Mutations in half baked/E-cadherin block cell behaviors that are necessary for teleost epiboly

Donald A. Kane*,‡, Karen N. McFarland† and Rachel M. Warga*

Department of Biology, University of Rochester, Rochester, NY 14627, USA

*Present address: Department of Organismal Biology and Anatomy, University of Chicago, 1027 East 57th Street, Chicago, IL 60637, USA
†Present address: University of Virginia Health Systems, Department of Pathology, 415 Lane Road, Charlottesville, VA 22908, USA
‡Author for correspondence (e-mail: ddke@mail.rochester.edu)

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Summary

Epiboly, the spreading of the blastoderm over the large yolk cell, is the first morphogenetic movement of the teleost embryo. Examining this movement as a paradigm of vertebrate morphogenesis, we have focused on the epiboly arrest mutant half baked (hab), which segregates as a recessive lethal, including alleles expressing zygotic-maternal dominant (ZMD) effects. Here we show that hab is a mutation in the zebrafish homolog of the adhesion protein E-cadherin. Whereas exclusively recessive alleles of hab produce truncated proteins, dominant alleles all contain transversions in highly conserved amino acids of the extracellular domains, suggesting these alleles produce dominant-negative effects. Antisense oligonucleotides that create specific splicing defects in the hab mRNA phenocopy the recessive phenotypes and, surprisingly, some of the ZMD phenotypes as well. In situ analyses show that during late epiboly hab is expressed in a radial gradient in the non axial epiblast, from high concentrations in the exterior layer of the epiblast to low concentrations in the interior layer of the epiblast. During epiboly, using an asymmetric variant of radial intercalation, epiblast cells from the interior layer sequentially move into the exterior layer and become restricted to that layer; they participate in subtle cell shape changes that further expand the blastoderm. In hab mutants, when cells intercalate into the exterior layer, they tend to neither change cell shape nor become restricted, and many of these cells ‘de-intercalate’ and move back into the interior layer. Cell transplantation showed all these defects to be cell-autonomous. Hence, as for the expansion of the mammalian trophoblast at a similar developmental stage, hab/E-cadherin is necessary for the cell rearrangements that spread the teleost blastoderm over the yolk.

Key words: Epiboly, Radial intercalation, E-cadherin, Epiblast, Teleost, Morphogenesis, Genetics, Antisense, Zebrafish

Introduction

Here we report on the molecular identity and the cellular basis for the epiboly defects in mutant alleles of the half baked (hab) locus, identified in the Tübingen large-scale genetic screen for mutations that affect morphogenesis in the zebrafish, carried out at the Max Planck Institut für Entwicklungsbiologie (Haffter et al., 1996). Epiboly is blocked in the mutants hab£HV43, lawine£x18, avalanche£m94, weg£x230 and volcano£n712 (Kane et al., 1996; Solnica-Krezel et al., 1996). In all these mutants, epiboly begins and continues normally during the blastula and early gastrula stage. The first phenotypes are seen at 70 to 80% epiboly, about 1.5 to 2 hours after the onset of gastrulation, when the mutants begin to arrest their vegetalward spreading. Curiously, the enveloping layer, a thin epithelium that covers the deep cells of the blastoderm, and the syncytial layer of the yolk cell are unaffected and both complete epiboly normally; only the movements of the deep cells are arrested. Hence, the mutants demonstrate that some aspects of the epiboly of the deep cells are under a separate control from that of the enveloping layer and the yolk cell.

Additionally, three of the five epiboly mutants display dominant phenotypes. The mutants hab£HV43, avalanche£m94 and lawine£x18 display a zygotic-maternal dominant (ZMD) effect that is expressed when both zygotic and maternal genomes are heterozygous for the mutant locus. This phenotype is displayed as an intermediate rate of epiboly between that of wild-type and homozygous mutant siblings. These embryos, termed ZMD mutants, complete epiboly about an hour after wild-type siblings, and later, during somitogenesis, cells dorsal to the developing neural tube round up and detach from the embryo (Kane et al., 1996). Also, hab£HV43 mutants display a semidominant trait of an enlarged hatching gland.

Although the epiboly mutants failed to complement one another, they were named separately because complementation testing was complicated by the dominant effects. In recent work, submitted elsewhere, we have shown that all the mutants (with the exception of vol) map to a single locus near the centromere of Linkage Group 7, and the locus is provisionally named the hab locus. Here we establish that all of the mutations at the hab locus are in a single gene. Hence, all the alleles have been renamed hab, following the precedent for the nomenclature of somitahab/captain hook (Kramer et al., 2002), which also includes dominant and recessive alleles.

In the first portion of this work, we demonstrate that the alleles of hab are mutants in a single gene that encodes a zebrafish homolog of E-cadherin, an important membrane
protein necessary for homotypic cell adhesion (Takeichi, 1987). In the mouse, E-cadherin mRNA is present maternally and E-cadherin function is necessary for the process of cell compaction in the 8-cell stage embryo (Ao and Erickson, 1992). Later, as zygotic expression of E-cadherin begins, it is necessary for the expansion of the trophectoderm (Larue et al., 1994). In the zebrafish, E-cadherin mRNA is present maternally (Babb et al., 2001), and experiments based on antisense oligonucleotides have shown that E-cadherin is necessary for blastomere adhesion during the cleavage stage, and later for aspects of morphogenesis during gastrulation and epiboly (Babb and Marrs, 2004). In frogs, based on dominant negative analysis, E-cadherin seems more important for tissue integrity during early development (Heasman et al., 1994; Levine et al., 1994), whereas the closely related C-cadherin is necessary for morphogenesis (Lee and Gumbiner, 1995). However, the two cadherins overlap in their expression patterns and possibly functionally as well. In all creatures, during and after gastrulation, E-cadherin is expressed in the epidermal ectoderm, in a pattern complementary to the expression of N-cadherin in the neural ectoderm, and in the endoderm (Rutishauser et al., 1988; Takeichi, 1995). Later, reflecting its diverse role in numerous developmental and epithelial tissue functions, E-cadherin is also expressed in many epithelial tissues, including some that are mesodermally derived.

At the structural level, the protein is characterized by five extracellular cadherin (EC) repeats, a single pass transmembrane domain and a cytoplasmic domain. The EC repeats are extremely conserved among their homologs in other species, and are necessary for the specific adhesion properties of E-cadherin (Blaschuk et al., 1990). The cytoplasmic domain of the protein binds α- and β-catenin, and indirectly actin, simultaneously anchoring the protein to the cytoskeleton of the cell and connecting the molecule to the WNT signaling pathway (Gumbiner and McCrea, 1993; Herrenknecht et al., 1991; Kintner, 1992; McCrea et al., 1991; Sanson et al., 1996). The protein is thought to function as a dimer, and the EC repeats are necessary for this dimerization (Brieher et al., 1996; Nagar et al., 1996). This fact has been the basis for the creation of transgenes that abate gene function: deleting one or two of the extracellular cadherin alleles were isolated in a large-scale mutagenesis screen (Haffter et al., 1996) and initially out-crossed to the polymorphic WIK (L11) strain of wild-type fish for mapping. Subsequent generations were out-crossed to various wild-type strains of fish that were prescreened for polymorphisms in the appropriate microsatellite markers surrounding the hab locus. Fish carrying the hab<sup>mur44</sup> allele were a gift from Dr Solnica-Krezel (Vanderbilt University). In this report, mutant embryos were always produced by mating two heterozygous parents. In the case of the ZMD alleles, this produced recessive mutants, ZMD mutants and wild-type embryos in a 1:2:1 ratio. Although there is a very slight maternal effect in the wild-type embryos produced from ZMD crosses, the phenotype is well within the variation seen in normal wild-type embryos.

In the second portion of this work, we examine the role of hab in the morphogenesis of the epiblast during zebrafish epiboly, focusing on differences in cell movement between hab mutants and wild-type embryos. The epiblast forms from all the deep cells of the blastoderm, and, somewhat similarly to the amniote epiblast, contributes cells to the hypoblast throughout gastrulation. Afterward, the epiblast forms the anlage for the ectodermal derivatives, whereas the hypoblast forms the anlage for mesodermal and endodermal derivatives. The epiblast thins as it spreads over the yolk cell, from about five cells thick at doming stage to about two cells thick at 100% epiboly, and finally to one cell thick in all but the axial region in the early segmentation stages. We hypothesized that this thinning may be due to the morphogenetic process of radial intercalation, the means where two or more layers of cells thin into a single layer, thus causing an expansion in surface area of the resulting layer. Using scanning electron micrographs of freeze-fractured blastulae, radial intercalation was classically described in the amphibian blastocoel roof (Keller, 1980), and this process is thought to be a driving force for the expansion of the amphibian animal cap. Radial intercalation is also known to occur in the zebrafish blastula as the blastoderm thins during the doming stage, and is renowned for its annoying property of mixing blastula cell lineages, thus causing the indeterminate early fate map of zebrafish (Helde et al., 1994; Kimmel and Warga, 1987; Warga and Kimmel, 1990; Wilson et al., 1995).

It is notable that in all the above examples, frog and fish, it is not clear if radial intercalation by itself is a force generating movement or if the movement is a passive response of a tissue to outside forces, e.g. being stretched or being compressed between two opposing tissues, as has been suggested by Wilson et al. (Wilson et al., 1995).

Here we describe the cell behaviors that drive late epiboly in the zebrafish embryo. We find two previously unrecognized layers in the zebrafish epiblast, the exterior layer of the epiblast and the interior layer of the epiblast, and we show that each of these layers have unique functions in this zebrafish version of radial interaction. In hab mutant embryos, these layers do not form completely and other cellular behaviors involved with radial intercalation do not occur normally. Finally, using cell transplantation, we show that the hab defects occur cell-autonomously, demonstrating that defects in radial intercalation are the most likely cause for the hab epiboly defect.

Materials and methods

Zebrafish strains

The hab<sup>des43</sup>, hab<sup>nod4</sup>, hab<sup>sl5</sup>, and hab<sup>M230</sup> alleles were isolated in a large-scale mutagenesis screen (Haffter et al., 1996) and initially out-crossed to the polymorphic WIK (L11) strain of wild-type fish for mapping. Subsequent generations were out-crossed to various wild-type strains of fish that were prescreened for polymorphisms in the appropriate microsatellite markers surrounding the hab locus. Fish carrying the hab<sup>mur44</sup> allele were a gift from Dr Solnica-Krezel (Vanderbilt University). In this report, mutant embryos were always produced by mating two heterozygous parents. In the case of the ZMD alleles, this produced recessive mutants, ZMD mutants and wild-type embryos in a 1:2:1 ratio. Although there is a very slight maternal effect in the wild-type embryos produced from ZMD crosses, the phenotype is well within the variation seen in normal wild-type embryos.

Positional cloning of the hab locus

hab was initially mapped to the centromere of Linkage Group 7 by half tetrad analysis (Johnson et al., 1995). For fine-resolution mapping we used a panel of 2916 haploid embryos and identified two closely linked microsatellite markers on either side of the hab locus (Knapik et al., 1998). These markers were used to isolate clones from a large-insert genomic library (Amemiya et al., 1999) and construct a contig, as has been described (Wei and Malicik, 2002). Clones spanning the critical region of hab were sequenced as part of the ongoing Zebrafish Genome Project (GenBank Accession numbers: PAC18005: AL929461; PAC10901L11: AL929295). To determine the site of the hab mutations we isolated total RNA from wild-type and hab embryo pools and generated E-cadherin cDNA by reverse transcription and PCR amplification using five overlapping primer pairs designed from the published cDNA sequence and compared sequence. We confirmed each mutation by isolating total RNA or genomic DNA from individual mutant and wild-type embryos to generate sequence for the site of lesion. Sequences of PCR primers are available from the corresponding author upon request.
**hab morpholino oligonucleotides**

*hab*E-cadherin splice site-targeting morpholino oligonucleotides were designed from trace sequences deposited by Sanger before the draft sequence was available. Exons were identified by comparison of the mRNA sequence to the genomic sequence and numbered according to mouse terminology (Ringwald et al., 1991). Morpholinos were purchased from Gene Tools, the sequence of MO1 is: 5′-GTACACACAGTAACCTTTACAGTG-3′; and the sequence of MO2 is: 5′-AAGCAATTTCATCCCTCTGTGAC-3′. We monitored splicing events by RT-PCR using pools of five embryos for each developmental timepoint. Sequences of PCR primers used for monitoring are available from the corresponding author upon request.

**Embryo manipulations and genotypic characterization**

For targeted gene-knockdown, we injected wild-type embryos with morpholino oligonucleotides (Gene-Tools) at the 1-cell stage. For transplants, we labeled donor embryos derived from crosses of *habmut4* heterozygotes with a mixture of 3% rhodamine-dextran and 3% biotin-dextran (Molecular Probes) at the 1- to 8-cell stage and transplanted donor cells into wild-type embryos at the onset of epiboly using standard procedures (Ho and Kane, 1990). Donor embryos were harvested and genotyped using the closely linked microsatellite marker Z20715 (F-5′-CATCCTGATAGTGCGCCAGCA-3′, R-5′-GTGTCGGGTAGGTGAC-3′). Host embryos were fixed at 80% epiboly and processed as below for the co-injected biotin-dextran. For in vivo cell labeling, we injected a single superficial blastomere with 3% rhodamine-dextran at the 2k- to 4k-cell stage using standard procedures (Warga and Nüsslein-Volhard, 1999) in embryos derived from crosses of *hab**dvi**4* heterozygotes. For these experiments we selected parental strains that gave less severe epiboly phenotypes so that survival of homozygous offspring persisted through the epiboly period. Embryos were genotyped by phenotype at the 100% epiboly stage based on the epiboly arrest trait of homozygous embryos and the detached cell trait of heterozygous embryos. Heterozygous embryos were reconfirmed at 24 hours by the enlarged hatching gland trait.

**Immunohistochemistry and RNA in situ hybridization**

Embryos injected with biotinylated-dextran were processed as described in *the Zebrafish Book* (Westerfield, 1993), cleared, mounted in Permount™ (Fischer Scientific) and photographed. Antibody staining was carried out as described (Warga and Nüsslein-Volhard, 1999); briefly, embryos derived from either wild-type parents or *hab**dvi**4* heterozygous parents were stained with anti-β-catenin (Sigma), embedded in 17% gelatin: 50% glycerol and bisected along the 90° meridian by hand using a razor blade. Afterward, these hand-sections were cleared, mounted in Permunt and photographed.

RNA in situ hybridization was carried out as described (Thiss et al., 1993) using embryos derived from wild-type parents. Afterward, hand-sections were cut as described above, cleared in 70% glycerol and photographed.

**Time-lapse and data analysis**

For in vivo observations, embryos derived from *hab**dvi**4* heterozygotes were mounted and recorded in multi-plane as previously described (Warga and Kane, 2003). Afterward, the recordings were analyzed using Cytos Software, looping half-hour segments from the time-lapse recording. In the case of Fig. 7, black and white images from one plane of the time-lapse video recording were imported into Adobe Photoshop and pseudo-colored to aid in presentation, matching the color of cells shown in the model in Fig. 9.

For analysis of cell shape, multiple planes from one time-lapse video segment were imported into Adobe Photoshop and reconstructed three-dimensionally so that individual cells in both the exterior and interior layer of the epiblast layer could be outlined as shown in Fig. 6. Both aspect ratio and cell surface area were calculated by importing these outline images into NIH Image and running macro programs, as previously described in Warga and Kane (Warga and Kane, 2003).

For analysis of cell-autonomy, chimeras were cleared, mounted and the entire donor population recorded using a Zeiss Axioptih II equipped with a CDC-300-RC camera (Dage MTI) with a digital output to a Power Macintosh 9600/350. Images were then imported into Adobe Photoshop, reconstructed three-dimensionally and analyzed in NIH Video, as described above. For the analysis of cell restrictions in the exterior layer of the epiblast, we examined clones of cells in face view when embryos reached 75% epiboly using a Zeiss DIC 40x water immersion lens on a Zeiss Axioskop equipped with a Sony F-707 Digital Camera. Using the EVL layer as a reference, cell depth was determined by counting the number of cells between the surface and the labeled cell. Clones in and near the axis were excluded because of the more complex layering in the neural keel.

**Results**

**The half baked mutations map to the gene encoding the zebrafish homolog of E-cadherin**

Using microsatellite markers, we mapped *hab* to a 0.2 M region near the centromere on Linkage Group 7 and, walking with overlapping P1-derived artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones (Amemiya et al., 1999), cloned the 500 kb region between flanking markers (Fig. 1). Clones that mapped within recombinants with *hab* were sequenced, revealing several cadherin-like sequences similar to the zebrafish homolog of E-cadherin cDNA (Babb et al., 2001). Further analysis showed severe rearrangements in the clones, which were probably artifacts that occurred in the library vectors or, in some cases, in the initial assembly of the sequence data. Nevertheless, one clone, PAC 71B9, contained the genomic sequence of zebrafish E-cadherin extending from exon3 to exon14.

All the *hab* alleles were sequenced from cDNA generated by RT-PCR (Fig. 2). Two of these alleles, *hab**dvi**20* and *hab**mut4*, were exclusively recessive and both contained nucleotide transversions that produced stop codons in the EC4 domain at amino acid positions 547 and 553, respectively. We noted in these experiments a reduction in the recovery of the RT-PCR product (data not shown), suggesting that nonsense-mediated transcript degradation was occurring. The other three alleles had ZMD effects. Two of these ZMD alleles, *hab**mut4* and *hab**dvi**18*, had mutations in the nucleotides that encoded the EC1 domain. In *hab**mut4*, the well-conserved valine162 was changed to an aspartic acid, and in *hab**dvi**18*, the well-conserved tyrosine176 was changed to an asparagine. The third dominant allele, *hab**dvi**4*, had an additional phenotype, a semi-dominant hatching gland enlargement trait; this allele possessed a mutation in the nucleotides that encoded the EC5 domain, changing the well-conserved isoleucine600 to a lysine.

**Phenocopy of the dominant and recessive *hab* phenotypes using antisense oligonucleotides**

To date, we have not completed the construction of an appropriate construct of *hab* to produce mRNA that could be used to rescue *hab* mutants. Therefore, to confirm that these mutations caused the *hab* phenotype, we injected antisense oligonucleotides into embryos to disrupt the zygotic gene function of *hab* and compared the knockdown phenotypes to that of *hab* mutants. Annealing antisense oligonucleotides to...
the splice donor site causes splicing defects, which in turn, disrupt specific regions of the protein product (Draper et al., 2001). Having no effect on already processed mRNAs, this approach knocks down the zygotic function of the targeted gene but not the maternally expressed mRNA product. Hence, the phenocopy should resemble the zygotic phenotype, not the potential maternal phenotype. The oligonucleotide MO1 (Fig. 3A) was designed to anneal with the exon9-intron9 junction. Embryos injected with MO1 produced a phenocopy that was very similar to the epiboly arrest of the hab recessive phenotype (Fig. 3B). At reduced doses, the phenocopy became less extreme, similar to the slowing of epiboly in the ZMD mutants, and no additional phenotypes appeared in the surviving embryos. Sequencing the RT-PCR product after the experiment showed that intron9 found a cryptic donor site within exon9 at gt1329, producing a small deletion in the mRNA (Fig. 3C). This caused a frameshift error upon entering exon10, and the conceptual protein (Fig. 3D) was truncated at stop codon in the downstream region of EC3, at approximately amino acid 420, a product 130 amino acids shorter than that predicted for the recessive hab alleles.

In a second experiment, the oligonucleotide MO2 was designed to target the exon7-intron7 junction. MO2 caused an extreme phenocopy of the hab recessive phenotype, displaying not only an epiboly arrest, but also causing the disaggregation of cells throughout the blastoderm (arrowhead in Fig. 3E), a phenotype often seen immediately before homozygous mutant embryos dissociate. At intermediate doses, we noticed that the blastoderm tended to thin unevenly before the epiboly arrest (arrow in Fig. 3E). At low doses, besides slowing epiboly, cells detached from the region dorsal of the neural tube, an aspect of the neural tube closure trait of the ZMD phenotype (Fig. 3E,F,G). Sequencing the knockdown product showed that MO2 caused an exon jump, producing a large in-frame deletion that removed all of exons 5 through 8 (Fig. 3H,I). In the conceptual protein, this resulted in a deletion from isoluecine165 to glutamic acid322, effectively removing the EC1 and EC2 domains of hab while leaving the signal sequence and pro domains intact, as well as the entire carboxy half of the protein. Thus, the MO2 protein product would be predicted to insert normally in the cell membrane but be lacking the function of the external EC domains of the protein, and may have similar effects to the dominant hab alleles.
alleles that possess transversions in the amino acids of the EC1 domain.

A radial gradient of hab expression in the non axial blastoderm during epiboly

To analyze the distribution of the hab gene product, we performed whole-mount in situ hybridizations, focusing our investigation on the embryonic epiblast before and during late epiboly, the time when the epiboly arrest trait of the mutant is most strongly expressed. At this stage, the epiblast is sandwiched between the very thin epithelium of the EVL, and, on the inside of the embryo, the yolk cell or, if present, the hypoblast.

Using a probe made from the full-length hab cDNA sequence, we found that cleavage stage embryos expressed hab ubiquitously, showing that the mRNA was present maternally (Fig. 4A), as shown previously (Babb et al., 2001). At 30% epiboly, hab expression began to fade, revealing a very subtle radial gradient of expression (Fig. 4B) from the deepest layers of the blastoderm (lowest expression) to the superficial cells of the blastoderm (highest expression). By shield stage, the epiblast began to clear on the dorsal side of the embryo and the gradient became steeper elsewhere (Fig. 4C). At the same time, hab was strongly expressed in the entire EVL layer (Fig. 4C,D), forming a ring of expression around the nuclei of these thin epithelial cells. Expression was especially high in a subpopulation of EVL cells that become the forerunner cells. At 70% epiboly, hab expression was maintained on the lateral and ventral sides of the epiblast, and, within those regions, formed a steep radial gradient that was highest in the cells just beneath the EVL (Fig. 4E,F), and was absent in the yolk cell. Expression of hab was maintained in the EVL and cleared in the axial portion of the epiblast (Fig. 4F,G); interior to this zone of clearing, expression was seen in the anterior edge of the hypoblast (Fig. 4F,G), as shown previously (Babb et al., 2001), and is presented as an ad hoc control to demonstrate the penetration of the whole-mount in situ probe.

Within these experiments, we also examined preliminary expression patterns of hab in the recessive mutants of the missense allele hab<sup>tx230</sup> and the nonsense allele hab<sup>tx230</sup>. In hab<sup>tx230</sup> mutants (Fig. 4I), we found almost complete absence of transcript. This is consistent with our difficulty in recovery of RT-PCR product for sequencing this mutant, as mentioned above, and further corroborates the idea that nonsense-mediated transcript degradation is occurring in the premature stop codon alleles.

Identification of the exterior layer and interior layer of the epiblast

During the examination of the above in situ experiments, there seemed to be slight differences in cell morphology that correlated with the concentration of hab in the superficial and deep cell layers of the epiblast. After fixing homozygous mutant and wild-type embryos at 80 to 90% epiboly, staining to visualize the cell membranes, and sectioning the embryos, two distinct layers of the epiblast could be distinguished on the ventral and lateral sides of the embryo (Fig. 5). The exterior layer of the epiblast was epithelial-like, containing thin cuboidal cells that spread under, and perhaps against, the EVL. In between the exterior layer and the hypoblast, there was an interior layer of the epiblast, which consisted of disorganized cells that tended to be oriented with their long axis...
perpendicular to the surface of the epiblast. In general, the border between the two layers was indistinct, and often individual cells were spanning the border. In homozygous hab mutants, cells in both layers were rounder than their wild-type counterparts: the cells of the exterior layer of the epiblast were less spread out, and the cells of the interior layer were less elliptical. Indeed, the mutant layers were so similar that it was difficult to say which layer contained the extra cells. Also, when compared with wild-type embryos, the exterior epiblast formed a less continuous epithelium in hab mutants (which can be seen nicely in comparisons among homozygous mutants and wild-type embryos in Fig. 8).

**Abnormal morphology of the epiblast layers in hab mutants is cell-autonomous**

To quantitatively measure differences between hab mutant and wild-type embryos, we examined the area and aspect ratio of cells in optical sections of live embryos at 80 to 90% epiboly and found highly statistically significant differences between the exterior and interior layers of the epiblast. When viewed obliquely, cells of the exterior epiblast were spread out and displayed a high aspect ratio (Fig. 6A), which tended to align with the dorsal side of the embryo, as previously shown (Concha and Adams, 1998). By contrast, the cells of the interior layer of the epiblast, which had their long axes perpendicular to the surface of the embryo, displayed a small diameter with no aspect ratio in the plane parallel to the cell surface. In homozygous mutants (Fig. 6B), cells in the exterior layer of the epiblast had smaller apparent diameters, consistent with their being less spread out, and cells in the interior layer of the epiblast had larger diameters, consistent with their loss of a radial aspect ratio. Calculations of cell volumes showed no difference between the volumes of interior and exterior cells, or cells in mutant and wild-type embryos (data not shown).
To test the cell-autonomy of hab, we transplanted cells from homozygous mutant and wild-type siblings into the epiblast of wild-type hosts, and fixed the embryos at 80 to 90% epiboly. Examination of the morphology of the donor cells in the fixed material showed that cells from the mutant donors had strikingly identical sizes and aspect ratios to the measurements in the live mutants (Fig. 6C,D), demonstrating that the mutation acts cell-autonomously. We also noted the relative contributions from the homozygous mutant and wild-type donors to the exterior and interior layers of the host epiblast. Wild-type cells tended to contribute to the exterior layer of the epiblast (286 exterior:104 interior), whereas homozygous mutant cells did not (111 exterior:196 interior). Hence, although mutant cells are able to contribute to the exterior layer of the epiblast, they tend not to, and this bias is cell-autonomous.

Abnormal radial intercalation in hab mutants

We hypothesized that a combination of radial intercalation and cell shape changes drive the spreading of the blastoderm during epiboly. To directly observe these processes, we recorded homozygous mutant, ZMD mutant and wild-type siblings during late epiboly and documented occurrences of cells intercalating into – and out of – the exterior layer of the epiblast (Fig. 7). In ZMD mutants and wild-type embryos, as newly intercalated cells entered the exterior layer, they flattened to the dimensions typical of cells in that layer. This is shown for a ZMD hab<sup>divd4/r</sup> mutant in Fig. 7A. The process of entering the exterior layer and flattening typically took about 15 minutes and is shown in the supplementary material (Movies 1-3).

In homozygous mutants, cells from the interior layer also intercalated into the exterior layer (Fig. 7B), although the cells never completely flattened out. This process occurred somewhat quicker than in wild-type embryos, taking about 8 to 10 minutes. Surprisingly, in the homozygous mutants, exterior layer cells often returned to the interior layer (Fig. 7C). Examining time-lapse records over a 15 minute interval, we measured intercalation and de-intercalation events in homozygous mutants, ZMD mutants and wild-type embryos (Table 1). De-intercalation events were never seen in wild-type embryos. However, in homozygous mutants, the rate of de-intercalation was almost equal to the rate of intercalation, suggesting that loss of the cells in the exterior layer of the epiblast is the physical basis for the arrest of epiboly. In ZMD mutants, there was approximately one de-intercalation event for every three or four intercalation events, which roughly correlates with the slowing of epiboly observed in the ZMD mutants.
Following the evidence above, it seems that individual cells must enter the exterior layer of the epiblast (via radial intercalation), then change cell shape, and afterward they become restricted to that layer. To test the restriction aspect of this hypothesis, we labeled individual cells near the animal pole when the blastoderm was at the 2000 to 4000 cell stage, shortly before the beginning of epiboly. Cells labeled in this period divided quickly over the next few hours, with the first division usually perpendicular to the plane of the surface, producing clonal descendents distributed between the EVL and the most superficial layer of the deep cell layer. Because cells of the EVL are restricted after the beginning of epiboly (Kimmel et al., 1990), they were disregarded for the remainder of the analysis. In other experiments, started slightly later, we labeled deep cells directly. At 80% epiboly, we examined the clones, counting the cells in the exterior layer of the epiblast and the approximate first and second interior layers. In general, clone sizes were about eight cells per embryo, and there was no difference in cell division between clones in mutant and wild-type embryos. Clones in wild-type embryos tended to remain in the exterior layer of the epiblast (Fig. 8A,E). Some cells were found in the interior layers, but because we did not follow the cells, e.g. by time-lapse analysis, we do not know when they entered that layer. It must have been quite early, because in our analysis during late epiboly (Table 1), we never saw wild-type cells leaving the exterior layer. Clones in homozygous mutant embryos tended to be found equally in all layers of the epiblast (Fig. 8C,D,E), and clones in ZMD mutants showed distributions intermediate between the homozygous mutant and wild-type embryos (Fig. 8B,E). Therefore, notwithstanding cells that are lost to the hypoblast, cells of the exterior layer of the epiblast normally remain in that layer throughout epiboly, and in mutant embryos, they do not.

**Cellular restrictions of the exterior layer of the epiblast**

Following the evidence above, it seems that individual cells must enter the exterior layer of the epiblast (via radial intercalation), then change cell shape, and afterward they become restricted to that layer. To test the restriction aspect of this hypothesis, we labeled individual cells near the animal pole when the blastoderm was at the 2000 to 4000 cell stage, shortly before the beginning of epiboly. Cells labeled in this period divided quickly over the next few hours, with the first division usually perpendicular to the plane of the surface, producing clonal descendents distributed between the EVL and the most superficial layer of the deep cell layer. Because cells of the EVL are restricted after the beginning of epiboly (Kimmel et al., 1990), they were disregarded for the remainder of the analysis. In other experiments, started slightly later, we labeled deep cells directly. At 80% epiboly, we examined the clones, counting the cells in the exterior layer of the epiblast and the approximate first and second interior layers. In general, clone sizes were about eight cells per embryo, and there was no difference in cell division between clones in mutant and wild-type embryos. Clones in wild-type embryos tended to remain in the exterior layer of the epiblast (Fig. 8A,E). Some cells were found in the interior layers, but because we did not follow the cells, e.g. by time-lapse analysis, we do not know when they entered that layer. It must have been quite early, because in our analysis during late epiboly (Table 1), we never saw wild-type cells leaving the exterior layer. Clones in homozygous mutant embryos tended to be found equally in all layers of the epiblast (Fig. 8C,D,E), and clones in ZMD mutants showed distributions intermediate between the homozygous mutant and wild-type embryos (Fig. 8B,E). Therefore, notwithstanding cells that are lost to the hypoblast, cells of the exterior layer of the epiblast normally remain in that layer throughout epiboly, and in mutant embryos, they do not.

**Discussion**

Based on identification of the mutant sequence and reproduction of the mutant phenotype with antisense oligonucleotides, we have shown that hab encodes the zebrafish homolog of E-cadherin. Here we discuss the nature of the mutations of hab. Then we consider the cellular behaviors that drive epiboly of the epiblast, which when disrupted, cause the hab phenotype.

**The recessive alleles of hab act as nulls for zygotically expressed E-cadherin**

The cell membrane protein E-cadherin requires the function of five EC domains, which facilitate the homophilic binding among cells, and a cytoplasmic portion necessary for interactions with the cytoskeleton. The recessive alleles of hab and the MO1 antisense oligonucleotide knockdown product all result in truncated proteins due to premature stop codons in the EC3 or EC4 domains. These mutations should produce

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### Table 1. Rates of intercalation in hab and wild-type embryos

<table>
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<tr>
<th>Genotype</th>
<th>Number of cells (number of embryos)</th>
<th>Intercalation events</th>
<th>De-intercalation events</th>
<th>Rate of intercalation</th>
<th>Rate of De-intercalation</th>
</tr>
</thead>
<tbody>
<tr>
<td>hab–/–</td>
<td>435 (3)</td>
<td>41</td>
<td>35</td>
<td>37.7/100 h⁻¹/²</td>
<td>32.2/100 h⁻¹/²</td>
</tr>
<tr>
<td>ZMD hab+/–</td>
<td>326 (3)</td>
<td>27</td>
<td>8</td>
<td>33.1/100 h⁻¹/²</td>
<td>9.8/100 h⁻¹/²</td>
</tr>
<tr>
<td>Wild-type</td>
<td>321 (3)</td>
<td>29</td>
<td>0</td>
<td>36.1/100 h⁻¹/²</td>
<td>&lt;0.6/100 h⁻¹/²</td>
</tr>
</tbody>
</table>

*All embryos are siblings from a single cross, hab<sup>dv43/6</sup> × hab<sup>dv43/6</sup>.

<table>
<thead>
<tr>
<th>Genotype&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Number of cells (number of embryos)</th>
<th>Intercalation events</th>
<th>De-intercalation events</th>
<th>Rate of intercalation</th>
<th>Rate of De-intercalation</th>
</tr>
</thead>
<tbody>
<tr>
<td>hab–/+</td>
<td>326 (3)</td>
<td>27</td>
<td>8</td>
<td>33.1/100 h⁻¹/²</td>
<td>9.8/100 h⁻¹/²</td>
</tr>
<tr>
<td>ZMD hab–/+</td>
<td>321 (3)</td>
<td>29</td>
<td>0</td>
<td>36.1/100 h⁻¹/²</td>
<td>&lt;0.6/100 h⁻¹/²</td>
</tr>
</tbody>
</table>

<sup>*</sup>Total number of cells at the beginning of the recording.

<sup>†</sup>Events measured over a 15 minute interval at approximately 75% epiboly stage.

<sup>‡</sup>Rates calculated as events per 100 cells per hour; The s.e.m. is ~±6/100 h⁻¹/².
proteins that contain the signal sequence and the pro domain and the first two or three EC domains, but lack the inner EC domains, the transmembrane domain and the cytoplasmic portion of the protein. Hence, we imagine that the hab recessive alleles are functional nulls. Interestingly, both of the hab recessive alleles were truncated in EC4, similar to two of the three recessive alleles of parachute (pac), which encodes the zebrafish homolog of N-cadherin (Lele et al., 2002); these pac alleles are thought to be recessive nulls. Nevertheless, in the cases of either hab or pac, it is possible that the truncated protein products are secreted and have some unknown function.

Even if null, probably none of the hab phenotypes display the lack-of-function phenotype of the gene, for hab is maternally supplied. The lack-of-function phenotype may resemble the knockdown phenotype of using 5′ antisense oligonucleotides seen by Babb and Marrs (Babb and Marrs, 2004), which should knock down maternal mRNA expression, or the phenotype could be more severe.

The ZMD alleles of hab act as dominant negatives

Our interpretation of the dominant phenotypes is that they are caused by mutations acting as antimorphic alleles. It is thought that cadherins act as dimers, and the EC repeats are necessary for dimerization (Briere et al., 1996; Nagar et al., 1996; Takeda et al., 1999). Based on sequence analysis, all the dominant alleles and the MO2 antisense knockdown product should produce proteins that are properly inserted into the cell membrane of the cells that normally express wild-type hab, but possess one or more nonfunctional EC domains. These mutant proteins should dimerize with wild-type proteins, and thus interfere with gene function in a dominant negative fashion.

The dominant negative effects of the ZMD alleles are consistent with the strengths and behaviors of their phenotypes. First, in the case of heterozygous embryos derived from a wild-type mother and a father heterozygous for a ZMD allele, there is no dominance. In this case, we imagine that the maternally encoded protein is capable of competing the effect of the one zygotic copy of the dominant allele. By this argument, hab is the strongest of the dominant alleles, as it alone has a dominant effect in the absence of a heterozygous mother. Second, when homozygous, the dominant alleles express more severe epiboly defects than the recessive alleles of hab (Kane et al., 1996). Here we imagine that the zygotic products of the dominant allele partially inactivate the maternally supplied products, and the phenotype is shifting toward what we would imagine is the maternal phenotype.

In the case of the ZMD alleles, the location of the mutation within the protein correlates with specific aspects of the dominant phenotypes, suggesting that domains of the protein may possess different functions in different regions of the embryo. For example, both hab<sup>p128</sup> and hab<sup>em94</sup> possess
mutations in the EC1 domain, and both their ZMD neural tube phenotypes look very similar. Both phenotypes express defects focused in the tail, and there, the defects are severe. However, the hab<sup>tv43</sup> allele possesses a mutation in the EC5 domain. Its ZMD neural tube phenotype is expressed the entire length of the neural axis, but, in general, the effect is not locally severe. Moreover, the hab<sup>tv43</sup> allele has the dominant hitching gland defect; hab<sup>mm63</sup> and hab<sup>tv91</sup> do not. Interestingly, the one pac allele that is reported to possess some dominant effects is a missense mutant in EC1 of N-cadherin (Malicki et al., 2003). This particular mutant also has allele-specific phenotypes, expressing a more severe phenotype in the eye than the recessive pac alleles.

In humans, gastric cancers that are particularly invasive have been traced to familial inherited mutations in E-cadherin (Hajra and Fearon, 2002), and some of these mutations are transversions that result in amino acid substitutions in the EC1 and EC5 domains. Many of our fish lines that carry the dominant alleles show reduced long-term survival, and it will be of interest to examine the fish that survive only to early adulthood.

A model for expansion of the epiblast in the zebrafish embryo

Our results suggest that a combination of radial intercalation with other cell behaviors drive the spreading of the non-axial epiblast during late epiboly. We have shown the existence of two cell layers in the epiblast, which we term the ‘interior layer’ and ‘interior layer’ (Fig. 9). Closely opposed to the well-organized epithelium of the EVL, cells of the exterior layer formed a continuous layer that roughly resembled a cuboidal epithelium. Underlying the exterior layer was the interior layer, a somewhat disorganized multilayer consisting of cells that tended to be aligned in a radial direction. Here we have documented that the cells of the interior layer of the epiblast moved into the exterior layer via radial intercalation. Once there, cells of the exterior layer tended to remain in the exterior layer, and became restricted to that layer.

We propose that the expansion of the epiblast has three components: first, cells of the interior layer radially intercalate into the exterior layer; second, the cells flatten as they join the epithelium of the exterior layer; third, these cells become restricted to the exterior layer.

Biomechanically, the model spreads the epiblast by two means. First, the process of radial intercalation increases the number of cells in the exterior layer of the epiblast, and thus expands the surface area of the epithelium, similar to the effect of radial intercalation in amphibians. Note that to secure this effect the cells should not leave the exterior layer. Second, the flattening of the cells further increases the surface area of the exterior epiblast. It is of interest that the exterior layer of the epiblast appeared to spread out against the EVL, as a substrate of sorts. Whereas this attachment was quite weak, for the epiblast freely slid under the EVL, the subtle interactions between the two layers might have mechanical consequences that are underappreciated.

The expansion of the zebrafish epiblast seemed to show a remarkable blend of Xenopus and mouse morphogenesis. The cell shape changes reported here for the exterior layer of the epiblast during teleost epiboly appeared very similar to those thought to occur during the expansion of the trophoderm epithelium (Barcroft et al., 1998; Fleming et al., 2001; Reima et al., 1993). Based on morphogenetic arguments, the formation of the mammalian trophoderm and the epiboly of the teleost blastoderm over the yolk have been suggested to be similar events (Kane and Adams, 2002). Interestingly, both these structures fail to form normally after zygotic E-cadherin is abrogated by mutation in either species (Kane et al., 1996; Larue et al., 1994), demonstrating that they are among the first structures that require the zygotic expression of E-cadherin. Observations such as these open questions as to the homology of these two structures.

Compared with radial intercalation in other model systems, teleost radial intercalation displays a number of unique differences. First, teleost epiboly may involve substrates, such as the EVL, that could organize radial intercalation. In urodeles, with the underlining of the animal cap by the void of the blastocoele, all the cell layers of the animal cap appear to participate equally in radial intercalation (Keller and Shook, 1994), resulting in a single layer at the completion of the process, and demonstrating that radial intercalation can be a substrate-free process. In this regard, zebrafish may have some similarities to Xenopus, which also possesses an outer epithelial layer that does not participate in radial intercalation (Keller, 1980). Second, teleost epiboly is markedly asymmetric. In amphibians, and especially in urodeles, there is an impression of two or three somewhat similar layers merging into a single epithelium. In teleosts, the exterior and the interior layers of the epiblast appear very different from one another. On the one hand, the exterior layer acts as a sink for the intercalation of cells and participates in cell shape changes, both processes that act to drive the epiblast over the yolk cell. On the other hand, the interior layer acts only as a reservoir, contributing cells as needed to the exterior layer. Such a mechanism might be more appropriate for teleost epiboly, where a relatively small but solid blastoderm must cover the huge yolk cell.

hab is necessary for restrictions and cell shape changes in the epiblast

Almost all the cell behaviors described by the above model exhibit defects in hab mutants. First, distinguishable exterior and interior layers of the epiblast did not form in the mutant. The cells of what would be the exterior layer fail to flatten and never form a continuous epithelium, and albeit more subtly, the cells of the interior layer failed to attain a radial aspect ratio. Second, radial intercalation in the mutant was negated by the inability of the cells to remain in the exterior layer. This aberrant cell movement was probably the direct cause for the arrest of epiboly.

The transplantation experiments demonstrate that hab acts in a cell-autonomous fashion. Hence, hab function is necessary within the cells themselves for their participation in the cell behaviors of the epiblast. More importantly, when mutant cells are placed in a wild-type embryo, both the cell shape phenotype of hab and the inability of hab cells to remain restricted to the exterior layer are virtually identical to the behavior of cells in mutant embryos. This result strongly indicates that the hab defects in cell behavior are not the result of other morphogenetic processes in the epiblast. We note that an important control experiment has not yet been done: that is, placing wild-type cells in mutant embryos. This experiment
would test whether wild-type cells can undergo normal cell behaviors in an embryo devoid of any global clues or forces that may be necessary for such cell behaviors, a non-autonomous effect. We had poor success with this experiment because of the general lethality of hab host embryos, which seems to be exacerbated by the process of transplantation itself. However, in other experiments, submitted elsewhere, we have seen that when wild-type cells are transplanted into neural tubes of ZMD mutants, the wild-type cells often assume mutant phenotype, suggesting that in certain situations, hab can act in a non-autonomous fashion. We are now breeding lines of the mutant hab<sup>n2210</sup>, which has the least severe epiboly phenotype, in order to produce a recessive mutant that reliably survives into early segmentation stages.

Our observations regarding the radial concentration gradient of hab mRNA are in line with the defects observed in hab mutants. hab mRNA is expressed in the exterior layer of the epiblast, and that layer is missing in hab mutants. Possibly, cells of the exterior layer require additional hab gene product because they are forming an epithelium, and if they are unable to produce additional gene product, they do not integrate into the exterior layer and slip back into the interior layer. The radial gradient could also control the ordering and layering of cells during epiboly. Cells sort into layers dependent on adhesive activity (Duguay et al., 2003; Steinberg and Takeichi, 1994). One could imagine that as the blastoderm thickens, cells with the highest concentration of hab gene product would move to the outside, against the EVL, which continuously expresses high levels of hab mRNA; cells with low concentrations would move to the yolk cell, which has downregulated hab mRNA. Indeed, in the MO2 antisense knockdown experiment we observed unevenness in the thicknesses of the blastoderm. Antisense injections are known to mix unevenly in the early cleavage embryo, and irregularities in oligonucleotide concentration would cause local differences in hab function. Hence, because cells would tend to adhere differently in regions of differing Hab concentrations, some regions would tend to be thicker than others.

In hab mutants, cells move, but many tissues do not. For example, radial intercalation occurs at the same rate in hab mutants as in wild-type embryos, but the mutant cells do not intercalate, unable to remain in the exterior epiblast, and the epiblast ceases movement. In work to be presented elsewhere, we have found that many aspects of global convergence are affected in hab mutants, but the cells themselves continue to move at the same rate as cells in wild-type embryos. Hab seems specifically required for the normal integration of cells into embryonic epithelia and continues to be required for the normal behavior of the cells within the epithelia. Hence, in hab mutants, such epithelia form poorly or not at all; when they do form, the epithelia behave abnormally, causing the slowing or arrest of large-scale movements. Unraveling the roles that Hab and other molecules play in these early cell interactions that bind together the cells of the gastrula will be instructive, as we untangle these subtle forces that act to shape the vertebrate embryo.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/5/1105/DC1

References


