

# A ubiquitin C-terminal hydrolase is required to maintain osmotic balance and execute actin-dependent processes in the early *C. elegans* embryo

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Accepted 18 March 2002

Journal of Cell Science 115, 2293-2302 (2002) © The Company of Biologists Ltd

## Summary

In the early *Caenorhabditis elegans* embryo, establishment of cell polarity and cytokinesis are both dependent upon reorganization of the actin cytoskeleton. Mutations in the *cyk-3* gene cause maternal effect embryonic lethality. Embryos produced by homozygous *cyk-3* mutant animals become multinucleate. We have further analyzed the *cyk-3* mutant phenotype and have found that *cyk-3* mutant embryos fail to properly polarize the actin cytoskeleton and fail to segregate germline determinants. In addition, they fail to assemble an intact cleavage furrow. However, we have found that *cyk-3* mutant embryos are intrinsically defective in osmotic regulation and that the cytokinesis

defects can be partially rescued by providing osmotic support. The *cyk-3* gene has been identified and found to encode a ubiquitin C-terminal hydrolase that is active against model substrates. These data indicate that the deubiquitination of certain substrates by CYK-3 is crucial for cellular osmoregulation. Defects in osmoregulation appear to indirectly affect actin-dependent processes.

Key words: Polarity, Cytokinesis, Cleavage furrow, Protein degradation, Endocytosis

## Introduction

In *Caenorhabditis elegans*, the actin cytoskeleton mediates important processes during development, including the establishment of embryonic polarity and the execution of cytokinesis. Upon fertilization, polarity is established along the anterior-posterior axis (a-p axis) and is later utilized to generate two daughter cells that differ in size and developmental potential. For instance, the posterior blastomere, P1, is uniquely endowed with P-granules, which are germline determinants that are segregated to the P-lineage and promote establishment of a functional germline (Kawasaki et al., 1998). Disruption of the actin cytoskeleton during a defined time window in one-cell stage embryos results in the loss of asymmetry along the a-p axis and in mislocalization of P-granules (Strome and Hill, 1988).

Another prominent actin-dependent process of early *C. elegans* embryos is cytokinesis. To divide a cell into two daughters, the cytoskeleton forms an actin-based contractile ring in the cell cortex. The contractile ring assembles between the two poles of the mitotic spindle so that the ingressing cleavage furrow endows each of the two resulting daughter cells with a complete set of chromosomes (for a review, see Glotzer, 2001). Mutations or disruption of either actin or myosin result in a failure of cytokinesis in animal cells (Carter, 1967; Guo and Kemphues, 1996; Mabuchi and Okuno, 1977; Strome and Hill, 1988).

The actomyosin cytoskeleton has diverse cellular functions in addition to its role in establishing cellular asymmetry and

performing cytokinesis. For example, the actin cytoskeleton is essential for cell motility, cell-substrate attachment and maintenance of cellular integrity. These diverse functions require a dynamic cytoskeleton that can be remodeled to accomplish these tasks. This flexibility, however, renders the actin cytoskeleton sensitive to perturbations. For example, merely preventing actin assembly by sequestering actin monomers (as with Latrunculin A) leads to the depolymerization of pre-existing actin structures (Coue et al., 1987).

Cell volume regulation is thought to be mediated by a host of transporters, pumps and channels that balance the intracellular concentration of ions, water and osmolytes (for a review, see Kwon and Handler, 1995). Upon hyperosmotic stress, cells lose water, which leads to a dramatic increase in the concentration of internal ions. Regulated volume increase (RVI) then occurs, involving the uptake of ions that are ultimately replaced by compatible osmolytes. Upon hypo-osmotic stress, cells take up water and swell, triggering a process termed regulated volume decrease (RVD). In RVD, both ions and osmolytes are actively exported to the extracellular environment, which leads to the concomitant loss of water and return to the normal cell volume. However, the molecular mechanism of cellular osmotic homeostasis remains poorly understood.

Exposing cells to dramatic changes in osmotic conditions leads to reorganization of the actin cytoskeleton, perhaps as a consequence of its intrinsic instability. For example, in human

cells, the total content of F-actin decreases in hypo-osmotic and increases in hyper-osmotic conditions concomitantly with cell volume changes (Hallows et al., 1996). In yeast, both actin cables and cortical actin patches are transiently disrupted upon osmotic shock (Chowdhury et al., 1992). *Dictyostelium discoideum* responds to hyperosmotic conditions by relocalizing myosin to the cortex and by significantly increasing phosphorylation of actin and myosin (Kuwayama et al., 1996; Zischka et al., 1999). Thus, the cellular osmotic state impinges on the actin cytoskeleton.

In this study, we have characterized a maternal effect embryonic lethal mutation in *C. elegans* called *cyk-3*. In utero, *cyk-3* mutant embryos fail to perform cytokinesis and become progressively multinucleated. Not only is cytokinesis defective, but partitioning of P-granules is also impaired. *cyk-3* mutant embryos are osmosensitive and appear swollen. Osmotic support can substantially rescue the cytokinesis defect. The *cyk-3* gene has been cloned by functional rescue and found to encode an ubiquitin C-terminal hydrolase. These data reveal that deubiquitination is required for cellular osmotic regulation and show that, in the *C. elegans* embryo, defects in osmoregulation can disrupt cytokinesis and some aspects of embryonic polarity.

## Materials and Methods

### Strains and alleles

The strains *cyk-3(t1525)*, *unc-32/qCI* and *cyk-3(t1535)*, *unc-32/qCI* were identified in a screen for maternal effect embryonic lethal mutations on chromosome III (R.S. and H.S., unpublished) (Gönczy et al., 1999). The strain MJ569 (*unc-79(e1068)*, MJ#Nec1, MJ#Nec2, *dpy-17(e164)III*) is described in Nishiwaki et al. (Nishiwaki et al., 1993). The strain *sma-4(e729) unc-36(e251)III* was kindly provided by Bruce Bowerman. The strain CB364 (*dpy-18(e364)III*) was obtained from the *Caenorhabditis* Genetics Center.

### Genetic mapping of *cyk-3*

*cyk-3* maps to LGIII and is not covered by several tested deficiencies, including sDf121 and sDf125 (Gönczy et al., 1999). A cross between the strains *cyk-3*, *unc-32/qCI* and *dpy-18/dpy-18* placed *cyk-3* at approximately -1.3 on the minus arm of chromosome III (17/669 *Unc-32* animals gave viable progeny, 0/17 segregated *Dpy-18* worms). Recombination mapping using the strain MJ569 positioned *cyk-3* proximal to *dpy-17* (0/7 *Dpy* non-*Uncs* carried the *cyk-3* mutation, 1/1 *Unc* non-*Dpys* did carry *cyk-3*). Recombination mapping using the strain *sma-4*, *unc-36* positioned *cyk-3* between *sma-4* and *unc-36*, close to *sma-4* (5/25 *Sma* non-*Uncs* and 15/17 *Unc* non-*Smas* carried the *cyk-3* mutation).

### Rescue experiments

Cosmids spanning the *cyk-3* candidate region (kindly provided by Alan Coulson, Sanger Center, Hinxton, UK), were injected in pools into the gonads of 10-15 *unc-32*, *cyk-3(t1525)/qCI* heterozygous animals, together with the *rol-6(su1006)* transgenic marker (Mello et al., 1991). Heterozygous F1 worms carrying the *rol-6* marker were transferred to individual plates to obtain lines segregating Rollers. From these lines, homozygous *unc-32 cyk-3(t1525)* worms were scored for viable progeny. Out of the injected cosmids, only cosmid ZK328 showed rescue activity (3/5 lines produced viable *Unc-32* progeny). A 7.5kb genomic *Bam*HI fragment containing only ZK328.1 and a promoter region was subcloned into pBS-KS+ and injected into *unc-32*, *cyk-3(t1525)/qCI* and *unc-32*, *cyk-3(t1535)/qCI*

as described above. Rescue activity was observed at similar levels to the whole cosmid (6/9 lines). The two *cyk-3* alleles (t1525 and t1535) were sequenced. One single point mutation was found in each allele, in both cases leading to a premature stop codon. We obtained cDNA clones yk9h1 and yk226b (kindly provided by Y. Kohara), which covered most of the *cyk-3* coding sequence except for a 5' prime region that had been predicted by genefinder. ESTs containing the predicted start codon preceded by a SL1 leader sequence have recently been released. The missing 5' prime end was amplified by PCR from a mixed stage cDNA library. Sequencing of this reconstructed full-length cDNA revealed that the genomic sequence of ZK328.1 was incorrect (4 Ts were read as 3 Ts at position 1920 of the cDNA). The revised cDNA sequence of ZK328.1 has been submitted to GenBank (accession number AF469173).

### RNA interference

We performed RNAi on the predicted genes contained in cosmid ZK328. Fragments of 280 to 350bp corresponding to the coding regions of these genes were amplified by PCR and subcloned into pGEM-T (Promega). In vitro transcription from both strands was performed (Ambion), the strands were annealed and injected into the gonads of wild-type hermaphrodites as described previously (Fire et al., 1998). Embryos were analyzed 18 hours and 24 hours post injection. Of the predicted genes of ZK328, only RNAi against ZK328.1 phenocopied *cyk-3* mutant embryos. The ZK328.1 PCR fragment used for RNAi was amplified with the primers CGTCGCTATGGTTTCCGATTG and CGATCAAAGGAAGTCCAAAACG.

### Antibody staining

Gravid hermaphrodites were placed into a drop of media A on a polylysine-coated slide. A coverslip was added, and sufficient pressure to extrude the embryos was applied. The embryos were immediately frozen in liquid nitrogen and fixed in -20°C methanol, and antibody staining was performed according to standard protocols. The monoclonal rat anti-tubulin antibody YOL 1/34 was used in a 1:300 dilution. The rabbit anti-PGL-1 antibody was kindly provided by Susan Strome and used in a 1:1000 dilution.

### Phalloidin staining

Gravid hermaphrodites were placed in a drop of fix and stain solution (4% Paraformaldehyde, 50 mM Pipes pH 6.8, 5 mM EGTA, pH 8 and 300 nM Rhodamine-labeled Phalloidin (Molecular Probes)) between thin spacers of vacuum grease on a siliconized slide. A coverslip was added and pressure was applied to extrude the embryos. Further gentle pressure was applied to permeabilize the embryos. After 20-30 minutes of incubation at room temperature, the embryos were washed with PBST (1×PBS and 0.05% Triton-X100), stained with Hoechst 33342 and analyzed.

### Time-lapse analysis and analysis of cytoplasmic flow

Time-lapse Nomarski analysis was performed as described previously (Jantsch-Plunger and Glotzer, 1999). To measure the cytoplasmic flow, time-lapse recordings of three wild-type and three *cyk-3* embryos at the pronuclear stage filmed in medium A were analyzed using Object Image, an extended version of NIH image software (developed by Norbert Vischer). The movement of individual yolk granules was followed for 30-40 seconds, and an average speed was calculated.

### Buffers for osmotic support and preparation of blastomeres

Medium A: 5 mg/ml Polyvinylpyrrolidone (PVP), 25 mM Hepes (pH 7.4), 0.5 mg/ml Inulin, 0.06 M NaCl and 0.025 M KCl. Blastomere

and embryonic growth media (EGM) was prepared as described previously (Edgar, 1995). The EGM used in this study was prepared according to the instructions for Minimal EGM. In order to vary the osmolarity, the medium was supplemented with either half (0.5×EGM) or double (2×EGM) concentration of stock salts. Calcein (Molecular Probes) was used at a concentration of 10  $\mu$ M and the membrane staining dye FM4-64 (Molecular Probes) at a concentration of 5  $\mu$ g/ml.

#### Deubiquitination assay

Ubiquitin (cosmid Z1010) and the ubiquitin-like proteins encoded by cosmid K12C11 (SUMO ortholog), H06I04 and F45H11 (NEDD8 ortholog) were amplified by PCR from a mixed stage cDNA library and subcloned into the LacZ expression vector pPD8.02 (Fire et al., 1990), thereby creating a N-terminal fusion of ubiquitin to  $\beta$ -galactosidase ( $\beta$ -Gal). To distinguish between bacterially expressed  $\beta$ -Gal and  $\beta$ -Gal expressed from the vector, a 620 bp *HpaI* fragment was removed, creating a truncated version of  $\beta$ -Galactosidase ( $\beta$ -Gal $\Delta$ ). Doa4p was amplified from *S.cerevisiae* (W303) genomic DNA by PCR and subcloned into pET28a. The *cyk-3* cDNA was also subcloned into pET28a. The  $\beta$ -Gal fusion proteins were co-expressed together with either Doa4p or CYK-3 in *Escherichia coli* BL21. Cleavage of ubiquitin or ubiquitin-like proteins from the ubiquitin- $\beta$ -Gal $\Delta$ *HpaI* fusion protein was assessed by probing for  $\beta$ -Gal with an anti- $\beta$ -gal antibody (Promega) on a western blot.

## Results

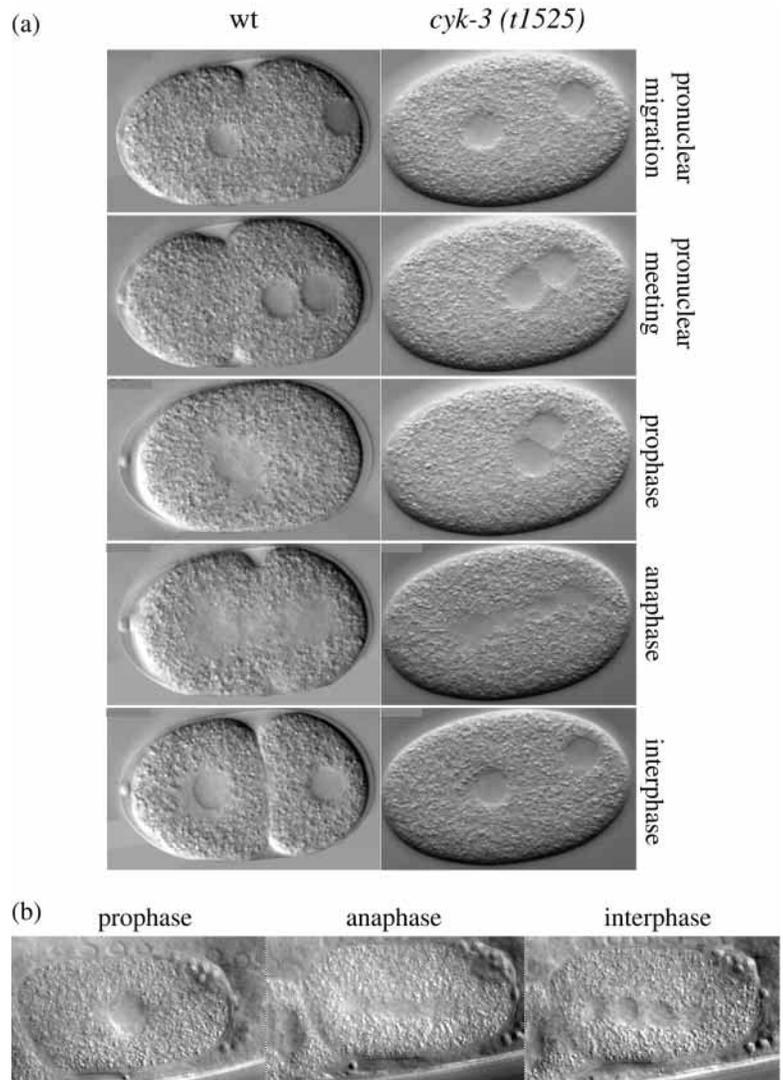
### *cyk-3* mutant embryos are defective in cytokinesis

A large-scale screen for maternal effect embryonic lethal (MEL) mutants on linkage group III led to the identification of two mutant alleles, t1525 and t1535, which failed to complement each other (H.S. and R.S., unpublished). Embryos from homozygous mutant hermaphrodites from either allele are osmosensitive in vitro. In utero, these embryos fail to perform cytokinesis and become multinucleate; this locus was thus named *cyk-3* for cytokinesis defective (Gönczy et al., 1999).

In order to analyze the mutant phenotype in more detail, we performed time-lapse Nomarski microscopy on embryos produced from *cyk-3* homozygous hermaphrodites ( $n=11$ ). Using osmotically balanced medium A (see Materials and Methods), we followed embryos from the onset of pronuclear migration to the completion of mitosis (Fig. 1). We observed a similar phenotype in dissected embryos (Fig. 1a) and on embryos in utero (Fig. 1b). The first defect observed is a lack of membrane contractions and a failure to perform pseudocleavage (100%). Pseudocleavage is a transient ingression of the plasma membrane that occurs during pronuclear migration. An additional penetrant defect observed is the inability of *cyk-3* embryos to extrude polar bodies, which contain the remnants of the meiotic divisions of the maternal pronucleus. Polar bodies are normally extruded from the embryo proper by a highly asymmetric form of cytokinesis.

Subsequent steps in early embryonic development,

such as pronuclear meeting, rotation of the nuclear-centrosome complex and nuclear division, appear to be normal in *cyk-3* embryos. We measured the time interval between the first and the second occurrence of nuclear envelope breakdown in wild-type and *cyk-3* mutant embryos and found that they complete one cell cycle in approximately the same time ( $17.5\pm 1.5$  minutes and  $20.3\pm 0.5$  minutes, respectively). In anaphase, however, *cyk-3* mutant embryos show a striking defect: they do not form a cleavage furrow, and they therefore fail to undergo cytokinesis (Fig. 1).



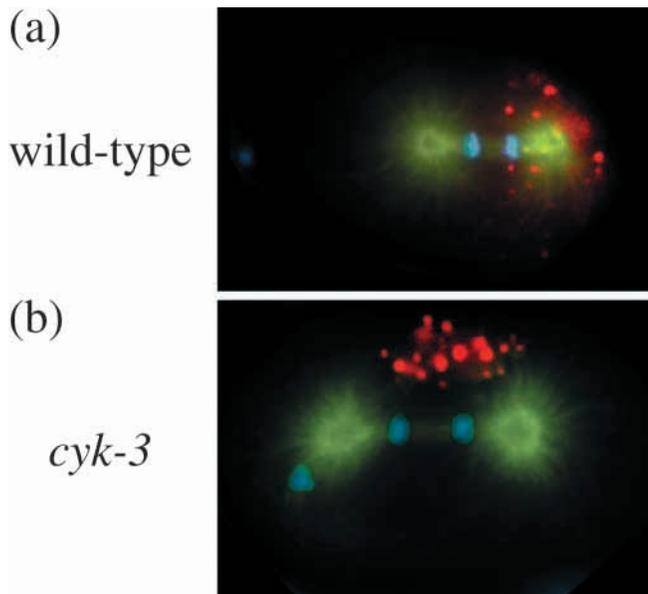
**Fig. 1.** *cyk-3* mutant embryos fail to perform cytokinesis. (a) *cyk-3* embryos were dissected from homozygous mutant hermaphrodites, and time-lapse microscopy was performed. During pronuclear migration, *cyk-3* embryos do not undergo any visible membrane contractions, and they fail to perform pseudocleavage. At the time of anaphase, when wild-type embryos initiate cytokinesis, *cyk-3* embryos show no sign of furrowing. At that stage, the mitotic spindle of both wild-type and *cyk-3* embryos is displaced towards the posterior hemisphere of the cell. While nuclear division occurs with normal kinetics, cytokinesis does not occur in these embryos. (b) Time-lapse analysis was performed on embryos within the uterus of homozygous mutant hermaphrodites. As dissected embryos, embryos inside the uterus do not show any furrowing at the time of anaphase. Nuclear division, however, occurs normally.

These observations suggest that several actin-dependent processes during early embryonic development are impaired. The lack of any visible membrane contractions, the absence of pseudocleavage and the absence of cytokinesis are reminiscent of phenotypes observed in early embryos upon treatment with actin-disrupting drugs such as cytochalasin (Strome and Wood, 1983). Processes that depend on microtubules, such as pronuclear migration and rotation of the pronuclei-centrosome complex, as well as chromosome segregation, appear normal in *cyk-3* embryos.

#### Embryonic polarity of *cyk-3* mutant embryos is partly disturbed

Since actin-dependent processes are impaired in *cyk-3* mutant embryos, we tested whether embryonic polarity, which requires a functional actin cytoskeleton, is also affected. Embryonic polarity was monitored in two ways, either by analyzing the localization of P-granules or by determining the position of the mitotic spindle in the first embryonic cell cycle.

We examined the localization of P-granules by staining fixed embryos with an antibody directed against PGL-1, a protein component of P-granules (Kawasaki et al., 1998). During pronuclear meeting, P-granules become localized to the posterior cortex of wild-type embryos where they remain during mitosis (Fig. 2). In subsequent divisions they are segregated into the P-lineage. In *cyk-3* embryos, P-granules are frequently mislocalized. From pronuclear meeting until telophase of the first mitosis, P-granules are not localized to the posterior cortex but cluster at approximately the middle of the embryos (11/12 embryos) (Fig. 2).

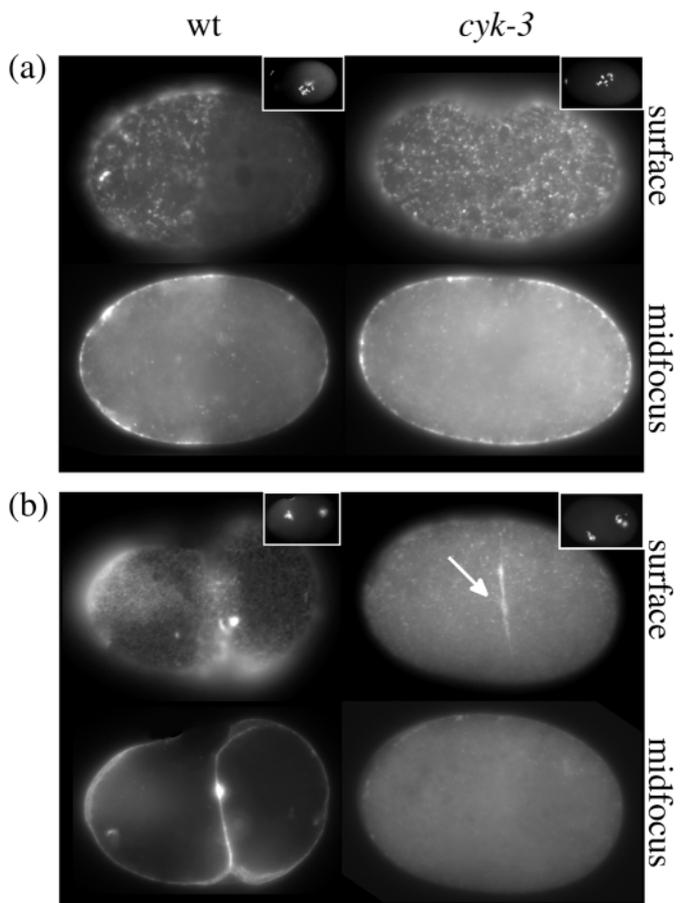


**Fig. 2.** P-granules are mislocalized in *cyk-3* mutant embryos. Embryos were dissected from homozygous mutant or wild-type hermaphrodites, fixed and stained with anti-tubulin (green), anti-PGL-1 (red) and Hoechst 33342 (blue). (a) In wild-type embryos, P-granules are localized to the posterior cortex during anaphase. (b) In *cyk-3* mutant embryos, P-granules are not localized to the posterior cortex during anaphase.

During the first mitotic division of wild-type embryos, the mitotic spindle is displaced towards the posterior hemisphere. To determine the extent of posterior displacement we measured anaphase spindles at maximal elongation relative to egg length and found that wild-type spindles are positioned at 0.55 egg length ( $n=5$ ). Mitotic spindles in *cyk-3* mutant embryos are also positioned at 0.55 egg length ( $n=5$ ). These findings show that different aspects of polarity are differently disturbed in *cyk-3* embryos. Whereas the mitotic spindle is positioned correctly, P-granules are mislocalized.

#### The actin cytoskeleton of *cyk-3* embryos is defective

In early *C. elegans* zygotes, actin forms a uniform cortical network of fibers and foci. During pronuclear formation and



**Fig. 3.** *cyk-3* embryos fail to redistribute f-actin but form an actin ring at the presumptive cleavage site. Embryos from either homozygous mutant or wild-type hermaphrodites were dissected directly into fixative containing rhodamine-labeled Phalloidin. DNA is stained with Hoechst 33342 and shown in the small insets. (a) At pronuclear meeting, the actin cytoskeleton of wild-type embryos is confined to the anterior hemisphere, forming the 'anterior cap' (surface view). In *cyk-3* mutant embryos of the same stage, the actin is homogeneously distributed (surface view). (b) In wild-type embryos at late telophase, a prominent actin-based contractile ring divides the cell into two. In *cyk-3* mutant embryos, the actin ring fails to divide the cell (midfocus). However, a ring of f-actin is formed at the right time and site, suggesting that cytokinesis is initiated (surface view, arrow).

migration, the actin cytoskeleton undergoes a dramatic redistribution that correlates with membrane contractions and pseudocleavage. At the time of pronuclear meeting, the majority of filamentous actin is localized to the anterior hemisphere, forming a structure called the ‘anterior cap’ (Strome, 1986) (Fig. 3a) ( $n=5/6$ ). Although *cyk-3* mutant embryos have an apparently normal uniform network of actin prior to pronuclear migration, the redistribution of the actin cytoskeleton does not occur. Instead, *cyk-3* embryos maintain a uniform distribution of filamentous actin around the cortex ( $n=4/4$ ) (Fig. 3a). The failure to redistribute actin might account for the lack of membrane contractions and pseudocleavage.

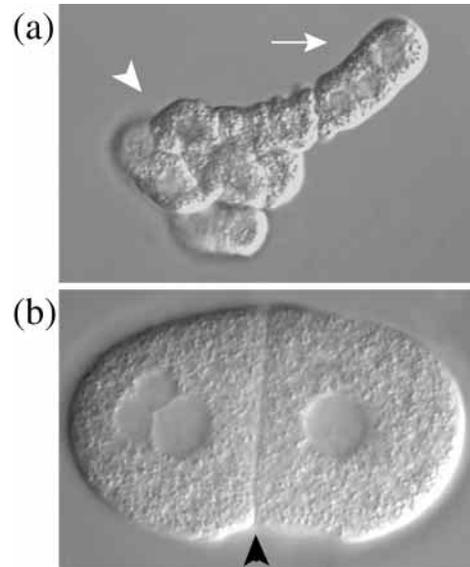
During late anaphase, a contractile ring containing actin and myosin is formed in wild-type embryos. As cells proceed through mitosis, this ring contracts, furrowing the overlying plasma membrane and thereby dividing the cell into two daughter cells (Fig. 3b) ( $n=6/6$ ). Focusing on the surface of late telophase *cyk-3* embryos, we found that a partial actin ring is formed at the correct position, slightly displaced to the posterior half of the embryo (Fig. 3b) ( $n=7/9$ ). This finding indicates that the initiation of cytokinesis is not impaired in *cyk-3* mutant embryos.

The rearrangement of the actin cytoskeleton in the early *C. elegans* embryo is concomitant with a cytoplasmic rearrangement, termed cytoplasmic flow, that is visualized by directed movement of yolk granules before and during pronuclear migration. Actomyosin-dependent cortical flow from the posterior to the anterior of the embryo induces streaming in the opposite direction in the interior of the embryo (Hird and White, 1993; Shelton et al., 1999). Since we observe defects of the actin cytoskeleton in *cyk-3* embryos precisely at the time when cytoplasmic flow occurs in wild-type embryos, we tested whether the movement of yolk granules is impaired in *cyk-3* embryos. We measured the rate of movement of the streaming of 90 individual granules from several wild-type or *cyk-3* mutant embryos. Although we observed a directed cytoplasmic flow in wild-type embryos with an average speed of  $6\pm 2$   $\mu\text{m}/\text{minute}$ , polarized movement of granules did not occur in *cyk-3* mutant embryos. Instead, in *cyk-3* embryos, granules moved with an average speed of  $1\pm 0.6$   $\mu\text{m}/\text{minute}$  in an apparently non-directional manner.

#### Optimized in vitro conditions partially rescue the cytokinesis defect observed in vivo

Apart from phenotypes related to the actin cytoskeleton, *cyk-3* mutant embryos are osmosensitive as well as sensitive to mechanical pressure. During the course of our actin staining, we also found that *cyk-3* mutant embryos are readily permeable to dyes. These characteristics are also found in wild-type embryos that are deprived of their eggshell.

In order to test whether mechano- and osmosensitivity can account for the cytokinesis phenotype observed in vivo, we analyzed the behavior of *cyk-3* mutant embryos under optimized in vitro conditions. We defined optimal in vitro conditions as conditions in which isolated wild-type blastomeres divide normally. Wild-type embryos were deprived of their eggshell and cultured in Embryonic Growth Medium (EGM) (see Materials and Methods). In this medium, wild-type cells perform cytokinesis normally (Fig. 4a). Cells of the P lineage divide along the a-p axes forming an elongated



**Fig. 4.** Optimized in vitro conditions partially rescue the cytokinesis defect of *cyk-3* embryos. (a) In EGM, wild-type blastomeres can divide normally. Cells of the P-lineage divide in a linear cleavage pattern (white arrow); cells of the AB lineage divide in a spiral cleavage pattern (white arrowhead). (b) *cyk-3* mutant embryos under the same conditions frequently show cleavage furrows (black arrowhead), indicating that cytokinesis is not a primary defect.

‘neck’ (Fig. 4a, arrow) (Edgar, 1995). AB daughter cells divide in a spiral cleavage pattern that results in a ‘ball’ of cells (Fig. 4a, arrowhead). In the same medium and in the absence of mechanical pressure, *cyk-3* mutant embryos are capable of forming cleavage furrows that constrict the membrane and divide the zygote (Fig. 4) ( $n=16/18$ ). However, the rescue of the cytokinesis defect is incomplete. Even though these embryos cellularize to some extent, individual cells often contain more than one nucleus, and the embryos remain swollen. Consistent with this observation, we did not find any mutant embryo that developed until hatching.

Even though optimal osmotic conditions together with the absence of mechanical pressure do not rescue the lethality of *cyk-3* mutant embryos, the process of cytokinesis itself is restored under these conditions. The cleavage furrows that form appear weak (in wild-type embryos, the ingressing cleavage furrow creates a prominent gap between the embryo and the eggshell; this gap is small or nonexistent in *cyk-3* mutant embryos), but they never regress and create distinct cells. We therefore conclude that the ‘cytokinesis machinery’ is not affected in *cyk-3* embryos. Thus, the cytokinesis defect is a secondary defect caused by osmosensitivity and sensitivity to mechanical pressure.

#### *cyk-3* mutant embryos have an intrinsic osmotic defect independent of defective extra-embryonic structures

Wild-type embryos are protected from their environment by an eggshell that is composed of an outer layer (vitelline), a chitinous middle layer and a lipid-rich inner layer (Rappleye et al., 1999; Schierenberg and Junkersdorf, 1992). The chitin layer provides mechanical support and gives the embryo its

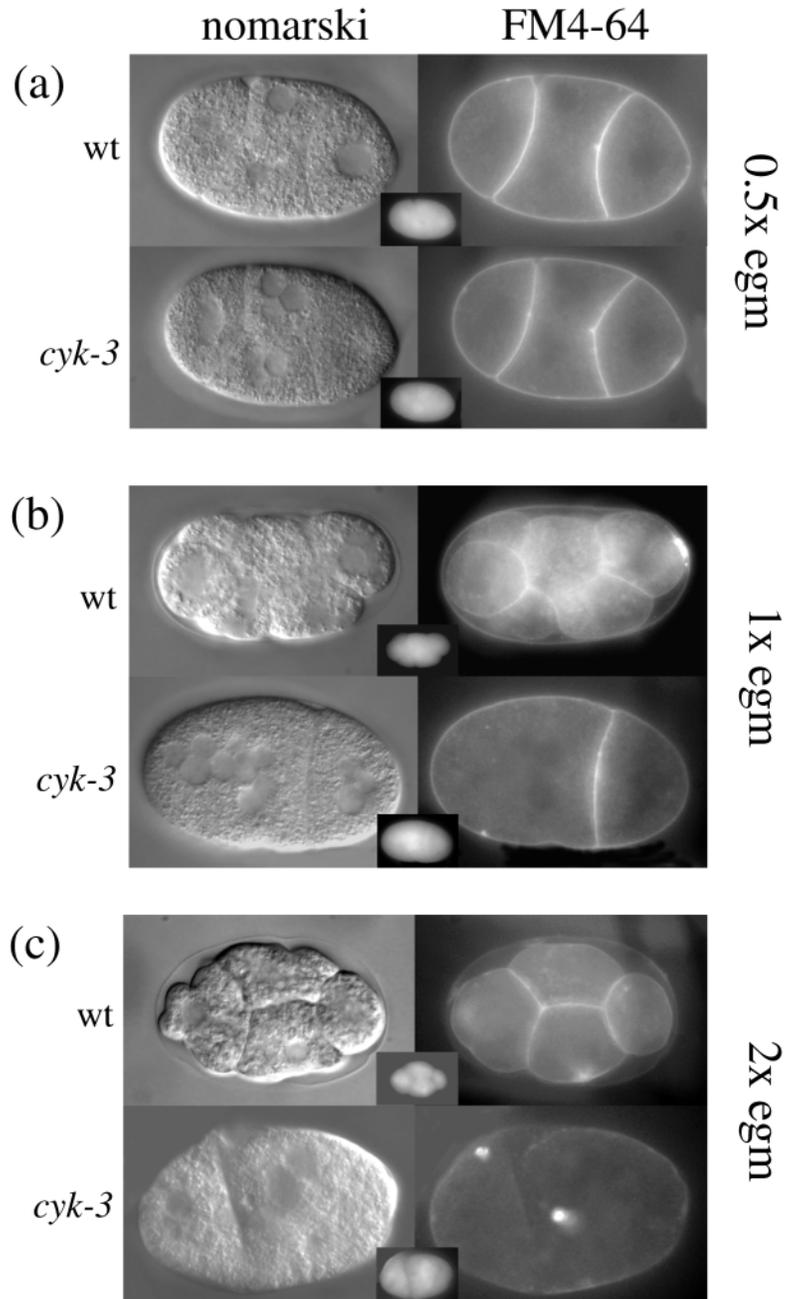
**Fig. 5.** Comparison of *cyk-3* embryos and wild-type embryos under different osmotic conditions. Embryos were dissected into EGM containing the vital dyes FM4-64 and calcein. FM4-64 stains membranes; calcein is a marker for viability (shown in the insets). 0.5×, 1× and 2× EGM refer to the increasing concentration of stock salts in the medium. (a) In hypotonic medium, wild-type and *cyk-3* embryos appear swollen, with few cleavage furrows. (b) In 1× EGM, under isotonic conditions, wild-type embryos have normal morphology; they neither shrink nor swell. By contrast, *cyk-3* embryos remain swollen, showing no difference from wild-type or *cyk-3* embryos observed in 0.5× EGM (compare with a). (c) In hypertonic medium, wild-type embryos respond to the higher ion concentration by shrinking. *cyk-3* embryos under the same conditions do not shrink but collapse. The collapsing does not involve membrane invaginations, since the ingression observed at the surface of the embryo does not correspond to membrane staining.

typical ellipsoid shape. Wild-type embryos whose chitin layer has been removed round up and become very sensitive to mechanical stress. The inner lipid-rich layer acts as a solvent barrier. Upon disruption or removal of this innermost layer, embryos are rendered permeable to dyes and sensitive to the osmotic conditions of their environment. Since *cyk-3* mutant embryos are both permeable and sensitive to their environment, it is likely that the inner lipid-rich layer is either absent or defective.

It is conceivable that defective extra-embryonic structures cause the osmosensitivity of the embryos. If this is the case, then *cyk-3* embryos should behave the same as wild-type embryos whose eggshell has been disrupted. To test this prediction, we disrupted the eggshell of wild-type embryos and compared the behavior of these permeabilized wild-type embryos with the behavior of *cyk-3* embryos under different osmotic conditions. To disrupt the lipid layer of wild-type embryos, pressure was gently applied to a coverslip, thereby cracking the eggshell of the embryos underneath. Permeabilization of the embryos was monitored using the vital dyes FM4-64 and calcein. FM4-64 was used to stain membrane structures, whereas calcein was used as a marker for viability. As a medium we used EGM, supplemented with different amounts of stock salts to change the osmolarity.

Wild-type embryos cracked in hypotonic medium (0.5×EGM) immediately swell. Additionally, cleavage furrows between two cells frequently disappear, and the remaining furrows constrict the membrane only weakly, presumably because of the higher turgor inside the embryo (Fig. 5a). Wild-type embryos under these conditions closely resemble *cyk-3* mutant embryos, which also appear swollen and furrow weakly (Fig. 5a).

Under isotonic conditions, in 1×EGM, wild-type embryos have a normal morphology; they neither swell nor shrink (Fig. 5b). The embryo consists of several viable, round cells. Under the same conditions, *cyk-3* embryos remain swollen, showing only weak furrowing (Fig. 5b). *cyk-3* embryos in 1×EGM appear similar to wild-type and *cyk-3* embryos in 0.5×EGM.



Wild-type embryos cracked in hypertonic medium (2×EGM) rapidly shrink (Fig. 5c). Large gaps appear between the remnants of the eggshell and the embryo. *cyk-3* embryos subjected to the same conditions do not react by shrinking. No gaps are observed between the eggshell and the embryo. *cyk-3* mutant embryos fail to adjust to the osmotic shock, and large creases are usually observed on the surface of the embryo (Fig. 5c).

In contrast to wild-type embryos whose eggshell has been disrupted, *cyk-3* mutant embryos barely respond to changes in the osmolarity of their environment. We therefore conclude that, apart from the apparent defect of the extra-embryonic structures, *cyk-3* embryos have an intrinsic cellular defect in regulating the response to osmotic stress.

*cyk-3* encodes an ubiquitin C-terminal hydrolase

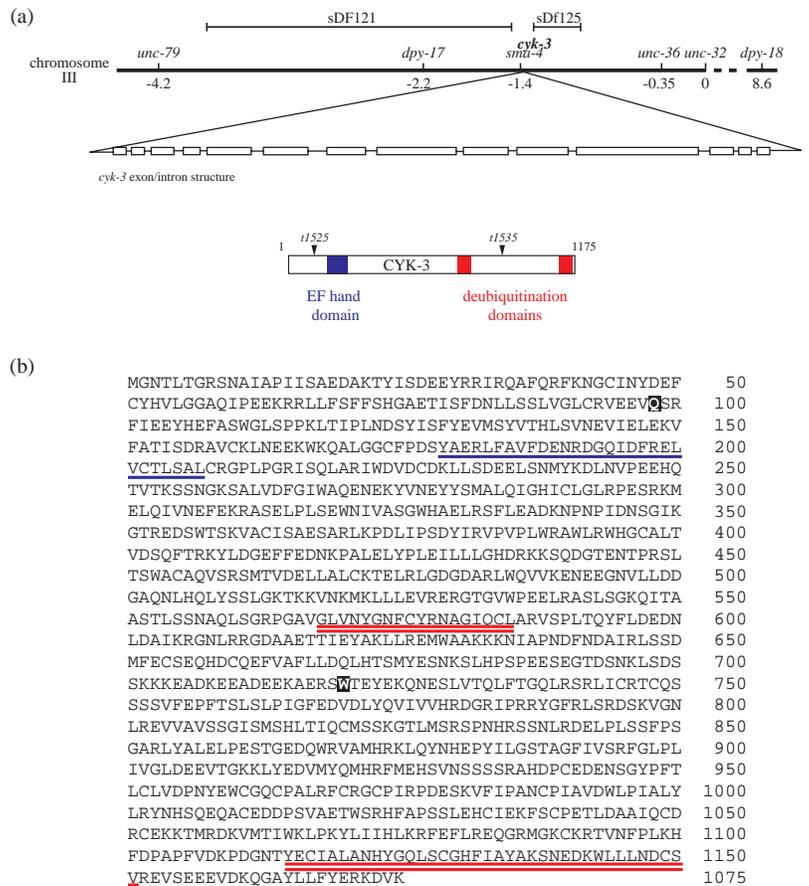
Using recombination mapping and testing of appropriate genomic deficiencies, we positioned *cyk-3* in the interval between *sma-4* and the breakpoint of sDf125 on LGIII, very close to *sma-4* (Fig. 6a). Functional rescue experiments were then used to further narrow the region. One cosmid from this region, ZK328, had rescuing activity. Using RNA interference against the predicted open reading frames of ZK328, we provisionally identified ZK328.1 as the gene encoding *cyk-3*, since its depletion caused both osmosensitivity and cytokinesis defects. To verify this candidate, we subcloned a genomic DNA fragment containing only the predicted gene and a promoter region into a plasmid and injected this construct. We obtained rescue activity to the same levels as with the whole cosmid. Sequence analysis of the two *cyk-3* alleles *t1525* and *t1535* revealed that each allele contained a single point mutation, in both cases leading to a premature stop codon (Fig. 6b). *t1525* is predicted to terminate the protein at amino acid 98 and is therefore probably a null allele (the other allele terminates the protein at residue 720; these alleles are phenotypically indistinguishable).

The sequence of ZK328.1 indicates that *cyk-3* is a ubiquitin C-terminal hydrolase, a deubiquitinating enzyme of the UBP family of deubiquitinating enzymes. CYK-3 contains a C-terminal UCH domain, consisting of a Cys (UCH1) and a His box (UCH2). In addition it contains an N-terminal EF-hand domain (Fig. 6a,b).

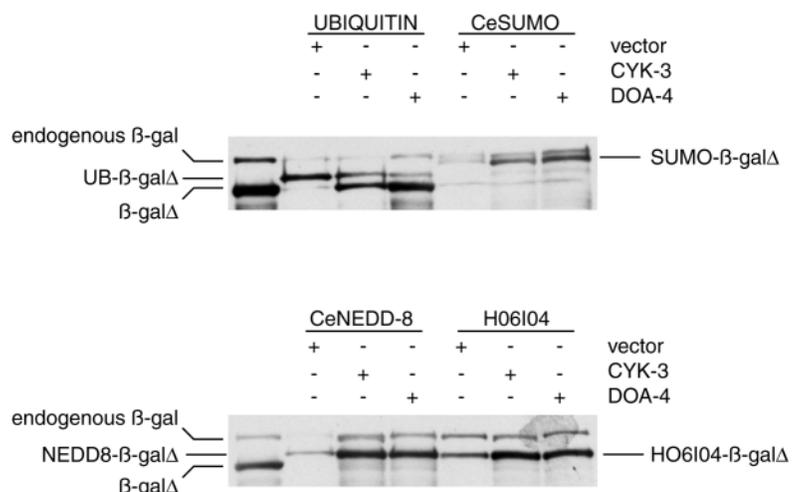
CYK-3 is a functional enzyme that specifically cleaves ubiquitin-fusion proteins

In order to determine whether CYK-3 is a functional enzyme capable of removing ubiquitin from a substrate, we co-expressed CYK-3 protein and, as a substrate, linear ubiquitin-β-galactosidase fusion proteins in bacteria. Ubiquitin was fused to the N-terminus of a truncated version of β-galactosidase (β-galΔ*HpaI*). We used truncated β-galactosidase in order to distinguish

**Fig. 7.** CYK-3 and DOA4 specifically cleave ubiquitin from linear protein fusions. Ubiquitin (UB) and the ubiquitin-like proteins CeSUMO, NEDD-8 and a ubiquitin-like protein encoded by cosmid H06104 were fused to a truncated version of β-Gal (β-GalΔ) and used as substrates. The fusion proteins were expressed in bacteria together with either CYK-3 or yeast DOA4. Cleavage activity was assessed by probing with an anti-β-Gal-antibody on a western blot. Both CYK-3 and DOA4 specifically cleave ubiquitin from β-GalΔ. In contrast, none of the ubiquitin-like proteins is cleaved from the β-GalΔ substrate in the presence of CYK-3 or DOA4.



**Fig. 6.** Map position and protein structure of *cyk-3*. (a) *cyk-3* maps to the central region of chromosome III, between the breakpoint of sDf125 and *sma-4*. The large genomic locus contains 14 exons coding for a 1175 amino-acid protein with two conserved domains, an N-terminal EF-hand domain and a C-terminal UCH domain consisting of a Cys-Box (UCH1) and a His-box (UCH2). (b) The CYK-3 protein sequence. The two residues marked with black boxes indicate the position of the point mutations leading to premature STOP codons in the alleles *t1525* (pos 98) and *t1535* (pos 720). The predicted EF-hand domain is underlined in blue; the predicted UCH domain is underlined in red.



our fusion protein from the endogenous  $\beta$ -galactosidase expressed in bacteria. We monitored deubiquitination by probing for  $\beta$ -gal with anti- $\beta$ -gal antibody on a western blot. As shown in Fig. 7, CYK-3 cleaves ubiquitin from the ubiquitin- $\beta$ -gal $\Delta$ HpaI fusion with a similar efficiency to yeast Doa4p. Doa4p has previously been shown to cleave ubiquitin in a similar assay (Papa and Hochstrasser, 1993).

Using the same assay, we also tested whether CYK-3 or Doa4p are specific for ubiquitin or whether either of them could also recognize a substrate targeted with a ubiquitin-like protein. We fused the *C. elegans* orthologs of the human ubiquitin-like proteins SUMO and NEDD8 to  $\beta$ -gal $\Delta$ HpaI and found that neither CYK-3 nor Doa4p cleave any of these fusion proteins (Fig. 7). Another ubiquitin-like protein encoded by cosmid H06108 is also not recognized by CYK-3 or Doa4p.

These results demonstrate that CYK-3 is a functional enzyme, a C-terminal hydrolase specific for recognizing and cleaving ubiquitinated substrates.

## Discussion

The *cyk-3* gene encodes a ubiquitin C-terminal hydrolase that is required for cellular osmoregulation. Loss of function mutations in this gene cause defects in actin-dependent processes, including cytokinesis and segregation of P-granules. We speculate that CYK-3 slows the degradation or endocytosis of an as yet unidentified factor that is essential for embryos to maintain osmotic balance.

### *cyk-3* mutant embryos are defective in osmoregulation

*C. elegans* embryos are normally protected from the outside environment by an extra-embryonic structure, commonly known as the eggshell. At present, not much is known about the origin of the eggshell or its molecular architecture. However, early studies on related nematodes (Wharton, 1980) combined with recent high resolution electron microscopy of *C. elegans* eggshells (Rappleye et al., 1999) show that the eggshell is composed of three different layers. An outermost layer, a chitinous layer and an inner lipid-rich layer. The most important layer for the embryo is the innermost layer. Embryos deprived of the two outer layers can still develop until hatching, but upon damage or removal of the innermost layer, embryos lose patterning information and develop into 'monsters' (Schierenberg and Junkersdorf, 1992). Additionally, the inner lipid-rich layer renders the embryo impermeable to vital dyes and provides protection from the osmotic conditions in the environment. We have found that *cyk-3* embryos are readily permeable to vital dyes and that they are exquisitely sensitive to osmotic conditions. Therefore, we conclude that the inner lipid-rich layer is compromised. Apart from the permeability defect, *cyk-3* mutant embryos respond differently to osmotic changes in the environment compared with wild-type embryos whose lipid-rich layer has been disrupted. Thus, *cyk-3* mutant embryos are defective in cellular osmoregulation. Moreover, we speculate that the internal pressure caused by the swelling may have prevented proper formation of the lipid-rich layer or, alternatively, disrupted it shortly after fertilization.

The defect in osmoregulation may or may not be the primary defect in *cyk-3* mutant embryos. However, it is certainly unlikely that CYK-3 acts directly in regulating the actin

cytoskeleton and that the defects in osmoregulation are a consequence of the disrupted actin cytoskeleton. Embryos with a highly defective actomyosin cytoskeleton owing to depletion, by RNAi, of non-muscle myosin or actin are not obviously osmosensitive (Gönczy et al., 2000; Guo and Kemphues, 1996; Shelton et al., 1999). Thus we conclude that the defects in actin-dependent processes in *cyk-3* mutant embryos do not cause the osmosensitivity phenotype.

Conversely, defects in osmoregulation perturb the actin cytoskeleton. When wild-type embryos are placed in hypotonic conditions, cytokinesis defects are observed, indicating that drastic changes in the cellular osmotic state can affect actin-dependent processes. Moreover, *cyk-3* embryos, either in utero or dissected into medium A, have many of the hallmarks of embryos treated with actin depolymerizing drugs; they are cytokinesis defective and fail to perform pseudocleavage. In addition, we do not observe polarized cytoplasmic flow. Regarding embryonic polarity, we find that the segregation of the P-granules to the posterior hemisphere is impaired in *cyk-3* mutant embryos. Asymmetric positioning of the mitotic spindle during the first cell cycle, on the other hand, occurs normally. Both processes have been shown to be actin dependent (Strome and Hill, 1988). At present, we do not know why one process is disturbed whereas the other is not.

The phenotype of *cyk-3* mutant embryos is somewhat similar to the phenotype of *pod-1* mutant embryos. *pod-1* encodes a coronin-like protein that binds to filamentous actin. Embryos lacking POD-1 display a variety of actin-related phenotypes, including loss of embryonic polarity and frequent failure of cytokinesis (Rappleye et al., 1999). Additionally, *pod-1* mutants are osmotically sensitive, indicating that osmoregulation and the regulation of the actin cytoskeleton are connected. In contrast to *cyk-3* embryos, however, *pod-1* embryos shrink in hypertonic medium, suggesting that the osmotic response in these cells is functional. Therefore, in contrast to *cyk-3* embryos, *pod-1* mutant embryos may be osmosensitive solely as a consequence of disrupted extra-embryonic structures.

Exposing *cyk-3* mutant embryos to optimized in vitro conditions allows cytokinesis to proceed, albeit not with high efficiency. This is in dramatic contrast to embryos observed in utero, which do not exhibit any furrowing activity. Thus, the uterus of *C. elegans* hermaphrodites is not an osmotically ideal environment. Moreover, the uterine contractions subject embryos to strong mechanical stress. Therefore, it may be beneficial to observe osmosensitive embryos both in utero and in optimized in vitro conditions.

### CYK-3 probably reverses the ubiquitination of a small number of substrates

The ubiquitin pathway for intracellular protein degradation is involved in a wide range of cellular functions, ranging from cell cycle regulation (Glotzer et al., 1991) to transcriptional regulation (Chen et al., 1993) to regulation of endocytosis (Hicke and Riezman, 1996) (for a review, see Hochstrasser, 1996). The biochemical machinery that comprises the ubiquitin pathway is complex. Proteins are targeted for degradation by the proteasome complex by chains of ubiquitin monomers. To build ubiquitin chains, a monomer is first activated by the E1 enzyme from which it is transferred to a ubiquitin-conjugating

enzyme, E2. Transfer to the target protein or to the end of a growing ubiquitin chain is catalyzed by E3 ubiquitin ligases. The ubiquitin C-terminal hydrolases (UCH) catalyze the reverse reaction - disassembly and/or removal of multiubiquitin chains. Although the ubiquitin conjugation machinery has been extensively studied, the ubiquitin C-terminal hydrolases are less well understood (for a review, see Wilkinson and Hochstrasser, 1998).

A loss of function mutation of an UCH would be expected to lead to increased amounts of one or more ubiquitin-modified target protein(s). If these ubiquitin moieties act as degradation signals then loss of function of the UCH would cause more rapid degradation of the target protein. This would affect the pathway(s) that require that specific target protein. There is one example of a UCH functioning in this manner. During development of the *Drosophila* eye, the UCH encoded by the *fat facets* (*faf*) gene is required for proper cell fate determination (Huang et al., 1995). Importantly, this function can be suppressed by mutations in a proteasome subunit gene, suggesting that *faf* acts to stabilize one or more substrates. Indeed, the epsin homolog encoded by the liquid facets is a good candidate for being a critical substrate of *faf* (Cadavid et al., 2000). However, UCHs can act in the protein degradation pathway in a different way. Yeast Doa4p is required for proteasome-dependent proteolysis and to recycle ubiquitin chains to maintain a pool of free ubiquitin (Papa et al., 1999; Papa and Hochstrasser, 1993; Swaminathan et al., 1999). Mutation of this type of UCH leads to pleiotropic defects in protein degradation. Since cell cycle timing and nuclear division occur with near normal kinetics in *cyk-3* mutant embryos, it appears unlikely that CYK-3 plays a role in general protein degradation. If CYK-3 is involved in ubiquitin-mediated protein degradation, we suggest that it functions by removing ubiquitin moieties from a specific protein (or from a small number of targets) that is involved in the osmoregulation of early *C. elegans* embryos.

A third possibility for CYK-3 function is that it regulates ubiquitin-mediated receptor internalization. A number of membrane proteins have been shown to be mono- or di-ubiquitinated upon ligand binding. This modification is essential for their internalization and subsequent degradation by the lysosome (for a review, see Hicke, 1999). A role for CYK-3 in endocytosis and degradation via the lysosomal compartment would also be consistent with the observation that general protein degradation is not impaired in *cyk-3* mutant embryos.

### Possible targets of CYK-3

At present, we can only speculate about the possible targets of CYK-3. Since *cyk-3* mutant embryos are mechano- and osmosensitive, ion channels and osmolyte transporters are possible CYK-3 targets. As mentioned above, there are reports of membrane proteins regulated by ubiquitination. The mammalian epithelial Na<sup>+</sup> channel is downregulated via ubiquitination of two of its subunits (Staub et al., 1997). Defective regulation of the epithelial Na<sup>+</sup> channel is implicated in several human diseases, including Liddle's syndrome, an inherited form of hypertension (Abriél et al., 1999). The *C. elegans* degenerins are orthologs of the mammalian epithelial Na<sup>+</sup> Channel proteins (Mano and Driscoll, 1999). Mutations in

these genes result in neuronal swelling and degeneration (Driscoll and Chalfie, 1991), leading to defects in locomotion. Since homozygous *cyk-3* adults move normally, we think it is unlikely that these particular Na<sup>+</sup> channels are targets of CYK-3 regulation. Indeed, animals homozygous for null mutations in *cyk-3* are indistinguishable from wild-type animals except that their progeny are inviable; CYK-3 appears essential only in the early embryo. Perhaps embryos require a specialized machinery for osmoregulation as the ratio of surface area to volume is particularly low in embryonic cells. Though technically demanding, one method to discover CYK-3 targets is to use electrophysiological methods to determine whether particular channels or transporters are absent in *cyk-3* mutant embryos. Recent analysis of membrane transporters in early *C. elegans* embryos and oocytes has shown the feasibility of such analysis (Rutledge et al., 2001).

We thank Bruce Bowerman and Johji Miwa and the *Caenorhabditis* Genetics Center (CGC) for providing strains. The CGC is funded by the National Center for Research Resources of the National Institutes of Health (NIH). We thank Susan Strome for providing the anti-PGL-1 antibody, Alan Coulson for providing cosmids and YACs, Yuji Kohara for providing us with cDNAs and Andy Fire for providing us with the vector pPD8.02. We are particularly grateful to Pierre Gönczy for helpful discussions and for critical comments on this manuscript. We want to thank Verena Jantsch for valuable advice and constant support throughout the project. This work was generously supported by Boehringer Ingelheim.

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