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Invadolysin: a novel, conserved metalloprotease links mitotic structural rearrangements with cell migration

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The cell cycle is widely known to be regulated by networks of phosphorylation and ubiquitin-directed proteolysis. Here, we describe IX-14/invadolysin, a novel metalloprotease present only in metazoa, whose activity appears to be essential for mitotic progression. Mitotic neuroblasts of Drosophila melanogaster IX-14 mutant larvae exhibit increased levels of nuclear envelope proteins, monopolar and asymmetric spindles, and chromosomes that appear hypercondensed in length with a surrounding halo of loosely condensed chromatin. Zymography reveals that a protease activity, present in wild-type larval brains, is missing from homozygous tissue, and we show that IX-14/invadolysin cleaves lamin in vitro. The IX-14/invadolysin protein is predominantly found in cytoplasmic structures resembling invadopodia in fly and human cells, but is dramatically relocalized to the leading edge of migrating cells. Strikingly, we find that the directed migration of germ cells is affected in Drosophila IX-14 mutant embryos. Thus, invadolysin identifies a new family of conserved metalloproteases whose activity appears to be essential for the coordination of mitotic progression, but which also plays an unexpected role in cell migration.

Introduction

Recent years have witnessed remarkable strides in our understanding of the control of cell cycle progression. It is now clear that cell cycle progression is driven by the successive activation and inactivation of the Cdk, and that abrupt transitions are often enforced by the cleavage of key targets by the proteasome after their ubiquitination by various E3 ubiquitin ligases (for review see Murray, 2004). Cdk phosphorylation of key components regulates the entry of cells into S phase and choreographs the subsequent firing of replication origins. Entry into mitosis is also determined by Cdk, and Cdk phosphorylation of the nuclear lamins drives the cycle of nuclear envelope disassembly and reassembly that is characteristic of mitosis in metazoa. The exit from mitosis is triggered not only by proteolysis of mitotic cyclins but also of securin leading to the activation of separase, a CD clan protease that cleaves the rad21/Scc1 non-structural maintenance of chromosomes cohesin subunit, thereby triggering the separation of sister chromatids.

One aspect of mitosis about which we still know relatively little is the process of mitotic chromosome formation. The compaction of chromatin into mitotic chromosomes is essential to avoid sister chromatid entanglement and cleavage of chromatin at cytokinesis. Recent breakthroughs, such as the discovery of the structural maintenance of chromosomes—containing condensin complex, at first appeared to provide the key, but recent analyses of condensin mutations and RNAi depletions have revealed that mitotic chromosomes form even in the absence of condensin subunits (Bhat et al., 1996; Steffensen et al., 2001; Coelho et al., 2003; Hudson et al., 2003). Further evidence suggests that correct mitotic chromosome condensation is linked to replication timing and checkpoint control (Loupart et al., 2000; Krause et al., 2001; Pflumm and Botchan, 2001). Thus, the search for factors that give the mitotic chromosome its characteristic form and integrity is still very much on.

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Abbreviations used in this paper: MMP, matrix metalloproteases; S2, Schneider 2.

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This work began as part of our ongoing characterization of Drosophila melanogaster mutations that are defective in mitotic chromosome condensation. Mitotic events are particularly amenable to study in Drosophila as many mutations affecting mitosis are lethal only at the late larval stages due to the maternal store of proteins in the embryo and the fact that larvae do not require mitotic activity for growth and development. This allows the use of mitotically active tissues such as larval brains and imaginal discs for studying proliferation in wild-type and mutant states (Gatti and Baker, 1989; Theurkauf and Heck, 1999). As a result, Drosophila has proven useful both for the characterization of known cell cycle regulators (O’Farrell et al., 1989; Edgar and Lehner, 1996; Fogarty et al., 1997; Sibon et al., 1997; Jager et al., 2001) and for the identification of novel factors, such as the polo and aurora kinases that have subsequently proven to be important for cell cycle regulation in diverse organisms (Sunkel and Glover, 1988; Llamazares et al., 1991; Glover et al., 1995).

We examined the l(3)IX-14 mutation because of its dramatic effects on mitotic chromosome formation, distinct from other mitotic mutations. Our detailed analyses have revealed the gene to be important for other aspects of mitosis, including spindle assembly and nuclear envelope dynamics. Furthermore, the identification of the gene and characterization of its higher eukaryotic counterparts led to several surprises. The IX-14 gene encodes a metalloprotease that is concentrated in cytoplasmic structures resembling invadopodia. Invasive tumor cells have the ability to elaborate invadopodia that facilitate extracellular matrix degradation, thus aiding metastasis (Bowden et al., 2001; Buccione et al., 2004). The IX-14 protein is structurally related to leishmanolysin, a major surface protease from Leishmania protozoa, which is thought to have a significant role in the pathogenesis of leishmaniasis (Yao et al., 2003). As a result of the subcellular localization and the sequence homology, we have termed the protein product of the IX-14 gene “invadolysin.”

We have discovered that invadolysin is highly concentrated at the leading edge of migrating macrophages. Consistent with a role in cell migration, we observe that the active migration of priorordial germ cells is affected in mutant fly embryos. Thus, IX-14/invadolysin, a member of a new class of metalloproteases, links mitosis with cell migration.

**Results**

**Chromosome structure is disrupted in the IX-14 mutation**

l(3)IX-14 is a late larval lethal mutation on the right arm of the third chromosome, generated in an ICR-170 chemical mutagenesis screen for imaginal disc mutations (Shearn et al., 1971). Preliminary analysis reported that the mutation was characterized by metaphase arrest and hypercondensed mitotic chromosomes (Gatti and Baker, 1989). We generated a transposon insertion allele of the gene, l(3)IX-1447T, by local hopping of a nearby P-element insertion. Here, we report a detailed phenotypic analysis of both alleles, investigating not only chromosome morphology but also other aspects of cell division.

Whole mount preparations of IX-14 third instar larval brains and imaginal discs showed that mutant tissues had proliferation defects resulting in much-reduced brain size and missing imaginal discs (unpublished data). Fig. 1 shows typical DAPI-stained mitotic figures observed in wild-type (Fig. 1 A) and in IX-14 mutant (Fig. 1 C) neuroblasts. The IX-14 neuroblasts show the length-wise hypercondensation of mitotic chromosomes initially observed after orcein staining, but additionally demonstrate that the mutant chromosomes appear loosely condensed with a ragged periphery. This phenotype differs from the extreme hypercondensation in other mutations that produce a mitotic arrest phenotype (Heck et al., 1993) or treatment of wild-type cells with microtubule poisons such as colchicine (Fig. 1 B). Allowing more time in mitosis with colchicine does not rescue the lateral condensation defect in IX-14 mutant neuroblasts (Fig. 1 D), thus we believe the phenotype represents a specific condensation defect rather than “conventional” hypercondensation in response to mitotic delay.

The IX-14 mutation affects interphase polytene chromosomes as well as mitotic chromosomes. Polytene chromosomes from salivary glands of wild-type third instar larvae are distinctively banded along the chromosome arms, and the centromeres of the endo-reduplicated chromosomes are clustered at the chromocenter (Fig. 1 E). This characteristic banding pattern is abolished in IX-14 mutant polytene and the chromosome arms appear twisted and frayed (Fig. 1 F). In many nuclei it is difficult to identify an obvious chromocenter. Additionally, mutant polytene were approximately half the size seen in wild type. This reduction in size may be due to under-replication, as BrdU incorporation is clearly reduced in IX-14 mutant neuroblasts, though the structural defects are not merely explicable by reduced replication.

To further investigate an effect of the IX-14 mutation on interphase chromatin structure suggested by the polytene phenotype, we addressed whether or not this mutation might act as a modifier in a position effect variegation assay. We examined the effect of one copy of each IX-14 allele on expression of the white gene in a white mottled 4 (wm4) background. In wm4, inversion of much of the X chromosome places the white gene (expression of which is responsible for normal red eye color) adjacent to heterochromatin, thereby reducing its expression. Mutations that enhance variegation, E(var)s, further reduce expression of the white gene (whiter eyes) potentially by increasing the “spreading” of heterochromatin. Suppressors of variegation, Su(var)s, result in greater expression of the white gene (redder eyes) by decreasing the heterochromatic environment of the reporter gene. Fig. 1 G shows a pronounced increase in the redness of flies’ eyes with one copy of each IX-14 allele in a wm4 background, indicating that IX-14 alleles are acting as Su(var)s and “opening” heterochromatin. By inference, one role of wild-type IX-14 may be to promote chromatin compaction or heterochromatin formation, which is consistent with the observed mitotic defects. Therefore, we conclude that IX-14 is required for chromosome architecture during both mitosis and interphase.
Spindle and centrosomal abnormalities are common in IX-14 mutants

The over-shortened mitotic chromosomes suggested that IX-14 mutants may experience a metaphase delay possibly caused by aberrant spindles. Neuroblasts of IX-14 mutants indeed exhibited abnormal spindle morphology (Fig. 2, B–D and F), and in fact only 2% of mitotic cells had a normal bipolar spindle. Spindle abnormalities included monopolar spindles (37%; Fig. 2 B, inset 1), disorganized spindles (34%; Fig. 2 B, insets 2 and 3), and mitotic figures where the spindle appeared bipolar, but asymmetric (27%, Fig. 2 C, inset 4). Mutant spindles also appeared to have thicker bundles of microtubules, compared with the finer fibers observed in wild type (Fig. 2 A). Almost no anaphase figures were observed in DAPI-stained neuroblasts from IX-14 mutant larvae, which is consistent with a metaphase delay or arrest.

Nearly 70% of mitotic figures in IX-14 neuroblasts appeared to have only one focus of centrosomal staining, as judged by CP190 localization (Fig. 2, B–D and F). Cells with monopolar spindles always had a single centrosome, whereas 75% of the asymmetric bipolar spindles also had only one CP190 “spot” (Fig. 2 G). This single centrosome phenotype was also verified with another mitotic centrosomal marker, centrosomin (unpublished data). In many cases, centrosomes appeared to have a dumbbell shape (Fig. 2 F, arrow), suggesting that centrosomes had duplicated but not separated. Although CP190 normally has a nuclear localization during interphase (binding to specific loci on polytene), the protein dissociates from chromosomes in mitosis (Whitfield et al., 1995). Mitotic chromosomes from IX-14 homozygous cells curiously appeared to have higher levels of CP190 than wild-type chromosomes (Fig. 2 F).

The aberrant spindles in the IX-14 mutation afford an explanation for the length-wise hypercondensation of chromosomes, as spindle defects would be expected to arrest cells in mitosis. However, the loose compaction and ragged edges of the chromosomes cannot be accounted for by spindle abnormalities as this is not observed when cells are arrested in mitosis in response to microtubule poisons or other spindle defects (Heck et al., 1993). Thus, IX-14 has essential roles both in chromosome and spindle architecture in Drosophila cells.

IX-14 mutant mitotic cells accumulate abnormally high levels of nuclear envelope proteins

As higher eukaryotic cells enter mitosis, chromosome condensation and spindle assembly are accompanied by the disassembly of the nuclear envelope. Surprisingly, the majority of IX-14 mitotic cells showed dramatically increased levels of lamin Dm0 (a B-type lamin), compared with wild-type mitotic cells (Fig. 3, A and B). Strikingly, the level of another Drosophila nuclear envelope protein, otefin, also appeared elevated in IX-14 mitotic cells (Fig. 3, C and D). When double immunofluorescence was performed, we observed a simultaneous increase of lamin and otefin in the same cells (unpublished data). We believe this elevation occurs before mitosis as late G2 cells (those positive for variegation assay using “white” as a reporter gene (wm)). Flies were sorted into categories based on redness of the adult eyes (white bars, 0–25% redness; yellow bars, 26–50% redness; orange bars, 51–75% redness; red bars, 76–100% redness). The distribution of eye color is shown for the original wm stock as well as for wm stocks containing one copy each of the two mutant l(3)IX-14 alleles. The majority of wmtl(3)IX-14 (both alleles) flies grouped toward the red end of the spectrum, implying that the mutant alleles act as suppressors of variegation (enhancing expression of the reporter gene) and, conversely, that the wild-type gene product acts to compact chromatin.

The increase in immunofluorescence corresponds to an actual increase in protein level. Immunoblotting for lamin Dm0 and otefin very clearly showed that these proteins accumulated to unusually high levels in IX-14 larval brains (compare with the α-tubulin loading control in Fig. 3 [E and F]). We additionally detected an increase in the level of Drosophila lamin C (unpublished data). We only detected increase of full-length forms by immunoblotting, with no evidence for accumulation of alternative forms. Merely arresting wild-type cells in mitosis with colchicine did not elevate lamin levels (unpublished data).
We conclude that the IX-14 mutation appears to affect multiple aspects of structural rearrangement as cells enter mitosis.

**The IX-14 gene encodes a novel metalloprotease**

We mapped the original IX-14 allele to the 85E10-F16 region by crossing to deficiency lines with known breakpoints. We then generated a P-element insertion allele by local hopping a nearby P-element, l(3)04017. The P-element allele allowed cloning of adjacent genomic DNA by inverse PCR; hybridization of this fragment to a *Drosophila* P1 array refined our mapping to 85F14-15. A candidate IX-14 gene was cloned by identifying a 3.6-kb full-length *Drosophila* adult head library EST whose sequence overlapped with the ~700-bp genomic fragment flanking the P-element. The gene was composed of nine exons, with the first exon separated from the remaining eight by a large (~8.6 kb) intron (Fig. 4 A, asterisk). This gene has been designated CG3953 in the *Drosophila* genome annotation database.

We mapped the P-element insertion site to 40 bp upstream of the start of transcription of the IX-14 gene. Precise excision of this P-element was shown to revert the mutant phenotype and restore viability. The insertion appeared to disrupt the IX-14 promoter, resulting in a strong hypomorphic or null mutation. To determine whether or not expression of this gene was affected in IX-14 alleles, Northern blot analysis was performed on total RNA from third instar larvae. This showed that the predicted 3.6-kb mRNA was indeed missing in larval extracts prepared from both IX-14 alleles (Fig. 4 B).
The 2,052 nucleotide ORF in the IX-14 cDNA encodes a 683 amino acid protein (predicted $M_r = 71$ kD) with homology to proteins in the M8 class of zinc-metalloproteases focused on the characteristic “HEXXHXXG[X]$_2$H” catalytic motif. The founding member of this family is the leishmanolysin cell surface protein (also called GP63) from the pathogen Leishmania major. In vitro mutagenesis of leishmanolysin has determined that the glutamic acid and three histidine residues are essential for protease activity (McMaster et al., 1994; Macdonald et al., 1995; McGwire and Chang, 1996). We have been able to identify orthologues in all higher eukaryotes examined, but conspicuously not in bacteria or yeasts, suggesting that the IX-14 gene product may only be required in metazoa.

The worm, human, mouse, and fly orthologues are shown in the alignment of Fig. 4 C, but other more divergent orthologues (e.g., Arabidopsis thaliana and Dictyostelium discoideum) are not included. Although the most obvious homology with leishmanolysin is centered on the conserved zinc-metalloprotease motif (Fig. 4 C, boxes) and immediate surrounding regions, the $\text{NH}_2$- and COOH-terminal regions are considerably more divergent. A potential signal sequence is present near the $\text{NH}_2$ terminus of all orthologues, although whether or not this sequence acts to target the protein is currently unknown. Intriguingly, there are at least nine blocks (Fig. 4 C, numbered double-headed arrows) shared among the worm, human, mouse, and fly orthologues that are absent from the leishmanolysin sequence. Despite this finding, the positions of 14 cysteines are remarkably conserved between leishmanolysin and the higher eukaryotic proteins (Fig. 4 C, asterisks). This result suggests strongly that the “core” of the IX-14 protease should resemble that of leishmanolysin (Schlagenhauf et al., 1998), and furthermore predicts that the indicated “insertions” should lie at the surface of this structure. Indeed, the sites of these insertions all map to the surface of the leishmanolysin structure (Fig. 4 D, black numbered spheres; the internal zinc ion essential for catalysis is represented by a magenta sphere). Therefore, we conclude that IX-14 is a member of a subgroup of the leishmanolysin protease group.

**RNAi depletion of IX-14 in cultured cells phenocopies neuroblast defects**

As further evidence that the gene we identified is responsible for the defects observed in IX-14 larval tissues, we performed dsRNA-mediated interference of IX-14 in Drosophila Schneider 2 (S2) cells. The abnormal spindle phenotypes seen in the mutant alleles, typically monopolar or disorganized, were phenocopied in roughly 25% of the IX-14 RNAi mitotic cells (Fig. 5 A). In addition, mitotic figures from RNAi cultures were frequently observed to have single centrosomes or what appeared to be two closely apposed centrosomes (Fig. 5 B). Although control S2 cells have a low level of aneuploidy and occasional abnormal numbers of centrosomes, neither the aberrant spindles nor unseparated centrosomes were observed in control cells (Fig. 5 A). Therefore, we are confident that loss of the IX-14 protein is responsible for the observed cellular phenotypes.

**IX-14 cleaves lamin in vitro**

To examine whether or not IX-14 has protease activity, suggested by its homology to the catalytic motif of leishmanolysin, we performed two complementary approaches. In the first, protein extracts prepared from wild-type and IX-14 mutant larval brains were examined for protease activity (Fig. 6 A). Protease activity was assayed in zymogram gels containing casein as substrate. Whole larvae harbor a high level of protease activity at numerous molecular masses when assayed by zymography (unpublished data). Therefore, we prepared protein extracts

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*Figure 3. Abnormal levels of nuclear envelope proteins in l(3)IX-14 mutant cells. Larval brains from wild-type and IX-14 homozygous mutant animals were processed for lamin and otefin detection. (A) Wild-type mitotic cells (arrow) show homogeneous lamin staining as the nuclear lamina becomes dispersed during mitosis. (B) In contrast, both IX-14 alleles have greatly increased lamin staining during mitosis (arrowhead). Bar, 5 $\mu$m. (C) Wild-type neuroblasts show distinct nuclear envelope staining similar to lamin. (D) Both IX-14 alleles also show greatly increased otefin staining during mitosis. Protein extracts from wild-type and IX-14 larval brains were probed for Dm0 lamin (E) and otefin (F). $\alpha$-tubulin (bottom) served as a loading control, and confirmed that the samples were similarly loaded. The levels of both lamin and otefin detectable by immunoblotting was significantly greater in the mutant tissues than in wild-type brains.*
Figure 4. **Molecular characterization of l(3)IX-14.** (A) Map of the DmIX-14 gene (CG3953), with exons depicted as black boxes and 5' and 3' UTRs as hatched boxes. The position of the P element insertion in l(3)IX-14(G7) is 40 bp upstream of the transcription start site (gray triangle). The first intron (8.6 kb, asterisk) is not shown to scale in this figure. (B) Northern blot of wild type and l(3)IX-14 heterozygous (+/+) and homozygous (+/+) total larval RNA probed with l(3)IX-14 full-length cDNA. The same blot probed for ribosomal protein RP49 mRNA is shown as a control. The l(3)IX-14 mRNA is undetectable in RNA obtained from homozygous l(3)IX-14 alleles. (C) T-COFFEE alignment showing homology between *Drosophila melanogaster* IX-14, metazoan orthologues, and Leishmanolysin, with the conserved HEXXHXXG (and third required H) zinc-metalloprotease motif boxed. Sequences shown are as follows: Ce, *Caenorhabditis elegans* (CAB16471); Hs, *Homo sapiens* (CAC42882); Mm, *Mus musculus* (NP 766411); Dm, *Drosophila melanogaster* (NP 652072); Lm, *Leishmania major* (AF039721). Although the homology between leishmanolysin and the other proteins appears to be largely limited to regions surrounding the metalloprotease motif, the placement of 14 cysteines (asterisks) is strikingly conserved. Nine regions shared among the higher eukaryotic ortho-
from larval brains only, as the phenotypes were clearly apparent in this proliferating tissue. Wild-type brain lysates had two visible bands of protease activity, migrating at 120 and 135 kD (Fig. 6 A, asterisks). Remarkably, these two bands of activity were almost completely absent in IX-14 brains, indicating that mutant extracts were indeed missing proteolytic activity. A separate stained gel documents that wild-type and mutant lanes were equivalently loaded (Fig. 6 B). As these zymogram gels are nondenaturing, the molecular mass of the observed activity is not necessarily related to predicted molecular mass, but may suggest that IX-14 migrates as a multimer or part of a complex. The lack of protease activity in mutant brains further suggests that the IX-14 gene product is responsible not only for the mutant phenotypes but also for the protease activity observed under these conditions (either directly or potentially through the activation of other proteases).

The increase in nuclear envelope proteins observed by immunofluorescence and immunoblotting in mutant brains suggested that lamin (or otefin) might be a substrate of the IX-14 metalloprotease. As shown in Fig. 6 C, in vitro synthesized Dm0 lamin is cleaved by in vitro synthesized IX-14. Furthermore, the cleavage of lamin can be inhibited by the inclusion of ortho-phenanthroline, a chelator of zinc (Fig. 6 C, asterisks). Although full-length lamin was detected by immunoblotting in this experiment (Fig. 6 C, arrowhead), we failed to detect any cleavage products (they may not be recognized by the antibody used). Thus, IX-14 is a novel essential protease capable of cleaving at least one component of the nuclear envelope.

**IX-14 localizes to distinct structures in the cytoplasm of Drosophila and human cells**

Several approaches were used to determine the subcellular localization of the IX-14 protein. *Drosophila* S2 cells transiently transfected with expression plasmids tagging either the NH2 or COOH terminus with EGFP showed cytoplasmic localization, whereas vector alone localized to both the nucleus and cytoplasm (Fig. 7 A). The human genome contains a single IX-14 gene, which we have shown by preliminary siRNA analysis to be essential for viability (unpublished data). Due to the limit of resolution with the relatively small and generally nonadherent S2 cells, we turned to examining IX-14 localization in human cells. A COOH-terminal EGFP fusion construct of HsIX-14 in HeLa cells was also localized...
in the cytoplasm, often as unusual ring-like structures (Fig. 7B). A control transfection with EGFP alone localized throughout both the nucleus and cytoplasm. From these experiments we concluded that the IX-14 protein localized predominantly in the cytoplasm of fly and human cells.

Two protein sequences for the human orthologue possibly representing alternatively spliced forms have been submitted: CAC42883–version 1 and CAC42882–version 2 (Fig. 4B). These forms differ in their NH2-terminal regions (upstream of the residues VINK) and by the presence of a 37–amino acid sequence in the COOH-terminal half of version 2 found in all the other eukaryotic orthologues so far (between the residues EDTG:RQML). We generated an antibody to amino acids 327 to 629 of HsIX-14.v1 (downstream of the catalytic motif). As this region is fully present in both predicted human versions, the antibody should recognize the two forms if they indeed both exist. Fig. 8 A shows a typical staining pattern observed with the HsIX-14327-629 antibody in HeLa cells. We observed unusual ring-like structures similar to those seen with the EGFP-tagged protein. These striking structures were also observed in two other human transformed cell lines, Jurkat and CF-PAC (unpublished data). These structures were observed in all interphase cells, and although their size remained fairly constant (<1 μm in diameter), the number of the structures varied on a cell to cell basis. Z-series of sections through cells showed that these structures were located in the lower third of the cells. Because the ring-like structures were dispersed in mitosis in all cell types examined, we believe that the localization (and/or activity) of IX-14 may be regulated during the cell cycle.

Although proteases are found in diverse structures within cells, this particular localization did not resemble any of the usual protease-containing complexes, e.g., 26S proteasome, the related COP9–signalosome complex, lysosomes, or aggresomes. Nonetheless, we performed colocalization immunofluorescence with antibodies to IX-14 and proteasome core subunits α5 and β5i, signalosome subunit Cgm3, chaperone protein Hsc70, and markers for Golgi, mitochondria, and lysosomes. We observed no significant colocalization between IX-14 and any of these proteins (unpublished data). However, numerous transformed cells contain invadopodia, which are believed to be important for extracellular matrix degradation and cellular migration (Bowden et al., 1999, 2001; Baldassarre et al., 2003; Buccione et al., 2004). Invadopodia resemble in size and distribution the structures labeled by the IX-14 antibody. As no proteins exclusive to invadopodia have yet been identified, definitive colocalization was not possible. However, we detected limited colocalization with markers shown to label invadopodia (e.g., phosphotyrosine, cortactin, and Dynamin 2), suggesting that these cytoplasmic ring-like structures very likely are invadopodia.

**A role for IX-14 in cell migration**

If IX-14 were involved in cell migration, as suggested by the invadopodia-like localization, one might predict that the protein should localize to regions of cells actively involved in migration. Thus, we examined the localization in stationary and migrating human macrophages (Fig. 8, B and C). Fig. 8 B showed that punctate, at times ring-like, cytoplasmic IX-14 structures were observed in cultured human macrophages (in the example shown, the cell is stationary). The morphology of macrophages is dramatically altered as they migrate. In the two examples shown, the IX-14 protein is strikingly mobilized from internal structures to the leading edge of cells (Fig. 8 C). This extraordinary relocalization of IX-14 protein suggests a very intimate involvement of this protein in cell migration.

Given a possible role for this protein in cell migration, we examined primordial germ cell migration in wild-type and IX-14 mutant *Drosophila* embryos (for review see Santos and Lehmann, 2004). After being the first cells to form very early in embryogenesis, primordial germ cells are passively carried along the dorsal side of the embryo in close association with the posterior midgut primordium. As this primordium invaginates, the germ cells are carried to the interior of the embryo. After this, they actively migrate away from the midgut toward...
the adjacent mesoderm where they associate with somatic gonadal precursor cells. These clusters of cells further migrate and coalesce into gonads later in embryogenesis. Using Vasa as a marker for primordial germ cells, we examined embryos both before and after the migration phases. As shown in Fig. 9, germ cells in both wild-type (Fig. 9 A, top) and mutant (Fig. 9 B, top) embryos are similarly dispersed at the stage before active migration. However, a dramatic difference is seen in embryos at the later stage of gonad coalescence, with gonads visible in wild type (Fig. 9 A, bottom), but not in the mutation (Fig. 9 B, bottom). Mutant larvae then lack gonads (unpublished data). Therefore, the IX-14 protease is playing a role in cell migration, as well as in mitosis, in Drosophila.

**Discussion**

*Drosophila* IX-14 mutations cause a variety of defects in chromosome, spindle, and nuclear envelope structure during mitosis. Here, we have shown that the IX-14 gene encodes a novel metalloprotease that is highly conserved in metazoa. Because of its homology to leishmanolysin, we suggest that the core of the IX-14 protease should structurally resemble that of leishmanolysin. In both *Drosophila* and human cells, the metalloprotease is concentrated in cytoplasmic organelles that we believe correspond to invadopodia. In recognition of the homology and intracellular distribution, we have termed the IX-14 enzyme invadolysin.

Three lines of evidence point to invadolysin activity as being crucially involved in the mitotic structural defects observed in *Drosophila* IX-14 neuroblasts: (1) The mRNA for the IX-14 gene is absent from larval tissues of two independently generated mutations, both of which have identical phenotypes; (2) dsRNA-mediated depletion of the IX-14 protein in cultured cells mimics the specific spindle and centrosome defects initially observed in the animal; and (3) in zymography experiments, mutant larval brains lack protease activity that is present in wild-type tissues.

Our analysis of the phenotype of IX-14 mutant alleles of *Drosophila* revealed that lamin (both Dm0 and C) and otefin proteins are present in elevated amounts in mutant neuroblasts. This finding suggests that invadolysin or one of its downstream targets normally promotes the turnover of these nuclear envelope proteins. In fact, cleavage may be due to invadolysin itself, as we have shown that the enzyme can cleave *Drosophila*...
lamin Dm0 in vitro. Many previous papers have demonstrated that lamina dynamics in mitosis are regulated by reversible phosphorylation by Cdk1:cyclin B and that the proteins are reused at nuclear envelope reformation during telophase (Gant and Wilson, 1997). Thus, although the mechanism and timing of the invadolysin-dependent nuclear envelope turnover has yet to be determined, the phenomenon is important because it reveals previously unsuspected complexities of nuclear envelope dynamics during the cell cycle. Identification of other invadoplysin substrates, e.g., ones essential for chromosome condensation and spindle assembly, will also illuminate further details of these mitotic rearrangements.

The IX-14 gene encodes a novel metalloprotease

Sequence analysis of the IX-14 gene suggested that it encodes a novel conserved zinc-metalloprotease with sequence homology to leishmanolysin and to orthologues that we have identified in a range of metazoan species. Leishmanolysin is a major cell surface protease that is required for *Leishmania*′s parasitic activity. It has been extensively studied due to its pathogenic role in leishmaniasis (Russell and Wilhelm, 1986; Yang et al., 1990; Connell et al., 1993; Xu and Liew, 1995; McGwire and Chang, 1996; Yao et al., 2003), and has also recently been shown to enhance migration of *Leishmania* through extracellular matrix (McGwire et al., 2003).

Like leishmanolysin, invadoplysin has the conserved residues of the metzincin protease “HEXXHXXG\[X\]H” motif. This motif has been characterized in detail by mutational analysis of the three conserved histidine (which coordinate the zinc ion) and glutamic acid residues, which are absolutely required for catalytic activity (McMaster et al., 1994; Macdonald et al., 1995; McGwire and Chang, 1996). We additionally noted the conserved spacing of 14 cysteines among leishmanolysin and the orthologues described in this paper. Molecular modeling studies revealed that the framework of the higher eukaryotic form of this protein is likely to closely resemble that of leishmanolysin, with differences being confined to surface features that likely mediate interactions with substrates, regulators, and/or other binding partners.

None of the identified invadoplysin orthologues has had any functions ascribed to them. Therefore, we propose that the IX-14/invadoplysin family is required for cell cycle structural rearrangements, a novel activity for this class of proteases.
Metalloprotease in mice (a member of the M48 family of zinc-metalloproteases) has a role in prelamin A cleavage (Pendas et al., 2002). Matrix metalloproteases (MMPs) have been implicated in remodeling of the extracellular matrix (Ishizuya-Oka et al., 2000) and in release of growth factors (Levi et al., 1996). MMPs have been shown to act in some cancers to digest the surrounding extracellular matrix and aid the process of metastasis (Ellerbroek and Stack, 1999; Horikawa et al., 2000; Johansson et al., 2000). Whereas the human genome encodes 24 MMPs, Drosophila has only two MMP genes: Dm1-MMP is expressed more specifically during embryonic stages and Dm2-MMP apparently throughout development (Llano et al., 2000, 2002). MMPs have been shown to act in some cancers to digest the surrounding extracellular matrix and aid the process of metastasis (Ellerbroek and Stack, 1999; Horikawa et al., 2000; Johansson et al., 2000). Whereas the human genome encodes 24 MMPs, Drosophila has only two MMP genes: Dm1-MMP is expressed more specifically during embryonic stages and Dm2-MMP apparently throughout development (Llano et al., 2000, 2002). Few mutations are available to study metalloproteases in a developing organism, although a study on double mutants of the two Drosophila MMPs has shown that whereas tissue remodeling is disrupted, embryonic development and larval mitosis are unaffected (Page-McCaw et al., 2003). Thus, no other metalloprotease affects cell proliferation as IX-14/invadolysin does.

Intracellular localization of IX-14 to invadopodia

Although a nuclear pool of invadolysin is detectable in human cells, protein is also localized to the cytoplasm in both fly and human cells, where it is concentrated in unusual ring-like structures. These structures are observed during interphase in all human cultured cells examined, but become dispersed during mitosis, when the protein adopts a more diffuse distribution. We believe the ring-like structures represent invadopodia, cytoplasmic structures that have been proposed to have an important role in cell migration and metastasis. Like invadopedia, the ring-like structures containing invadolysin are localized to the lower third of the cell close to the substratum. They also contain cortactin, paxillin, and dynamin 2, previously shown to be enriched in invadopodia. Intriguingly, dynamin 2, which is also found in invadopodia, has been recently shown to play a role in centrosome cohesion (Thompson et al., 2004), thus tying in with our results linking migration with mitosis.

Strikingly, although invadolysin has a punctate cytoplasmic localization in stationary macrophages, when actively migrating macrophages are visualized, invadolysin becomes strongly concentrated at the leading edge of the cell. How this relocation occurs will be investigated in living cells, coupled with an analysis of extracellular matrix degradation. Invadolysin appears to be the first specific marker for invadopodia as a discrete cytoplasmic compartment, and, as such, will be a key potential target for drugs designed to limit cell migration.

Analysis of invadolysin mutants identifies a previously unsuspected role for metalloproteases in cell division and development

The IX-14 mutation in Drosophila causes cells to arrest in mitosis with hypercondensed mitotic chromosomes surrounded by poorly condensed chromatin, a phenotype that is clearly dis-
tinct from the chromosome hypercondensation commonly observed in mutations exhibiting mitotic delay (Heck et al., 1993; Theurkauf and Heck, 1999). Invadolysin also appears to have a role in chromosome architecture during interphase because mutations exhibit poorly structured polytene chromosomes and have compromised heterochromatin, as assayed by position effect variegation. The levels of nuclear lamin and otefin proteins are dramatically increased in mutant mitotic cells. One possibility suggested by these results is that invadolysin might regulate interactions between chromatin and the nuclear envelope that are important for gene expression and/or mitotic chromosome condensation. Invadolysin could have a direct role on components such as topoisomerase II or condensin, or the effects we have observed could result from disruption of a novel pathway in which proteolysis plays a role.

In addition, invadolysin has nonchromosomal roles, as both centrosome duplication and/or separation and mitotic spindle formation are aberrant in mutant neuroblasts. Thus, the phenotypes resulting from loss of invadolysin are novel and distinct from other previously described mitotic defects. We have presented evidence that the protein is involved in migration of human macrophages and fly primordial germ cells. Thus the identification and study of this gene has provocatively linked mitosis with cell migration.

A simplistic hypothesis is that different forms of the protein may be important for structural rearrangements during the cell cycle and for cellular migration. Clearly, the generation of reagents recognizing specific forms would be required to address this question. Differential regulation of the expression or localization of these forms could be used during development and/or disease pathogenesis. It is clear from the striking phenotype displayed by IX-14 mutations that invadolysin illuminates a previously unsuspected pathway essential for cell division and development in metazoans. Future identification of invadolysin substrates will help to clarify the role of this protein and to lead to better understanding of the ways in which structural changes during the cell cycle may be coordinated with cell movements.

Materials and methods

**Drosophila stocks**
The wild-type strain used was Canton S. We received I(3)IX.14 from M. Gatti (University of Rome, Rome, Italy); it was originally generated in an ICR-170 screen by A. Shearn (Johns Hopkins University, Baltimore, MD). We generated I(3)IX.14 by local hopping a nearly P transposon inser- tion, I(3)J04017, obtained from the Bloomington Stock Center.

**DAPi staining of Drosophila neuroblast squashes and polytene chromosomes**
Brains or salivary glands from third instar larvae were processed as described previously (Heck et al., 1993; Lopour et al., 2000) and mounted in Mowiol/glycerol. Fluorescence was observed using a fluorescence microscope (model AX-70 Provis; Olympus) fitted with epifluorescence filters (Chroma Technology Corp.). Dured (at ~18°C) using a camera (model Orca II; Hamamatsu) with Vysis QUIPS software and processed using Adobe Photoshop 4.0.

**Immunofluorescence of third instar larval brains**
Analysis of α-tubulin (Sigma-Aldrich), CP190 (provided by W. Whitfield, University of Dundee, Dundee, Scotland), lamin (provided by P. Fisher, State University of New York at Stony Brook, Stony Brook, NY), otefin (provided by Y. Gruenbaum, Hebrew University of Jerusalem, Jerusalem, Israel), cyclin B (provided by J. Raff, University of Cambridge, Cambridge, England), and P–H3 (Upstate Biotechnology) antibodies in third instar larval brains was performed as described previously (Bonaccorsi et al., 2000). BrdU incorporation and subsequent detection by a rat anti-BrdU antibody (Harlan Sera) in larval brains was performed as described previously (Lopour et al., 2000). Imaging was performed as detailed in the previous section.

**Immunoblotting**
Protein extracts were electrophoresed on Novex SDS-PAGE gels (Invitro- gen), and then transferred to nitrocellulose membrane (Schleicher and Schuell). Membranes were rinsed for 5 min with PBS-Tw (PBS + 0.1% Tween 20), and then blocked for 1 h with Safeway dried skimmed milk (5% in PBS-Tw) at RT with shaking. Primary and secondary antibody incubations were performed in PBS-Tw for 1 h at RT with shaking, with washes of 2 × 3 min, 1 × 15 min, and then 2 × 5 min after each incubation. Chemiluminescent detection of HRP-conjugated secondary antibody was performed using ECL reagents from Amersham Biosciences, according to the manufacturer's instructions.

**Northern blotting**
Northern blots were performed as described in Sambrook et al. (1989). Total RNA was prepared from homogenized third instar larvae using the RNeasy RNA extraction kit (Qiagen). 10 µg of total RNA per lane was electrophoresed on a 1% agarose gel. RNA was transferred to nitrocellulose membrane by capillary action with 20× SSC overnight. RNA was subsequently UV-cross-linked to the membrane using a Strata-linker (Strategene). Hybridization of 32P-dCTP (AP Biotech)-labeled probes at a specific activity of at least 106 counts per milliliter was performed at 65°C overnight as described in Church and Gilbert (1984). The HighPrime kit (Boehringer) was used for all random primer labeling. Autoradiography was done at 80°C using XAR-5 film (Kodak).

**dsRNA-mediated interference**
 dsRNAi was performed on Drosophila S2 cells as described previously (Clemens et al., 2000; Vass et al., 2003), using 15 µg/ml of double stranded IX-14 RNA per 2 ml of cells at 10°C cells/ml.

**Immunofluorescence of Drosophila S2 and HeLa cultured cells**
S2 cells were grown on either Permanox Chamber Slides (Lab-Tek) for the RNAi experiments or on poly-l-lysine coverslips in 6-well plates for the EGFP/fusion detection using a rabbit anti-GFP antibody (Molecular Probes). HeLa cells were grown on coverslips in 6-well plates for transfection with EGFP fusion constructs. For immunofluorescence, cells were fixed with 4% PFA in PBS for 3 min, permeabilized in PBS + 0.5% Triton X-100 for 1 min, and washed 2 × 10 min incubations in PBtx. The cells were blocked for 1 h in 3% BSA in PBS at RT. Primary antibody incubation was overnight at 4°C in 0.3% BSA in PBS. Cells were washed 3 × 5 min in PBtx, followed by secondary antibody incubation for 1 h at 37°C (secondary antibodies incubated separately). Final washes were performed in PBtx, with the penultimate wash containing 0.1 µg/ml DAPI, and the coverslips were mounted in Mowiol/glycerol. Imaging was performed as detailed in the section DAPI staining of Drosophila neuroblast squashes and polytene chromosomes.

**Detection of protease activity by zymography**
Zymogram gels (Novex) containing casein as a substrate were used for all zymography. Wandering third instar larvae were dissected in 1× EBR. Brains were transferred to 50 µl of chilled EBR, and then homogenized for ~30–60 s with a handheld homogenizer. 50 µl of 2× Tris-glycine gel sample buffer (Novex) plus 1 mM DTT was added, and the samples were incubated for 10 min at RT. Samples (without boiling) were loaded onto 12% Tris-Glycine Zymogram gels and electrophoresed at 125 V constant, generally for 3–4 h, at least until the dye front had run off the gel. After electrophoresis, gels were rinsed in renaturing buffer (2.5% Triton X-100) for 30 min, equilibrated in Developing buffer (Novex) for 30 min, and incubated overnight at 37°C in Developing buffer. Gels were stained with Coomassie blue (Sigma-Aldrich) to visualize the casein in the gel.

**in vitro proteolysis of lamin by invadolysin**
375 ng of Lamin in T7-7 vector, IX-14 in POT2 vector, or GFP control vector was added to one 24-µl aliquot of the RTS 5000 Escherichia coli Circular Template kit (Roche). Each expression aliquot was incubated in a water bath at 30°C for 1 h. 10 µl of IX-14 was mixed with 1 or 4 µl of lamin or GFP before being incubated in a water bath at either 29 or 37°C for
15 min, 50 min, or 1 h. In some cases, the incubation was with ZnCl₂ or ZnSO₄ or the zinc chelator 1,10-phenanthroline. After the reaction, samples were boiled at 100°C for 5 min in SDS-PAGE sample buffer. Lamin was detected after blotting to nitrocellulose using an mAb generated to the Drosophila N-terminal head region (amino acids 22–28).

**Macrophage isolation and culture**

Macrophages were isolated from normal human blood following the procedure of Giles et al. (2001). After isolation, the mononuclear cells were plated at 4 × 10⁶/ml in Iscoves’ modified Dulbecco’s medium (with L-glut and 25 mM Heps) onto glass coverslips in 12-well tissue culture plastic plates for 1 h. Adherence to the glass coverslips was used to enrich for the monocytes out of the lymphocytes/monocytes mix (lymphocytes do not attach). After the plates, the plates were washed in Hank’s Balanced Salt Solution (without divalent cations) to remove nonadherent lymphocytes, and then 2 ml of Iscoves’ modified Dulbecco’s medium + 10% autologous serum was added and the cells were cultured at 37°C, 5% CO₂ for 5 d. After 5 d, the cells were washed again to remove any nonadherent cells and fixed in 3% PFA at RT for 20 min. After washes in PBS, 50 mM NH₄Cl was added to the wells for 15 min. Coverslips were permeabilized in 0.1% Triton X-100 for 4 min. After washes, the cells were blocked in 10% heat-inactivated human serum and processed for immunofluorescence as usual.

**Detection of germ cells**


**Online supplemental material**

Further details on TUNEL labeling of larval brains, dsRNA-mediated interference in S2 cells, and cultured cell transfections (Drosophila and Helia) are provided online. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200405155/DC1.

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**References**


