

The zebrafish epiboly mutants

Donald A. Kane^{*,†}, Matthias Hammerschmidt[‡], Mary C. Mullins[§], Hans-Martin Maischein, Michael Brand[¶], Fredericus J. M. van Eeden, Makoto Furutani-Seiki, Michael Granato, Pascal Haffter, Carl-Philipp Heisenberg, Yun-Jin Jiang, Robert N. Kelsh[†], Jörg Odenthal, Rachel M. Warga[†] and Christiane Nüsslein-Volhard

Max-Planck Institut für Entwicklungsbiologie, Abteilung für Genetik, Spemannstrasse 35, 72076 Tübingen, Germany

*Author for correspondence (e-mail: kane@uoneuro.uoregon.edu)

†Present address: Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403, USA

‡Present address: Harvard University, Department of Molecular and Cellular Biology, 16 Divinity Avenue, Cambridge, Massachusetts 02138, USA

§Present address: University of Pennsylvania, Department of Cell and Developmental Biology, 605 Stellar-Chance, Philadelphia, PA 19104-6058, USA

¶Present address: Institut für Neurobiologie, Universität Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany

SUMMARY

Epiboly, the enveloping of the yolk cell by the blastoderm, is the first zebrafish morphogenetic movement. We isolated four mutations that affect epiboly: *half baked*, *avalanche*, *lawine* and *weg*. Homozygous mutant embryos arrest the vegetal progress of the deep cells of the blastoderm; only the yolk syncytial layer of the yolk cell and the enveloping layer of the blastoderm reach the vegetal pole of the embryo. The mutations *half baked*, *avalanche* and *lawine* produce a novel dominant effect, termed a zygotic-maternal dominant effect: heterozygous embryos produced from heterozygous females slow down epiboly and accumulate detached cells over the neural tube; a small fraction of these mutant individuals are viable. Heterozygous embryos produced from heterozygous males crossed to

homozygous wild-type females complete epiboly normally and are completely viable. Additionally, embryos heterozygous for *half baked* have an enlarged hatching gland, a partial dominant phenotype. The phenotypes of these mutants demonstrate that, for the spreading of cells during epiboly, the movement of the deep cells of the blastoderm require the function of genes that are not necessary for the movement of the enveloping layer or the yolk cell. Furthermore, the dominant zygotic-maternal effect phenotypes illustrate the maternal and zygotic interplay of genes that orchestrate the early cell movements of the zebrafish.

Key words: epiboly, dominant, maternal, yolk syncytial layer, blastoderm, gastrulation, zygotic-maternal effect

INTRODUCTION

The study of morphogenesis in the early embryo is one of the most interesting but one of the least understood questions in developmental biology. How cells establish a trajectory through an embryo is virtually unknown. We have approached this problem by identifying mutations in the zebrafish that affect morphogenesis; our hope is that in understanding the genes disrupted by these mutations we might better understand vertebrate morphogenesis. This report describes mutants that affect epiboly, the movement during which the cells of the blastoderm envelop the yolk cell.

Epiboly is the first morphogenetic movement of the embryo. Before epiboly commences, the organization of the zebrafish blastula reflects the specialization of cells for the ensuing morphogenesis (Fig. 1). In the late blastula, the embryo divides into three cellular domains, the enveloping layer of the blastoderm, the deep cells of the blastoderm, and the yolk cell (Kane, et al., 1992). Each of these domains acquires distinct mitotic rhythms during and after the midblastula transition and each of these domains acquires distinct roles during and after epiboly. The enveloping layer forms an epithelial monolayer covering the blastoderm and later becomes the embryonic periderm, the

embryo's first skin. The deep cells of the blastoderm rearrange to form the hypoblast and the epiblast, which form the embryonic anlagen. The nuclei of the yolk cell, termed the yolk syncytial layer, lead the blastoderm towards the vegetal pole.

The function of the zebrafish yolk cell during epiboly seems very similar to that of the more thoroughly studied *Fundulus* yolk cell. In both species, the first mitotic arrest of the embryo occurs in the yolk cell just before the beginning of epiboly (Kane et al., 1992; Trinkaus, 1992, 1993). In *Fundulus*, elaborate connections develop between the yolk cell and the enveloping layer of the blastoderm, suggesting that the yolk cell is towing the enveloping layer (Betchaku and Trinkaus, 1978); if the blastoderm is removed, then the vegetal movement of the yolk syncytial layer continues, demonstrating that the movement of the yolk syncytial layer is independent of the blastoderm and autonomous to the yolk cell (Trinkaus, 1951). Consistent with a function as a towing motor, the yolk cell has microtubules and microfilaments in the proper orientation to effect such movement, and treatments that interfere with these structures interfere with epiboly (Strähle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994).

No experiment has demonstrated the epibolic role of blastoderm. The enveloping layer of the blastoderm seems to be a

passive component of epiboly given its tight attachment to the yolk cell and its stretched out appearance late in epiboly (although see Keller and Trinkaus, 1987). The dependence of the deep cells of the blastoderm on the yolk cell is less clear, especially given the transient and changing connections between the deep cells and the yolk cell. For instance, at any one time, individual deep cells are moving relative to the enveloping layer, relative to the margin of the blastoderm, relative to the yolk cell and relative to other deep cells. In *Xenopus*, which has no yolk cell and no yolk syncytial layer, epiboly occurs albeit to a lesser extent; this movement is thought to be mediated entirely by radial intercalation (Keller, 1980). Does the teleost blastoderm accomplish some component of epiboly autonomously?

This report presents the mutations that arrest epiboly which were found in the Tübingen screen for new zebrafish mutants. We found four mutations that, when homozygous, arrested the epiboly of the deep cells of the blastoderm but not the yolk syncytial layer or the enveloping layer. Three of these mutations produce a phenotype described here as zygotic-maternal dominant effect, a novel genetic effect. These mutants will be important for the genetic dissection of cell movement in zebrafish.

MATERIALS AND METHODS

Production and isolation of mutations: the Tübingen screen

The detailed procedures for the mutagenesis and isolation of new and novel zebrafish mutants is described elsewhere (Mullins et al., 1994; Haffter et al., 1996). Briefly, male zebrafish were mutagenized with ethyl nitrosourea and outcrossed; their progeny were raised to adults en masse, producing an F₁ generation. Random incrosses between the F₁ progeny produced individual families; these F₂ families were raised to adults. Random incrosses within each family produced F₃ embryos which were screened for the presence of mutants. If a family was carrying a particular mutation, one quarter of the incrosses, on average, had mutant embryos; in each such cross, one quarter of the embryos were mutant. The individual pairs of parents that produced mutant embryos were outcrossed to carry the mutations to subsequent generations.

Stocks

Fish were maintained as described elsewhere (Westerfield, 1993; Mullins et al., 1994). Stocks of all the epiboly mutants were maintained as outcrosses against, in alternate generations, Tübingen and TL, both stock lines maintained in Tübingen. Stocks of *ava* and *law* were maintained by sorting and raising zygotic-maternal dominant mutants; about 5 to 10% of these heterozygotes are viable. Stocks of *hab* were maintained by sorting and raising partial dominant mutants; these heterozygotes are completely viable.

Embryos

Eggs were produced for experiments by natural crosses and initially maintained in E3 medium (Mullins et al., 1994). For most experiments, embryos were dechorionated manually and incubated at 28±2°C in E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 0.150 mM KH₂PO₄, 0.050 mM Na₂HPO₄, and 0.7 mM NaHCO₃, pH 7.1-7.4).

DAPI staining

Embryos were fixed in 4% buffered paraformaldehyde for 2 hours at room temperature, washed in PBST, and incubated in a solution of

0.01% DAPI for 1 to 2 hours. Photographs were taken within 2 hours of staining using a Hoechst filter set.

Alpha amanitin injections

Alpha amanitin (Sigma Chemical Company) was maintained as a frozen 2 mg/ml solution in water. Soaking embryos in alpha amanitin leads to general effects that can be duplicated with hypertonic salt solutions (0.5 M NaCl). Therefore, approximately 2 nl of a 1:10 dilution of the alpha amanitin stock was pressure injected into dechorionated embryos at the 4 cell stage, giving an effective cytoplasmic concentration of 8 µg/ml. Control embryos were injected with distilled water.

Transplantations

Transplantations were done as described by Ho and Kane (1990). In the cases where chimeric embryos were produced, large numbers of cells (more than 100 blastomeres) were transplanted from embryos homozygous for *hab* into embryos homozygous for *gol*, a pigmentation mutant that has reduced melanin; these were raised to adulthood and tested by intercrossing tank mates. Such intercrosses normally yielded crosses that were 100% *gol*; the presence of a chimera was revealed by the presence of wild-type embryos; these individuals were heterozygous for *hab* and *gol*.

Microscopy and time lapse recordings

Observations were made on either a Zeiss Axioskop or Axiophot microscope equipped with Nomarski differential interference contrast optics and UV epilumination. Viewing chambers were constructed of two 60×24 mm no. 1 coverslips separated with three pairs of 18×18 mm no. 1 coverglass spacers, or with tape spacers of the same thickness; the chamber edges were sealed with Vaseline to prevent evaporation. Low light images were captured on an CCD Camera attached to an Intensifier (Videoscope KS-1381) and computer enhanced before recording.

For time lapse recordings, the microscopes were equipped with a motor driven focus controller and motor driven shutters for the white light and UV sources, all computer controlled (Applied Scientific Instrumentation, Eugene, Oregon). Embryos were immobilized with 0.06% agarose + 0.01% agar in E2 medium. A high resolution camera (Newvicon, model VS2000N, from Videoscope) was used to record single frame images to a Laser Videodisc Recorder (SONY, model LVR-4000P) using a computer to control the plane of focus, UV and white light shutters. A Quadra Mac II Computer (Apple Computer Corporation) equipped with a digitizing board (PixelPipeline, Perceptics Corporation) and an auxiliary monitor output (RasterOps) running AxoVideo 2.0 software was used for time lapse control, image enhancement, and generating playback sequences. All figures were produced using Adobe Photoshop 3.0.

RESULTS

A block in epiboly is the earliest phenotype expected due to a recessive zygotic mutation

To identify the earliest phenotype expected from mutating a zygotic gene, cleavage stage zebrafish embryos were injected with the transcriptional inhibitor alpha amanitin to an effective cytoplasmic concentration of 8 µg/ml. This is about 10 times the concentration that arrests RNA polymerase II in *Drosophila* (Edgar et al., 1986) and *Xenopus* (Newport and Kirschner, 1982), and about 100 times the minimum concentration that causes an observable effect in zebrafish (data not shown).

Injected embryos developed normally during the cleavage period and the early blastula period, an expected result, given

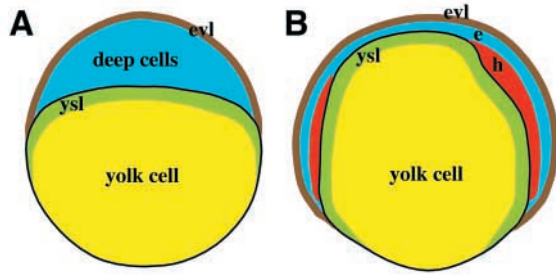


Fig. 1. Schematic of epiboly. (A) Structure of the embryo at high blastula stage, drawn from Fig. 2A. The enveloping layer (brown) covers the deep cells (blue); both structures comprise the blastoderm. The yolk cytoplasmic layer (green), exaggerated in thickness, covers the animal pole hemisphere of the yolk cell. The cell membrane of the yolk cell is shown in black. (B) Structure of the embryo at 70% epiboly, drawn from Fig. 2E. The dorsal side is to the right. The cytoplasmic layer (and yolk syncytial layer) move toward the vegetal pole. The enveloping layer follows the cytoplasmic layer of the yolk, remaining on the surface of the embryo. At the margin of the blastoderm, the deep cells turn inward to form the hypoblast (red), the presumptive mesoderm and endoderm; the non-involuting deep cells become the epiblast (blue), the presumptive ectoderm. Abbreviations: ysl, yolk syncytial layer; evl, enveloping layer; e, epiblast; h, hypoblast.

that there is little or no transcription before the zebrafish midblastula transition (Kane and Kimmel, 1993). The earliest effect of the alpha amanitin injection was an arrest at 4.5 hours, 1.5 hours after the onset of midblastula transition. This is the stage when control embryos were at dome stage, just prior to the beginning of epiboly. At 8 hours, when control injected embryos had reached 80% epiboly, the alpha amanitin-injected embryos retained the shape of a midblastula embryo (Fig. 2). The experimental embryos began to die at 8 to 10 hours, about the time that unfertilized oocytes begin to lyse. A comparison of cell size between 8 hour experimental and control embryos revealed that the size of the blastomeres was very similar (Fig. 2D), suggesting that the alpha amanitin-injected embryos had completed approximately the same number of rounds of cell division as the control embryos. Hence, mutants that affect the cell cycle are likely to exhibit their phenotypes after the beginning of epiboly (see Kane et al., 1996).

Identification of early mutations

The experiments with alpha amanitin indicated that, with care, it was possible to identify mutations that affected epiboly. For example, because the alpha amanitin-injected embryos died at 8-10 hours, along with the unfertilized eggs in a cross, mutants with similar phenotypes might be obscured by the moderate background infertility. Therefore, to avoid missing early mutants, we strove to sort fertile from infertile embryos before the beginning of epiboly, and, the next day, we saved all pairs of F₂ parents that yielded F₃ screening crosses in which one quarter of the embryos were dead at 30 hours. These F₂ pairs were mated again and their offspring checked more carefully during the gastrula and segmentation stages; this 'rescreen' procedure yielded a number of early mutants, including members of the Dorsal Specification Group (Mullins et al., 1996), the Early Arrest Group (Kane et al., 1996), and one in the Epiboly Group, described in this paper as *weg*.

The remainder of the epiboly mutants were isolated based on the diverse aspects of their dominant phenotypes. Initially, *avalanche* (*ava*) was characterized as a neural tube mutant and *lawine* (*law*) was characterized as a notochord mutant; both these phenotypes are novel dominant phenotypes, described later in this report as zygotic-maternal dominant effect phenotypes. The mutant *half baked* (*hab*) was isolated based on its partial dominant phenotype, an enlarged hatching gland. In all these cases, the recessive phenotypes were not seen until the re-identification of the mutants in the generation after the primary screen.

To summarize, four mutations arrest epiboly when homozygous (Table 1). Three of the four – *hab*, *ava*, and *law* – also cause delayed epiboly and neural tube defects as a zygotic-maternal dominant effect phenotype. One of these mutations, *hab*, also causes a partial dominant zygotic phenotype, an enlarged hatching gland. To date, *hab* has been the best characterized and has been the focus of the experiments in this paper; as available, we add observations from the other mutants.

The epiboly arrest phenotype

The recessive phenotypes of *hab*, *ava*, *law*, and *weg* segregate as simple Mendelian lethal traits (Table 2). These phenotypes slow down and arrest the epiboly of the deep cells of the blastoderm, stopping all vegetal progress by about 60±10% in *ava*, 70±10% in *law* and by about 80±10% epiboly in *weg* and *hab* (Fig. 3A-F). This arrest is followed by a slow retraction toward the animal pole during which time the yolk cell begins to lyse near the vegetal pole, killing the embryos, with the exception of occasional surviving *hab* mutants. The processes of involution and convergence seem to be only mildly affected if at all, for cells begin to involute at 50% epiboly and, with the exception of *ava*,

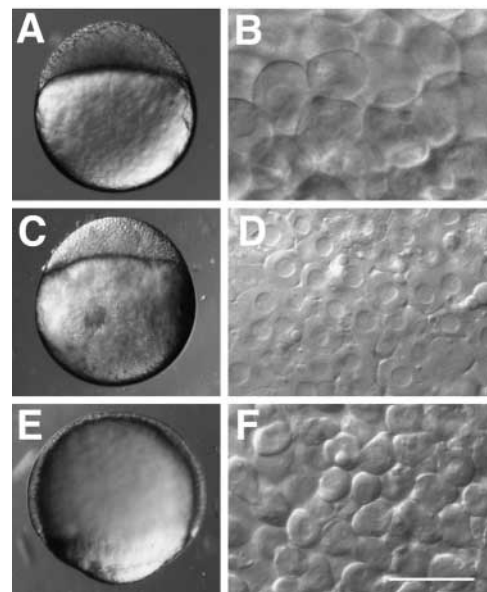


Fig. 2. Alpha amanitin-treated embryos do not initiate epiboly. (A) Untreated embryo at high blastula stage. (B) Blastomeres in A. (C) Alpha amanitin treated embryo at 8 hours. The embryo has the same shape as the blastula stage embryo. (D) Blastomeres in C. Note that the cells are smaller, indicating that several rounds of cell division have occurred. (E) Untreated sibling at 8 hours. (F) Blastomeres in E. Scale bar, 200 μ m for A,C,E; 50 μ m for B,D,F.

Table 1. Description of mutants

Genes	Alleles	Recessive phenotype*	Dominant phenotype
<i>half baked (hab)</i>	<i>dtv43</i>	Epiboly arrest at 8 hours; lethal at 10-15 hours but with exceptional survivors until 20-30 hours	Zygotic-maternal†: epiboly slowed; detached cells dorsal of the neural tube Partial‡: enlarged hatching gland
<i>avalanche (ava)</i>	<i>tm94</i>	Epiboly arrest at 7 hours; lethal at 8-10 hours	Zygotic-maternal: epiboly slowed; detached cells dorsal of the neural tube
<i>lawine (law)</i>	<i>ts18</i>	Epiboly arrest at 7 hours; lethal at 10-14 hours	Zygotic-maternal: epiboly slowed; detached cells dorsal of the neural tube
<i>weg (weg)</i>	<i>tx230</i>	Epiboly arrest at 8 hours; lethal at 9-12 hours	—

*Recessive phenotype: segregates with homozygotes; a simple Mendelian recessive.
†Zygotic-maternal dominant effect phenotype: segregates with heterozygotes produced from crosses between heterozygous females and homozygous wild-type males; phenotype is not seen in heterozygotes produced from reciprocal crosses between homozygous wild-type females and heterozygous males.
‡Partial dominant phenotype: segregates with heterozygotes.

the prechordal plate reaches the animal pole on schedule (Fig. 2C-E). Mutant *ava* embryos, which have only been partially characterized to date, may have a mild retardation of the hypoblast.

Occasionally, a small fraction of *hab* mutant embryos survive and develop until 20 to 30 hours. In these cases, a rudimentary embryo forms in the animal hemisphere (Fig. 4A) and many differentiated tissues appear in approximately the location predicted from the blastula fate map (Kimmel et al., 1990). A kinked twisted notochord (Fig. 4B) forms on the dorsal margin, a pronephric tubule (Fig. 4C) and somites (Fig. 4D) form along the lateral margin, and a tiny little tailbud forms on the ventral margin. Overlying the somites is neural tissue, presumably the spinal cord (data not shown). The ear (Fig. 4E) and sometimes eyes form near the animal pole; often the elementary divisions of the brain form (data not shown). Somites form and muscle cells differentiate; a rudimentary nervous system forms and seems to be functioning causing rhythmic twitching. One defect in these surviving mutants is the large collection of detached cells over the mutant neural tube (Fig. 3A,F), which, in some cases, can extend completely around the stalled germ ring. While the origin of these cells is presently unclear, their final position is where epidermis normally forms.

Separation of the movement of the yolk syncytial layer and enveloping layer from the movement of the deep cells

To understand the details of how cell movement is changed in mutants arrested for epiboly, we recorded the blastoderm and yolk cell of *hab* mutant embryos during epiboly using timelapse video microscopy, focusing on the relationships between the margin of the blastoderm and the nuclei of the yolk syncytial layer (Fig. 5A,B). The yolk syncytial layer normally migrates toward the vegetal pole at about 100 µm per hour and the deep cells of the blastoderm follow typically 50 to 100 µm behind. In *hab* embryos this gap between the yolk syncytial layer and the deep cells begins to widen at 80% epiboly. As the deep cells stall, the yolk syncytial layer continues toward the vegetal pole, completing the epiboly of the yolk cell. A comparison of the rates of the vegetalward progress of the yolk syncytial layer shows few, if any, differences among *hab/hab* embryos, *hab/+* embryos, and *+/+* siblings (Fig. 5C). Mutant embryos, fixed at 10 hours and stained with DAPI, show the deep cells of the blastoderm covering the animal hemisphere and a thin layer of nuclei covering the vegetal hemisphere (Fig. 5D).

Close examination of live mutant embryos revealed an epithelium covering the yolk cell, demonstrating that the enveloping layer had completed epiboly along with the yolk cell. Fig. 5E, from a live *hab* embryo at 90%, shows the cells of the enveloping layer extending vegetalwards over the yolk cell. The enveloping layer closes over the yolk plug almost completely, leaving a small 20 to 30 µm purse string like opening at the vegetal pole (Fig. 5F). In wild-type embryos this opening ultimately closes as it is carried with the forming tailbud; in mutant embryos the opening may close imperfectly, for it often corresponds to the initiation point for the ensuing lysis of the yolk cell.

At 10 hours a striking morphology develops at the margin of the arrested blastoderm of mutant embryos, a situation with no comparison in control embryos which, being well into tailbud or early segmentation stages, have no margin. In *hab* mutants the marginal deep cells send blebs and lamellipodia out from the margin (Fig. 5G). In *ava* many deep cells escape from the blastoderm margin and move into the space between the yolk cell membrane and the enveloping layer cells (Fig. 4H). These cells are rapidly moving and seem to behave as hypoblast cells, however, their fate is not yet known.

Survival of transplanted homozygous *hab* cells

Mutant embryos that arrest during epiboly typically die during

Table 2. Segregation analysis

Cross	Female×male	n†	Phenotypes*(percentage embryos)				
			A	B	C	D†	E†
<i>hab/+</i> × <i>hab/+</i>		285	26	22	9	1	42
<i>hab/+</i> × <i>+/+</i>		319	47	0	39	2	12
<i>+/+</i> × <i>hab/+</i>		721	53	0	0	47	0
<i>ava/+</i> × <i>ava/+</i>		453	19	25	56	0	0
<i>ava/+</i> × <i>+/+</i>		1320	50	0	50	0	0
<i>+/+</i> × <i>ava/+</i>		150	100	0	0	0	0
<i>law/+</i> × <i>law/+</i>		226	75‡	25	0	0	0
<i>law/+</i> × <i>+/+</i>		461	50	0	50	0	0
<i>+/+</i> × <i>law/+</i>		77	100	0	0	0	0
<i>weg/+</i> × <i>weg/+</i>		453	77	23	0	0	0

*Phenotypes: (A) Wild type. (B) Epiboly arrest. (C) Epiboly slowed; detached cells over the neural tube. (D) Enlarged hatching gland. (E) Epiboly slowed; detached cells over the neural tube; ragged hatching gland.

†n is for phenotype classes A-C; because of clutch to clutch lethality, a variable feature, less embryos were scored for phenotypes D and E.

‡Embryos were scored only for the recessive phenotype; therefore, Category A also includes unscored epiboly slowed and detached cells over the neural tube phenotypes.

somite stages. Could epiboly be slowed and stopped by a mutation in a gene necessary for cell maintenance? Because such genes could be required cell autonomously for cell survival, we transplanted mutant cells into a wild-type host and recorded their survival, similar in strategy to the approach taken for the analysis of the Early Arrest Group mutants (Kane et al., 1996).

Mutant cells survive irrespective of their final position in the host and contribute to a variety of tissues. Fig. 6A,B shows labeled *hab* cells in a 30 hour host; these cells survived at least 7 days, the limit of our examination with lineage tracer. In long term experiments, we transplanted mutant cells into hosts that were *golden (gol)*. Because the mutant *hab* cells were phenotypically wild type for *gol*, the dark *gol*⁺ melanocytes of the *hab* donor were easily distinguished from the lighter *gol* melanocytes of the host. These cells survived through adulthood, based on the retained changes in the adult pigment pattern (data not shown), similar to that seen in adults chimeric for *albino* (Culp et al., 1991).

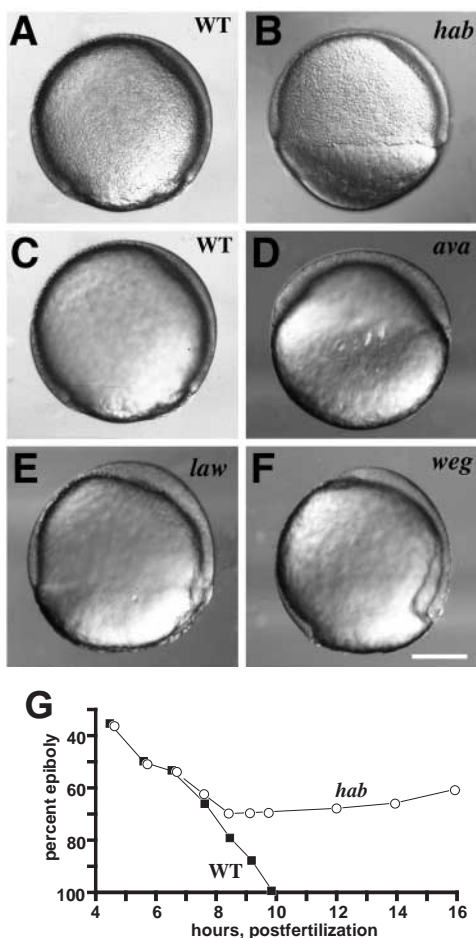


Fig. 3. Arrest of the blastoderm in *half baked* mutants. (A) Surface view of wild type at 8 hours. (B) Surface view of *hab* recessive phenotype at 8 hours. Note that the extent of the movement of the prechordal plate towards the animal pole is the same in both wild type and mutant. (C) Optical section of wild type at 8 hours. (D) Optical section of *ava* recessive phenotype at 8 hours. (E) Optical section of *law* recessive phenotype at 8 hours. (F) Optical section of *weg* recessive phenotype at 8 hours. (G) Relationship between progression of the deep cells of the blastoderm in normal embryos (filled squares) and *hab* embryos (open circles). Scale bar, 200 μ m.

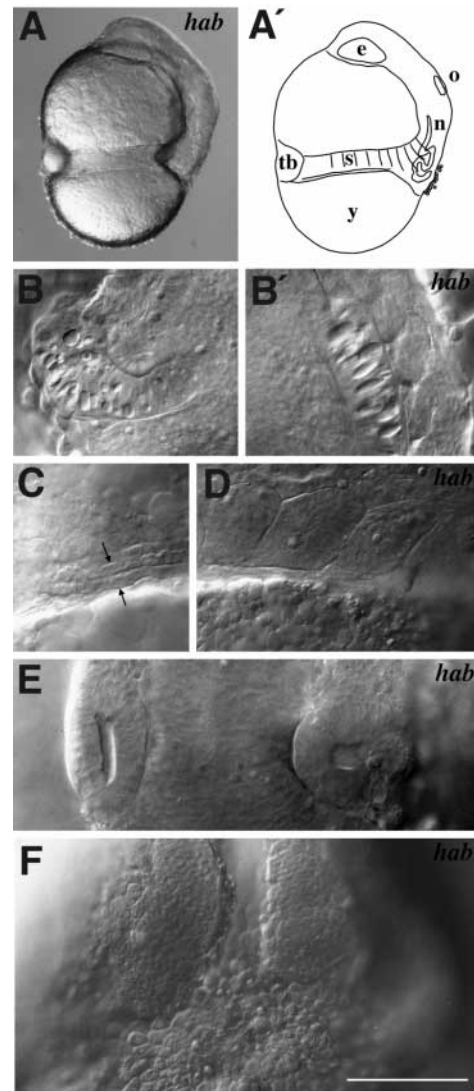


Fig. 4. Formation of differentiated tissues in surviving homozygous *hab* mutants. (A) Rare surviving embryo at 24 hours. (A') Drawing of embryo in A. e, eye; o, otic vesicle; n, notochord; s, somite; tb, tailbud; y, yolk. (B,B') Two focal planes of twisted notochord in one embryo. (C) Pronephric tubule, marked by arrows. (D) Somites of same embryo as in C in an adjacent superficial optical section. (E) Dorsal view of otic vesicles. (F) Dorsal view of detached cells over the neural tube at the approximate level of the hindbrain. Scale bar = 300 μ m for A; 70 μ m for B-E; 100 μ m for F.

In a continuation of these experiments, we raised the chimeric embryos to adulthood and tested them for the presence of *hab/hab* mutant cells in the germline. Of 45 fish tested, 3 male fish had small clones in the germline revealed by the presence of pigmented *gol*⁺ phenotypes within *gol/gol* clutches (Fig. 6C). Such *gol*⁺ embryos should be heterozygous for *gol* and *hab*. To confirm that chimerae in the fish were indeed *hab/hab*, we scored for the *hab* dominant phenotype, the enlarged hatching gland phenotype described below; all embryos that were phenotypically *gol*⁺ also had the enlarged hatching gland (Fig. 6D, 15/15). Thus cells homozygous for *hab* are capable of surviving in the male germline. A chimera for *hab* has not yet been identified in a female.

Zygotic-maternal dominant effect phenotype of *hab*, *ava* and *law*

Females that are heterozygous for *hab*, *ava* and *law*, when mated to homozygous wild-type males, produce clutches in which a portion of the embryos display slowed epiboly and neural tube defects (Table 2). Females heterozygous for *ava* and *law* produce clutches in which exactly half of the embryos have the mutant phenotype; *hab* females have variable numbers but never more than 50% mutant. This dominant phenotype segregates with heterozygous carriers (Table 3). Males that are heterozygous for *hab*, *ava* and *law*, when mated to homozygous wild-type females, produce normal embryos (Table 2) except for the dominant hatching gland phenotype of *hab* heterozygotes. Thus these mutations produce a dominant maternal phenotype that is only revealed when both the mothers are heterozygous and the offspring are heterozygous.

The zygotic-maternal dominant phenotypes express weak attributes of the recessive phenotypes. Epiboly is not arrested but slowed, proceeding at about 90% the normal rate (Fig. 7A-C). Mutant embryos accumulate detached cells dorsal of the neural tube (Fig. 7E) accompanied by small variably sized and variably placed furrows in or over the neural tube (Fig. 7F). These phenotypes range from weak cases where a few extra detached cells appear over the tip of the tail to extreme cases where the detached cells cover the entire length of the embryo, however, the range never extends to the degree seen in the recessive phenotype. Embryos with the zygotic-maternal dominant phenotypes develop into a variety of disparate phenotypes by 30 hours (Fig. 7G), some of which survive. For *ava*, for which most of the heterozygote survival data has been collected, the surviving fraction averages about 10% and varies from 0% to 100%, depending on both the genetic background and the individual female (Table 3).

The *hab* dominant zygotic phenotype

The *hab* mutation produces a partial dominant phenotype, an enlarged hatching gland (Fig. 8). This phenotype is found in up to 50% of the progeny of any one clutch (Table 2) and segregates with heterozygotes (Table 3). The

phenotype is completely viable (Table 3); thus, sorting for the dominant phenotype is an efficient method to produce stocks of heterozygous carriers.

The hatching glands consist of the hatching gland cells themselves, which are derivatives of the prechordal plate, and epidermal support cells, which are derivatives of the ectoderm (Warga, 1996). Counts of the number of hatching gland cells fail to show any difference between mutants and siblings, and the mutants themselves hatch no earlier than siblings (data not shown). In embryos which have also the zygotic-maternal dominant phenotype, the hatching gland is ragged, giving the appearance that cells are non-adhesive. The interaction

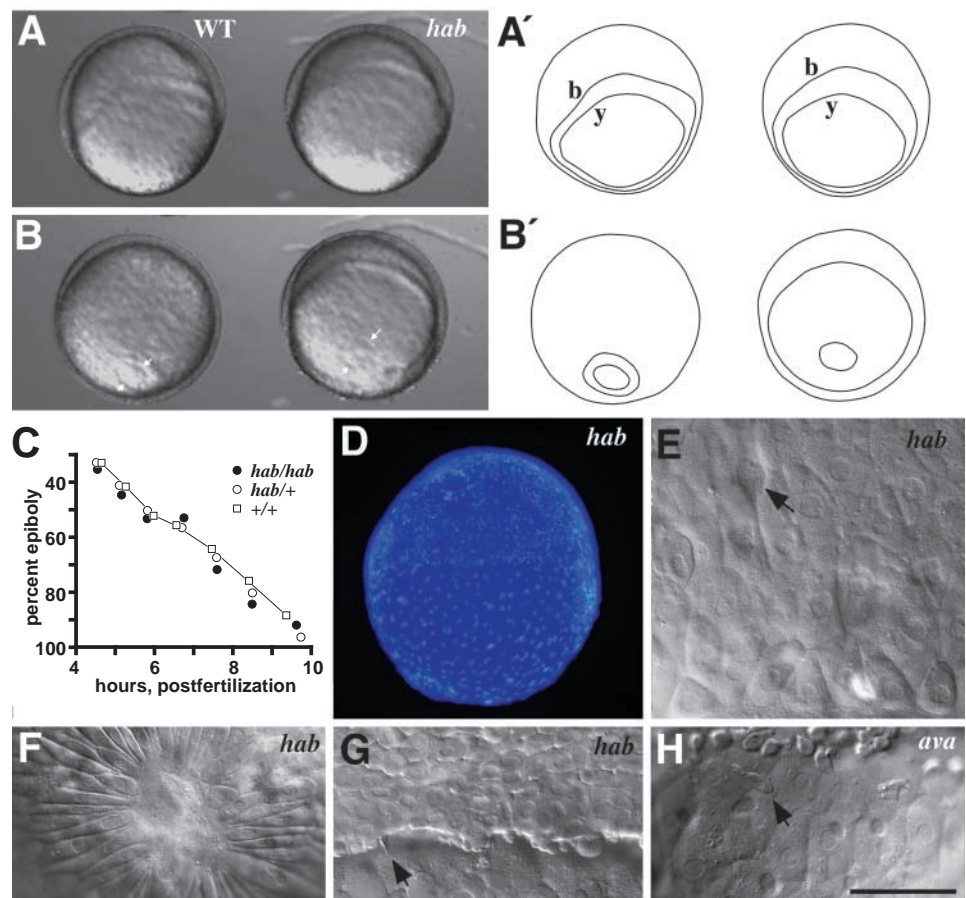


Fig. 5. Separation of the movement of the deep cells of the blastoderm from the vegetalwards movement of the yolk syncytial layer and the enveloping layer. (A,B) Video pictures from a time lapse recording of a *hab* mutant (right) embryo and a wild-type sibling (left). The embryos are obliquely oriented to give a partial side view and a vegetal pole view. A' and B' are line drawings of A and B, adding details seen from the playing of the time lapse movie. The innermost circle (y) indicates the margin of yolk syncytial layer and the enveloping layer; the next larger circle (b) indicates the edge of the deep cells of the blastoderm. (A) 7 hours, at 60% epiboly. (B) 9.5 hours, at 95% epiboly. In the *hab* mutant the progression the blastoderm is delayed; the vegetal movement of the yolk syncytial layer is normal. (C) Relationship between the progression of epiboly of the yolk syncytial layer of *hab* homozygotes, *hab* heterozygotes produced by females heterozygous for *hab*, and wild-type siblings. (D) DAPI stained embryo at 10 hours. The scar of nuclei to the left of the vegetal pole is the closure point of the enveloping layer. (E) Enveloping layer cells out on the yolk cell. The arrow shows a yolk syncytial layer nucleus, slightly out of focus. The vegetal margin of the enveloping layer cells is at the bottom edge of the photograph. (F) Closing of the yolk plug by the enveloping layer in *hab* mutant embryos. (G) The margin of the deep cells of the blastoderm in *hab* mutant embryos, showing cells extending blebs and lamellipodia. (H) Rogue deep cell in *ava* embryos migrating out into the space between the enveloping layer and the yolk cell. Scale bar, 300 μ m for A and B; 200 μ m for D; 40 μ m for E and F; 60 μ m for G and H.

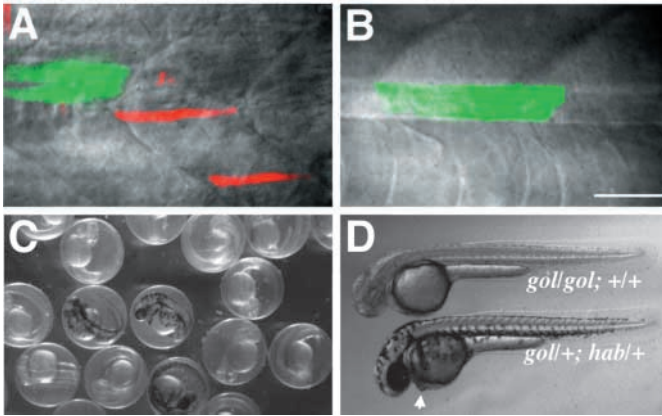


Fig. 6. Survival of *hab* cells in wild-type hosts. (A) *hab* (green) and wild-type (red) muscle cells transplanted into wild-type hosts. (B) *hab* (green) floor plate cells transplanted into wild-type hosts. (C) Wild-type embryos produced from a test cross between a *gol* female and a *gol* male containing a chimeric germ line for *gol* and *hab*. In this cross, 6 of 250 embryos were wild type. (D) Verification that the wild-type embryos shown in C are heterozygous for *hab*. The arrow indicates the enlarged hatching gland, indicating the embryo is heterozygous for *hab*. Scale bar: 30 μ m for A,B.

between the two phenotypes may be related to a common element affected by both phenotypes.

Complementation testing

All the mutants fail to complement each other. Table 4 shows that, in all of the test crosses accomplished, one quarter of the embryos displayed the recessive phenotype. However, because it is unclear what a double dominant phenotype is, it is difficult to interpret the results of complementation testing. Since each mutant has different aspects of the described phenotype, we are provisionally considering the four mutations as disruptions in separate genes.

DISCUSSION

The four mutations described in this paper all arrest the epiboly of the deep cells of the blastoderm. Homozygotes of *hab*, *ava*, *law* and *weg* display a recessive phenotype that results in the arrest of the vegetal movement of the deep cells in mid-epiboly. Heterozygotes of *hab*, *ava*, and *law*, when produced from an heterozygous female, display a zygotic-maternal dominant effect phenotype that slows epiboly and causes detached cells to accumulate dorsal to the neural tube. Also, heterozygotes of *hab* have a partial dominant effect phenotype, an enlarged hatching gland.

Separation of the epiboly of the deep cells of the blastoderm from the epiboly of the enveloping layer and yolk cell

The recessive phenotypes of the mutants in this report affect the deep cells of the blastoderm, slowing and stopping their spread over the yolk cell, but do not affect the morphogenetic behavior of the yolk cell and the enveloping layer, both of which spread to the vegetal pole of the embryo. Thus, in the mutants, the yolk cell completes epiboly without the deep cells. This is similar to the results of an experiment by J. P. Trinkaus (1951) where he

removed the blastoderm of the *Fundulus* embryo and observed the yolk cell autonomously complete epiboly.

The experiment by Trinkaus demonstrates that the epibolic behavior of the yolk cell is independent of the blastoderm, because the yolk cell completes epiboly after the blastoderm was removed. The mutant phenotypes demonstrate that the behavior of the enveloping layer also is independent of the deep cells of the blastoderm, because the enveloping layer completes epiboly even when the deep cells of the blastoderm do not. All the observations together are consistent with the notion that the yolk cell autonomously completes epiboly and that the enveloping layer follows the yolk syncytial layer; furthermore, we learn from the mutant phenotypes that there are additional mechanisms necessary to spread the blastoderm over the yolk cell. Note that these observations shed little light on the autonomous location of gene function; these genes could act in the yolk cell, the enveloping layer, or the deep cells themselves to somehow control their movement towards the vegetal pole of the embryo.

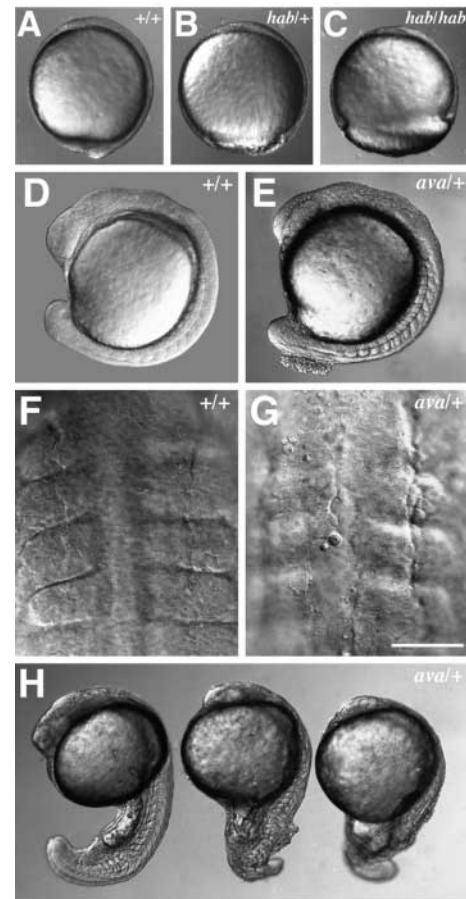


Fig. 7. Zygotic-maternal dominant phenotype of *ava* and *hab*. (A) Wild type, (B) *hab*/+, (C) *hab*/*hab*. (D) Wild-type at 15 hours, (E) *ava*/+ at 15 hours. Note detached cells dorsal of the neural tube of the tail. (F) Dorsal view of homozygous wild-type neural tube at the level of somite 5 to 10. (G) Dorsal view of mutant *ava*, showing small gaps in the surface over the neural tube. (H) The variety of phenotypes that develop from *ava*/+. The mutant embryo on the left, which has almost a wild-type appearance, developed a swim bladder and grew into an adult; the others died. Scale bar, 300 μ m for A-C; 200 μ m for D,E; 70 μ m for F,G; and 300 μ m for H.

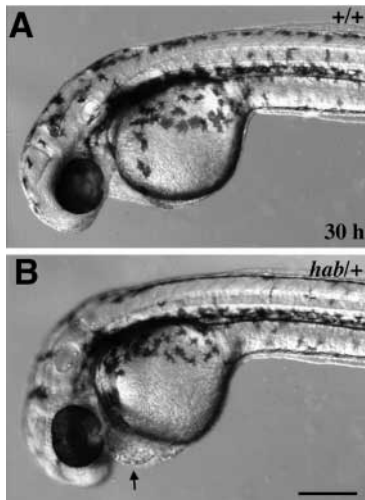


Fig. 8. Zygotic dominant hatching gland phenotype of *hab* heterozygotes. (A) Homozygous wild-type embryo at 30 hours; (B) *hab*/+. Arrow indicates the enlarged hatching gland. Scale bar, 100 μ m.

The interaction of maternal and zygotic genes controlling early cell movements

This report describes three mutations that have a novel maternal-zygotic interaction; an accompanying report (Mullins et al., 1996) describes an additional gene, *piggytail* with a similar interaction. These phenotypes are termed **zygotic-maternal dominant effects** because the dominant effect is dependent on both the genotype of the mother and the embryo, both of which must be heterozygous to produce the mutant phenotype. The mutations have a dominant maternal effect in that females must be heterozygous to produce the mutant phenotype; however, the mutations have also a partial dominant effect in that the embryos themselves must be heterozygous to show the mutant phenotype. Though not dominant effect mutations, a number of loci near the *zeste-white* region of *Drosophila* were shown to have a 'maternal-zygotic' lethal interaction in particular genetic backgrounds (Robbins, 1983).

These zygotic-maternal dominant effect phenotypes are reminiscent of the *Drosophila* paternal rescue maternal effect mutations, *deep orange*, *rudimentary* and *fused* (Wright, 1970). In these mutants, eggs produced from homozygous mothers fertilized with mutant sperm show the mutant phenotype; eggs fertilized with wild-type sperm show a wild-type phenotype, that is, the eggs are 'rescued' by the paternal contribution. That is,

Table 3. Lethality and genotype of embryos sorted for dominant phenotypes

Cross female \times male	Sorted embryos*	% Survival \dagger	% Carriers \ddagger
<i>ava</i> /+ \times +/+	477§	18	97 (33/34)
<i>law</i> /+ \times +/+	592§	11	95 (18/19)
+/+ \times <i>hab</i> /+	226¶	85	96 (51/53)

*Embryos were sorted for dominant phenotypes and raised.

\dagger Percentage of sorted embryos that survived to adults.

\ddagger Percentage of heterozygous carriers in survivors, as tested by incrosses.

§Sorted for detached cells over the neural tube at 10 to 15 hours.

¶Sorted for enlarged hatching gland at 30 hours.

Table 4. Complementation analysis

Cross	Female \times male	<i>n</i>	Phenotypes*: Percentage embryos				
			A	B	C	D	E
<i>hab</i> /+ \times <i>weg</i> /+		127	60	22	1	0	17
<i>weg</i> /+ \times <i>hab</i> /+		154	46	24	0	30	0
<i>hab</i> /+ \times <i>ava</i> /+		138	23	19	38	2	18
<i>ava</i> /+ \times <i>hab</i> /+		452	26	22	29	3	20
<i>hab</i> /+ \times <i>law</i> /+		294	28	24	31	2	1
<i>weg</i> /+ \times <i>ava</i> /+		61	73	27	0	0	0
<i>weg</i> /+ \times <i>law</i> /+		127	79	21	0	0	0
<i>ava</i> /+ \times <i>law</i> /+		233	24	22	54	0	0
<i>law</i> /+ \times <i>ava</i> /+		54	19	29	52	0	0

*Phenotypes: A, wild type; B, epiboly arrest; C, epiboly slowed – detached cells over the neural tube; D, enlarged hatching gland; E, epiboly slowed – detached cells over the neural tube; enlarged often ragged hatching gland.

to rescue a paternal rescue maternal effect phenotypes, only one wild-type copy of a gene is necessary among four possible copies, the two maternal copies and the two zygotic copies; hence, the function of such a gene can be achieved either maternally or zygotically. However, the zygotic-maternal dominant effects of *hab*, *ava* and *law* suggests that the additive doses of both the maternal and the zygotic gene products are essential. To rescue the zygotic-maternal dominant effect phenotype, three wild-type copies are necessary among the two maternal copies and the two zygotic copies.

One prediction from these result is that there would be a strong maternal recessive phenotype for females homozygous for any of these mutations. Although homozygous embryos are lethal, at least in males it is possible to produce germ line clones of homozygous mutant cells. This approach will hopefully yield females that possess germ line clones.

The relationship of the *hab* recessive and dominant effect phenotypes

The *hab* mutation segregates two dominant phenotypes: a zygotic-maternal dominant effect phenotype, and a partial dominant effect phenotype. Like the recessive phenotype, the zygotic-maternal dominant effect phenotypes affect epiboly: the recessive phenotype arrests epiboly; the zygotic-maternal dominant effect phenotype slows epiboly. When mutants survive to 20 hours or later, the recessive and zygotic-maternal dominant effect phenotypes also seem related: the recessive phenotype causes a large accumulation of detached cells to form on the dorsal side; the zygotic-maternal effect phenotype causes detached cells to form dorsal to the neural tube. Thus, in both of these examples, the zygotic-maternal dominant effect phenotypes may be weakened recessive phenotypes.

The partial dominant phenotype of *hab*, an enlarged hatching gland, has no obvious recessive attributes; for instance, there is no associated slowing of epiboly. However, an enlarged hatching gland phenotype could be related to the detached cells dorsal to the neural tube seen in the recessive and zygotic-maternal dominant effect phenotypes. Although the origin of the detached cells has not yet been conclusively established, based on their dorsal location relative to the neural tube, they are likely to originate in the ventral ectoderm, near the fate map position of the epidermis (Kimmel et al., 1990). This location is close to the anterior extension of the prechordal plate, cells of which have a role in the formation of the hatching gland (Warga, 1996).

Perhaps the lack of spreading of the deep cells of the blastoderm slows the clearing of cells from the ventral region of the blastoderm, thus allowing an abnormally higher number of cells to interact with the anterior edge of the prechordal plate, causing the hatching gland to be abnormally large.

Four mutants, four genes?

The complementation data is consistent with the idea that the four epiboly mutants described in this paper are at one locus. Each intercross segregates one quarter embryos possessing the arrested epiboly phenotype; if the mother is *ava*, *law* or *hab*, the intercross segregates an additional fraction possessing the detached cells over the neural tube phenotype, and if either parent is *hab*, the intercross segregates an additional fraction possessing the enlarged hatching gland phenotype. However, three of the mutations have zygotic-maternal dominant effect phenotypes, phenotypes for which little is known. At least in *Drosophila*, intra-allelic non-complementing mutations are common for dominant mutations, the most extensive example being the lack of complementation between mutations at many of the *Minute* loci (Lindsley and Grell, 1968). Therefore, it is unclear what differences to expect in distinguishing a recessive phenotype from a double dominant phenotype, or, in the case of the *weg* trans-heterozygotes, what differences to expect in distinguishing a recessive phenotype from a phenotype due to inter-allelic interactions with a dominant allele.

If the mutations are in one gene, then the mutant phenotypes suggest a complicated allelic series. The *hab* mutant has the weakest recessive phenotype: homozygous embryos of *hab* survive long past the segmentation period, whereas *ava*, *law* and *weg* die; homozygous embryos of *ava* have the strongest recessive phenotype. However, of the zygotic-maternal dominant effect phenotypes, the *hab* one is the strongest; heterozygous embryos derived from heterozygous mothers rarely survive. Finally, *hab* alone has a partial dominant effect phenotype. Hence the mutant with the weakest recessive phenotype would have the strongest dominant effect phenotype.

We have provisionally named the mutants separately until mapping confirms or contradicts our complementation data.

The Tübingen screen found neither mutants that resembled the alpha amanitin blocked embryos nor mutants in which the epiboly of the yolk cell itself was arrested or slowed. If the mutants described in the report are typical, then many epiboly genes may be both maternally and zygotically active. In many, perhaps most, cases the maternal dose might be sufficient to cover the zygotic deficiency; such mutants could be difficult to detect in the screen, especially if the phenotype was transient. Additionally, three of the four mutations reported here are dominant; dominant mutations in other early genes may be lethal.

The mutations here described will increase our understanding of how epiboly and cell morphogenesis works. We are now engaged in mapping the genes to resolve the complementation testing results; this approach will lead the way to the cloning of these genes and a molecular understanding of cell movement.

We thank Drs John Postlethwait and Sigfreid Roth for their helpful comments on earlier drafts of this paper. This work was supported in part by a grant from the National Institutes of Health to D. A. K.

REFERENCES

Betchaku, T. and Trinkaus, J. P. (1978). Contact relations, surface activity,

and cortical microfilaments of marginal cells of the enveloping layer and of the yolk syncytial and yolk cytoplasmic layers of *Fundulus* before and during epiboly. *J. Exp. Zool.* **206**, 381-426.

Culp, P., Nüsslein-Volhard, C. and Hopkins, N. (1991). High frequency germline transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc. Nat. Acad. Sci. USA* **88**, 7953-7957.

Edgar, B. A., Kiehle, C. P. and Schubiger, G. (1986). Cell cycle control by the nucleocytoplasmic ratio in early *Drosophila* development. *Cell* **44**, 365-372.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J. M., Jiang, Y.-J., Heisenberg, C.-P., Kelsh, R. N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C. and Nüsslein-Volhard, C. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1-36.

Ho, R. K. and Kane, D. A. (1990). Cell-autonomous action of zebrafish *spt-1* mutation in specific mesodermal precursors. *Nature* **348**, 728-30.

Kane, D. A. and Kimmel, C. B. (1993). The zebrafish midblastula transition. *Development* **119**, 447-456.

Kane, D. A., Maischein, H.-M., Brand, M., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.-P., Jiang, Y.-J., Kelsh, R. N., Mullins, M. C., Odenthal, J., Warga, R. M. and Nüsslein-Volhard, C. (1996). The zebrafish early arrest mutants. *Development* **123**, 57-66.

Kane, D. A., Warga, R. M. and Kimmel, C. B. (1992). Mitotic domains in the early embryo of the zebrafish. *Nature* **360**, 735-737.

Keller, R. E. (1980). The cellular basis of epiboly: an SEM study of deep cell rearrangement during gastrulation in *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **60**, 201-234.

Keller, R. E. and Trinkaus, J. P. (1987). Rearrangement of enveloping layer cells without disruption of the epithelial permeability barrier as a factor in *Fundulus* epiboly. *Dev. Biol.* **120**, 12-24.

Kimmel, C. B., Warga, R. M. and Schilling, T. F. (1990). Origin and organization of the zebrafish fate map. *Development* **108**, 581-94.

Lindsley, D. L. and Grell, E. H. (1968). *Genetic Variations of Drosophila melanogaster*. Washington, D.C.: Carnegie Institute Wash. Publ. No. 627.

Mullins, M. C., Hammerschmidt, M., Haffter, P. and Nüsslein-Volhard, C. (1994). Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr. Biol.* **4**, 189-202.

Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C.-P., Jiang, Y.-J., Kelsh, R. N. and Nüsslein-Volhard, C. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* **123**, 81-93.

Newport, J. and Kirschner, M. (1982). A major developmental transition in early *Xenopus* embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686.

Robbins, L. (1983). Maternal-zygotic lethal interactions in *Drosophila melanogaster*: *zeste-white* region single-cistron mutations. *Genetics* **103**, 633-648.

Solnica-Krezel, L. and Driever, W. (1994). Microtubule arrays of the zebrafish yolk cell: organization and function during epiboly. *Development* **120**, 2443-2455.

Strähle, U. and Jesuthasan, S. (1993). Ultraviolet irradiation impairs epiboly in zebrafish embryos: evidence for a microtubule-dependent mechanism of epiboly. *Development* **119**, 909-919.

Trinkaus, J. P. (1951). A study of the mechanism of epiboly in the egg of *Fundulus heteroclitus*. *J. Exp. Zool.* **118**, 269-320.

Trinkaus, J. P. (1992). The midblastula transition, the YSL transition and the onset of gastrulation in *Fundulus*. *Development Supplement* **75-80**.

Trinkaus, J. P. (1993). The yolk syncytial layer of *Fundulus*: Its origin and history and its significance for early embryogenesis. *J. Exp. Zool.* **265**, 258-284.

Warga, R. M. (1996). *Origin and Specification of the Endoderm in the Zebrafish, Danio rerio*. PhD Thesis. Tübingen, Germany: Universität Tübingen.

Westerfield, M. (1993). *The Zebrafish book*. Eugene, Oregon: University of Oregon Press.

Wright, T. (1970). The genetics of embryogenesis in *Drosophila*. *Adv. Genet.* **15**, 261-395.