs toward the APLM and render them competent to initiate cardiogenesis.

FUTURE DIRECTIONS

In addition to being expressed in hESCs, ELA mRNA is also found in the adult human prostate and kidney. It will be interesting to examine its role in these tissues. Although it is quite speculative at this stage, several forward-looking statements can be made with respect to the possible therapeutic value of recombinant ELA. Assuming that ELA signals through APLNR in adults, it will be important to assess whether this hormone is endowed with potent cardioprotective and vasodilatory properties, as observed for APLN (Ashley et al., 2005; Maguire et al., 2009), and therefore can serve as a therapeutic peptide for cardiac

repair/regeneration and blood-pressure control. In this regard, ELA may serve as a potent inducer of cardiac lineages in vitro, and along this line, potential loss-of-function *ELA* alleles may be associated with cardiovascular diseases in the general human population. Coincidentally, since APLNR permits entry of HIV-1 by serving as a coreceptor (Zou et al., 2000), recombinant ELA may serve as a competitive inhibitor of HIV-1 when APLNR is solicited for viral entry.

EXPERIMENTAL PROCEDURES

Accession Codes

The human *ELABELA* gene and its vertebrate homologs are accessible with the following Ensembl IDs: *Homo sapiens:* ENSG00000248329; *Mus musculus*: ENSMUSG0000007430; *Gallus gallus*: ENSGALG00000023444; and *Danio rerio*: ENSDARG00000094729.

Antibodies and Recombinant Peptide

Polyclonal antibodies were raised against a human N-terminal epitope (nh2-QRPVNLTMRRKLRKHNC), C-terminal epitope (CMPLHSRVPFP-cooh), or whole mature ELABELA peptide (nh2-QRPVNLTMRRKLRKHNCLQRRCMP LHSRVPFP-cooh) and peptide-affinity purified from rabbit sera. For western blotting, ELA antibodies were used at 1 μg/ml. α TGN-46 was purchased from Serotec (#AHP500GT). Recombinant mature ELA was synthetically produced and consists of the last 32 aa of ELA.

Developmental RT-PCR and Quantitative PCR Analysis

Total RNA was extracted from a group of 30 embryos at the designated stage using RNA Easy Kit (QIAGEN). cDNAs were synthesized from 1 μg of total RNA using random hexamers (Promega) and Superscript III reverse transcriptase (Invitrogen). RT-PCR was performed for 24 cycles and zebrafish *actin* was used as a loading control. Quantitative PCR (qPCR), normalized to *actin* expression levels, was performed with the SYBR Green Master Mix (Roche). The reactions were carried out in triplicate for each experiment and data were expressed as means ± SE. All qPCR primers used are listed in Supplemental Experimental Procedures.

Cell Culture and Immunofluorescence

The Shef4 cell line was used and exhibited the standard morphological and surface marker characteristics of hESCs and a normal 46XY karyotype (Inniss and Moore, 2006). Feeder-free hESCs were cultured in mTSER1 (Stem Cell Technologies) on Matrigel (BD 354277). For immunofluorescence, hESCs were dissociated and plated onto Matrigel-coated chamber slides (Millipore) and cultured for 48 hr. Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized in 0.5% Triton X-100 with 5% fetal calf serum in PBS, and stained overnight with α C antibody at 1 μg/ml.

Embryological Methods

The protocols used for fertilization, microinjections, secretion assay, and WISH are available at our protocol website (http://www.reversade.com-a.googlepages.com/protocols/). Zebrafish were maintained and used according to guidelines approved by the Singapore National Advisory Committee on Laboratory Animal Research. ZFNs were purchased from Sigma-Aldrich, and 250 pg of mRNAs encoding the ZFN pair was injected into 1-cell-stage embryos. The ZFN-binding sites within exon 1 are as follows: 5′_TCCACCCGCTGTA TCT_3′ and 5′_GCTGCTGCTGACAGT_3′. The sequencing primers flanking the mutation sites are as follows: forward 5′_AACACTTGCTGAGAGCGA CAG_3′, reverse 5′_AGATGTGGTGGTGTTGAGTAGC_3′. For *ela* overexpression, 200 pg of SP6-transcribed zebrafish *ela* capped mRNA (Applied Biosystems) was injected into 1-cell-stage zebrafish embryos. The translation-blocking morpholino oligonucleotides (MOs) used for *aplnra* and *aplnrb* have been described previously (Scott et al., 2007; Zeng et al., 2007) and were purchased from Gene Tools. Embryos were injected at the 1-cell stage with a combination of 1 ng of *aplnra* MO and 0.5 ng of *aplnrb* MO (Zeng et al., 2007). Information on the probes used for WISH can be found in the Supplemental Experimental Procedures. Zebrafish embryos at 100% epiboly were dechorionated and processed for western blotting as previously described (Link et al., 2006). For the *Xenopus* secretion assay, 16 ng of SP6-transcribed human *ELA* capped mRNA (Applied Biosystems) was injected into each cell of 4-cell-stage embryos. At stage 8, the embryos were dechorionated and dissociated in calcium magnesium-free medium (CMFM) with 5 mM EDTA. Cell pellets of ten embryos were transferred into 1.5 ml tubes and allowed to secrete for 24 hr at room temperature in 40 μl of fresh CMFM medium with or without BFA. The conditioned supernatant was carefully depleted of all cells before SDS-PAGE electrophoresis was conducted on 16.5% Tris-Tricine precast gels (BioRad).

AP-ELA Binding Assay

An AP-ELA fusion construct was generated by cloning the mature C-terminal 32-mer of ELA in frame with the carboxy terminus of the AP ORF, which contained an N-terminal signal sequence from *Xenopus chordin* (Reversade and De Robertis, 2005). This construct was transfected into 293T cells with Fugene HD (Promega) and allowed to secrete for 48 hr into serum-free media (Pro293a CDM; Lonza). The resulting supernatant was incubated with test cells for 3 hr, washed three times with PBS, lysed, and heated at 65°C to inactivate endogenous APs. Lysates were then incubated with BM Purple (Roche) at 37°C for chromogenic development.

DNA Constructs and Site-Directed Mutagenesis

Zebrafish *aplnra*, *aplnrb*, and *elabela* ORFs were cloned from 80% epiboly cDNA into vector pCS2+ between restriction sites BamHI and XbaI. Human *APLNR* and *GPR15* ORFs were cloned from human genomic DNA into pCS2+ between restriction sites BamHI and XbaI. The primers used for amplification are listed in the Supplemental Experimental Procedures. *aplnrb*^{grinch} (*W90L*) was generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) with the following primer pair: 5′_CTTTGTGGTGACCCTGCCCCT GTTGGCCGTCTACACTGCTCTG_3′ and 5′_CAGAGCAGTGTAGACGGCCA ACAGGGGCAGGGTCACCACAAAG_3′.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.11.002.

ACKNOWLEDGMENTS

We are grateful to all members of the Reversade laboratory for support. We also extend special thanks to May Aun Loh, Hui Yi Khoo, and Simone Laupheimer for technical support. We thank Davor Solter, Barbara Knowles, and Chin Yan Lim for constructive feedback. B.R. is a fellow of the Branco Weiss Foundation and an A*STAR and EMBO Young Investigator. This work was funded by a Strategic Positioning Fund for Genetic Orphan Diseases and an inaugural A*STAR Investigatorship from the Agency for Science, Technology and Research in Singapore.

Received: August 9, 2013
Revised: October 6, 2013
Accepted: November 4, 2013
Published: December 5, 2013

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