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Drosophila morgue influences cell numbers and positions in the embryonic nervous system

BARBARA A. SCHREADER, YIQIN WANG, STEPHANIE CARTER, JOANNA GRIGAS and JOHN R. NAMBU*

Biology Department, University of Massachusetts, Amherst MA, USA

ABSTRACT Morgue is a unique multi-domain protein that contains a zinc finger motif, an F box, and a variant E2 conjugase domain. The presence of these domains suggests potentially complex and novel functions for Morgue in ubiquitination pathways. Morgue was originally identified via its gain-of-function enhancement of eye cell death phenotypes in *Drosophila* and ectopic expression of Morgue also influences circadian rhythms. However, there is as yet little known about Morgue's normal developmental or physiological functions. To address this issue, we generated several *morgue* loss-of-function mutants via P element excision mutagenesis and analyzed the mutant phenotypes during the fly life cycle. These studies revealed that *morgue* null mutants are viable, though approximately 10% of the mutants exhibit defects in pupal spiracle eversion and malformations in the adult abdominal cuticle. In addition, a similar subset of *morgue* mutant embryos exhibited alterations in the normal number, position, or morphology of specific neurons and glia. Analysis of Morgue protein localization was addressed through generation of a transgenic fly strain that expresses a GFP::Morgue fusion protein. Use of this strain revealed Morgue protein localization in multiple cellular compartments, including nuclei, cytoplasm and membranes. Taken together, these diverse phenotypes and distribution patterns suggest pleiotropic functions for Morgue.

KEY WORDS: *Drosophila, morgue, cell death, ubiquitin, nervous system*

Introduction

Protein ubiquitination is a highly conserved and tightly regulated post-translational modification that is mediated by an enzymatic cascade where the sequential functions of an E1 activator, E2 conjugase, and E3 ligase transfer ubiquitin onto specific substrate proteins (Pickart and Eddins, 2004; Ciechanover 2005). Formation of a poly-ubiquitin chain targets a substrate to the 26S proteasome complex that can degrade or process the protein while mono-ubiquitination can result in endocytic engulfment. Ubiquitination can influence protein stability, activity, or localization and is critical in a wide array of developmental and physiological processes. In addition, several human pathologies are associated with abnormal accumulations of ubiquitinated proteins. Ubiquitination plays a crucial role in cell survival decisions by regulating the relative levels of antagonistic death activators and death inhibitors (Lee and Peter 2003). Significantly, members of the Inhibitor-of-Apoptosis (IAP) protein family contain both caspase-binding BIR domains and an E3 ubiquitin ligase RING domain and can repress apoptosis by preventing the activation or function of bound caspases as well as promoting their ubiquitination and proteasome-mediated turnover (Vaux and Silke 2005; Steller, 2008). In *Drosophila*, IAPs are essential for cell survival; loss-of-function *diap1* mutants exhibit embryonic lethality and massive ectopic cell death, and cell lethal effects of *diap1* loss is observed in somatic tissues (Hay 2000). In mammals, IAPs are also important for cell survival and mis-regulation of IAP expression is associated with the immortality of transformed oncogenic cells (Wright and Duckett 2005). In both flies and mammals, IAP activities are strongly influenced by a set of related antagonists. In *Drosophila*, this includes 4 related RHG proteins, Reaper,

Abbreviations used in this paper: BIR, Baculovirus IAP Repeat; Cys, cysteine; DBD, dorsal bipolar dendritic; EST, expressed sequence tag; GFP, Green Fluorescent Protein; Gly, glycine; hid, head involution defective; His, histidine; HRP, horseradish peroxidase; IAP, Inhibitor of Apoptosis Protein; Lys, lysine; Morgue, Modifier of reaper and grim ubiquitously expressed; PBS, phosphate buffered saline; PG3, peripheral glial cell 3; RHG, reaper, hid, grim; RING, Really Interesting New Gene; s.d., standard deviation; UTR, untranslated region.

***Address correspondence to:** John R. Nambu. Department of Biological Sciences, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431, USA. e-mail: jnambu@fau.edu

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Grim, Sickle, and Hid, that can each antagonize IAP-caspase interactions by competing with caspases for binding to the IAP BIR domain (Bergmann *et al.* 2003; Kornbluth and White 2005). In mammals, the related Smac/Diablo and HtrA2/Omi proteins similarly bind IAP BIR domains and repress caspase neutralization (Vaux and Silke 2003). These observations suggested a double-repression model of cell death, whereby death-inducing caspase actions are normally held in check by the IAPs. This inhibition is removed by expression of IAP antagonists, which are either transcriptionally regulated (e.g. *Drosophila reaper*) or selectively released from the mitochondria (e.g. mammalian Smac/Diablo and HtrA2/Omi) in response to pro-death stimuli. Importantly, IAP antagonists can induce IAP auto-ubiquitination and proteasome-dependent degradation to facilitate apoptosis (Ryoo *et al.* 2002; Yoo *et al.* 2002; Fu *et al.* 2003; Yang *et al.* 2004). In addition, IAPs can ubiquitinate and promote degradation of their antagonists (MacFarlane *et al.* 2002; Olson *et al.* 2003; Wilkinson *et al.*, 2004). Thus, the regulation of IAP levels and ubiquitination activities is under complex proteolytic control that is crucial for appropriate cell survival decisions.

In addition to IAPs, several other ubiquitination proteins also influence cell survival decisions and may modulate IAP functions. These include both well-defined types of ubiquitination proteins, such as the E2 conjugase UbcD1/Effete (Ryoo *et al.* 2002; Yoo *et al.* 2005), as well as several novel ubiquitination proteins including Scythe (Thress *et al.* 1998; Desmots *et al.*, 2005), Bruce (Hauser *et al.*, 1998; Bartke *et al.* 2004), and Morgue (Hays *et al.* 2002; Wing *et al.*, 2002). To help clarify functional interactions between the ubiquitin/proteasome pathway and programmed cell death, we have focused attention on the *Drosophila morgue* gene. Morgue was initially identified in genetic modifier screens based on the eye cell death induced by targeted R/Grim gene expression or mutations in the *irreC-rough*est gene (Hays *et al.*, 2002; Wing *et al.* 2002). A function for *morgue* in cell death pathways was further implied by demonstration that *morgue* P element insertion alleles or a *morgue* deletion mutant dominantly suppressed RHG (Reaper, Hid, Grim)-mediated eye cell death and exhibited ectopic lattice cells in the developing ommatidia. Furthermore, Morgue expression induced apoptosis in cultured insect cells and downregulated DIAP1 levels; Morgue was also shown to directly associate with DIAP1 and downregulate DIAP1 levels (Hays *et al.* 2002; Wing *et al.*, 2002). In addition to its role in programmed cell death, Morgue has also been implicated in circadian rhythms pathways as targeted Morgue expression rescues behavioral arrhythmicity exhibited by flies reared in conditions of constant light (Murad *et al.* 2007). Consistent with potentially diverse functions, the *morgue* gene exhibits widespread expression throughout the fly life cycle.

Significantly, the 491 amino acid Morgue protein contains a unique combination of functional domains. This includes an NH_2-terminal $CX_2CX_8CX_2C$ zinc finger motif, a centrally-located F box that includes five stereotypic Tryptophan residues, and a COOH-terminal variant E2 conjugase domain where the active site Cysteine is replaced by a Glycine residue. The putative Cys4 type zinc finger is of unknown activity but is well conserved in all Morgue homologs (Zhou *et al.* 2009). F box proteins serve as adaptors in SCF E3 ubiquitin ligase complex that bind to a Skp protein and a specific substrate protein that is targeted for ubiquitination by an E2 ubiquitin conjugase (Ho *et al.* 2006). Consistent with potential functions as an F box protein, Morgue associates with *Drosophila* SkpA in an F box-dependent manner (Wing *et al.* 2002; Giot *et al.* 2003) and contains potential substrate binding regions. The Morgue E2 conjugase domain shows strong sequence similarity to other E2s, including *Drosophila* UbcD1/Effete, which also associates with DIAP1 and promotes DIAP1 turnover (Ryoo *et al.* 2002). However, the Gly/Cys active site substitution implies that Morgue cannot form a covalent thioester linkage to ubiquitin and lacks the catalytic capability to directly mediate ubiquitination. Morgue may thus also/instead act as a Ubiquitin E2 Enzyme Variant (UEV). UEVs all lack the active site cysteine although some UEVs, such as MMS2 or UEV1, can associate non-covalently with ubiquitin and may form catalytically active heterodimers with another bona fide E2, such as Ubc13 (Eddins *et al.* 2006; Hau *et al.* 2006; Lewis *et al.* 2006). All Morgue homologs share the active site Gly/Cys substitution (Zhou *et al.* 2009), suggesting a specific functional requirement for Glycine in this position. Interestingly, no other known UEV contains a Glycine in the active site. This unique combination of both an E2 and E3 functional domain on one polypeptide suggests Morgue may provide novel ubiquitination activities.

Despite an apparent role for Morgue in regulating cell survival, there is as yet limited information on its normal functions in living and dying cells during development. These analyses have been hampered both by a lack of specific *morgue* null alleles and lack of knowledge on the subcellular sites of action for Morgue protein. In this study we address these issues. First, specific *morgue* loss-of-function alleles were generated via P element excision mutagenesis. These mutations delete portions of the Morgue coding

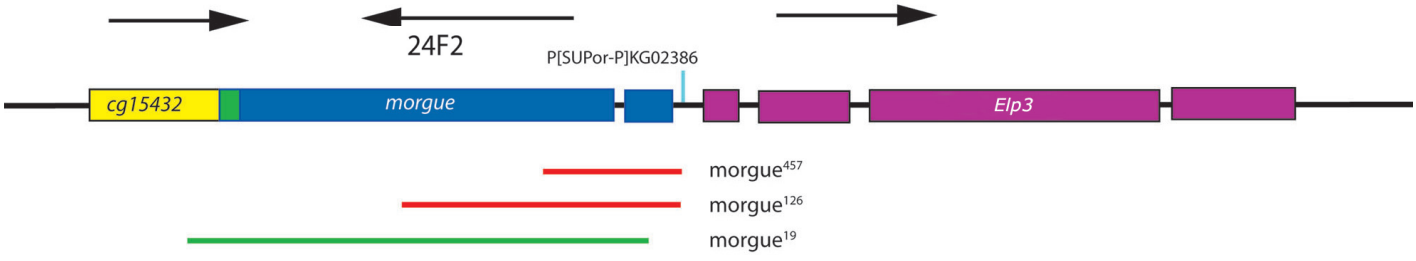

Fig. 1. Generation of morgue-specific P element excision alleles. *The* morgue *gene is flanked by the* cg15432 *and Elp3 genes at 24F2 on chromosome 2L. Both* cg15432 *and* Elp3 *are transcribed in opposite directions with respect to* morgue *(black arrows). The 3′-untranslated regions of* morgue *and* cg15432 *overlap over 119 nucleotides (green). Excision of the P[SUPor-P]KG02386 insertion (light blue line) located just upstream of* morgue *resulted in generation of the* morgue^{126} *and* morgue^{457} *deletions (red lines). The* morgue^{19} *deletion (green line) was generated previously (Wing* et al. *2002) and removes the entire coding region of* morgue *as well as a portion of* cg15432.

region but do not disrupt sequences of the nearby *cg15432* or *Elp3* genes. These *morgue*-specific deletion mutants are homozygous viable and fertile. However, some mutant animals exhibit disruptions in anterior spiracle eversion during pupariation and abnormal tergite elaboration during formation of the adult abdomen. As high levels of cell death normally occur in the developing nervous system, embryos lacking maternal and zygotic *morgue* function were examined using several markers that label specific sets of neurons and glia. This analysis revealed that *morgue* functions influence the numbers, positions, and morphologies of discrete CNS and PNS cell types. Finally, to gain insight into the distribution of Morgue protein within cells we examined the localization of GFP::Morgue and Morgue::6XHis fusion proteins. Morgue was localized in several cellular compartments in distinct cell types. Taken together, these findings suggest widespread and diverse functions for *morgue* in multiple developmental processes.

Results

Morgue-specific mutants are viable and exhibit anatomical defects

Previous genetic analyses of *morgue* function utilized either the EP2367 and EP1184 *morgue* P element insertions, or a small *morgue* deletion, *morgue*^{19} (Hays *et al*. 2002; Wing *et al*. 2002). The EP2367 and EP1184 strains are homozygous viable and respectively contain insertions of the EP P element in the 5'-UTR or 3'-UTR of *morgue*. Neither of these alleles corresponds to a *morgue* null mutation. In contrast, *morgue*^{19} is a null allele that deletes the entire protein-coding region; these mutants exhibit a fully lethal phenotype (Wing *et al*. 2002). However, *morgue*^{19} also deletes a portion of the nearby *cg15432* gene (Fig. 1). Analysis of a *cg15432* EST (GenBank BT032808.1) indicated that the proximal breakpoint of the *morgue*^{19} mutation extends through the 134 base pair 3'-UTR and final 12 amino acids encoded by *cg15432*. The 118 amino acid CG15432 protein contains a lysine-rich NH_2-region and a COOH-terminal DUF1674 domain (see: http://www.flybase.org/reports/FBgn0031603.html). CG15432 corresponds to a member of the Fam32A protein family that is very highly conserved in a wide range of invertebrate and vertebrate organisms, including *C. elegans* and humans (Lai *et al*. 2000), suggesting important, though as yet unknown functions. The *morgue* gene is also closely flanked on the 5' (proximal) side by the *Elp3* gene that encodes a 552 amino acid protein resembling an Acyl-CoA N-acyltransferase (see: http://www.flybase.org/reports/FBgn0010235.html). To fully understand *morgue* function it is important to obtain *morgue*-specific mutations that do not disrupt *cg15432* or *Elp3* and we therefore sought to generate *morgue*-specific null alleles. A P element excision screen was performed on the P[SUPor-P]KG02386 insertion located in the *morgue* 5'-UTR, 51 base pairs upstream of the Morgue start codon (Fig. 1). Approximately 100 excisions were analyzed for viability and subjected to PCR assays using primers in and around the *morgue* gene to identify and characterize deletion events. From these analyses, two *morgue*-specific deletions were identified, *morgue*^{126} and *morgue*^{457}. The two alleles share the same 5' breakpoint that corresponds to the site of the P element insertion. The *morgue*^{457} deletion extends for 595 base pairs and removes the coding region up to the Asp145 residue located between the zinc finger and F box (Fig. 1). The *morgue*^{126} deletion extends for 1,131 base pairs and ends at Ala321 between the F box and conjugase domain (Fig. 1). Neither deletion impacts the transcribed portions of *cg15432* or *Elp3*. Both mutant alleles remove the native initiator Methionine as well as significant portions of the *morgue* coding region. This suggests that they act either as a strong hypomorph or null allele to greatly impair or eliminate Morgue functions. In addition to these loss-of-function *morgue* alleles, several precise excision alleles, including *morgue*^{100}, were obtained.

Unlike *morgue*^{19}, homozygous *morgue*^{126} and *morgue*^{457} mutants are viable, although the mutant flies are somewhat lethargic. Both new alleles are also viable when placed *in trans* to *morgue*^{19}. We first analyzed the new *morgue* alleles for their ability to suppress the phenotype of P[GMR-*hid*] flies. Heterozygosity for the *morgue*^{126} or *morgue*^{457} allele resulted in a dominant suppression of ectopic eye cell death (Fig. 2A) that is comparable to that seen for the *morgue*^{19} null allele (Wing *et al*. 2002). The extent of suppression was not significantly enhanced by homozygosity for either the *morgue*^{126} or *morgue*^{457} mutation (data not shown). This indicates that a specific reduction of *morgue* function is sufficient to attenuate the death-inducing activities of RHG genes.

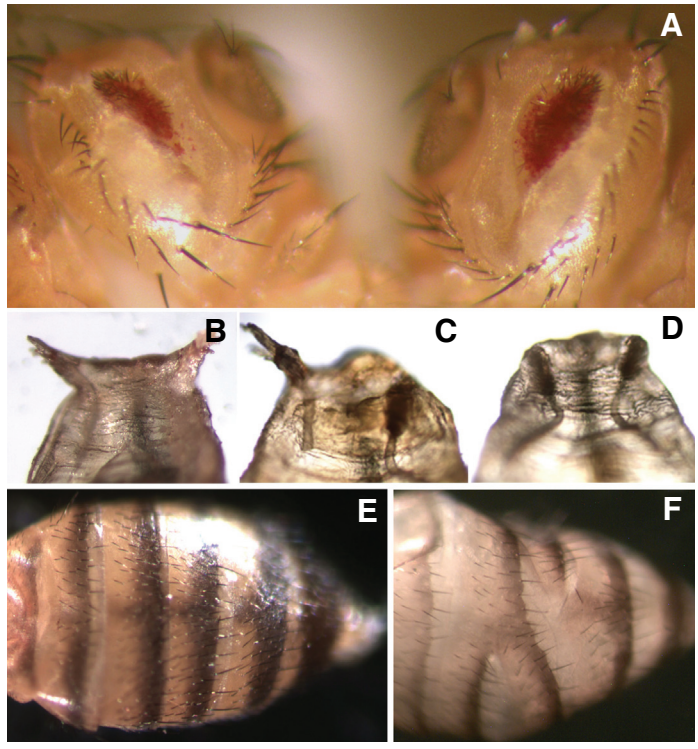

Fig. 2. *Morgue* mutants exhibit dominant suppression of HID-induced eye cell death and defects in pupal case and adult cuticle formation. (A) *The ectopic eye cell death in P[GMR-*hid*]/+ flies (left) is dominantly suppressed by heterozygosity for the* morgue^{126} *allele in P[GMR-*hid*]/*morgue^{126} *flies (right).* **(B-D)** *In contrast to a wild type pupae* **(B)**, *13% of homozygous* morgue^{457} *mutant pupae fail to normally evert either one* **(C)** *or both* **(D)** *of the anterior spiracles.* **(E,F)** *Wild type adult flies* **(E)** *possess well-organized tergite plates on the dorsal abdomen that exhibit clear segmental reiteration. 10% of homozygous* morgue^{126} *adults exhibit disruptions in the normal elaboration of tergites that include misalignment and aberrant segmental organization* **(F)**.

A subset of *morgue*^{126} and *morgue*^{457} mutants exhibited anatomical abnormalities in the pupal and adult stages. Specifically, in the pupal cases of 10.0% (51/511) of *morgue*^{126} mutants and 13.0% (68/523) of *morgue*^{457} mutants, one or both of the anterior spiracles failed to evert and extend (Fig. 2 B-D). While these defects were incompletely penetrant, no such eversion defects were observed in wild type (n=530) or *morgue*^{100} pupae (n = 498). Furthermore, 10.2% of both eclosed *morgue*^{126} (52/507) and *morgue*^{457} (50/488) adult flies exhibited malformations in the tergite plates on the dorsal abdomen (Fig. 2 E,F). These disruptions varied in severity and included disruptions, fusions, or truncations, along the midline of the tergites. These defects were also not observed in wild type (n=559) or *morgue*^{100} (n=474) flies. No other anatomical defects were detected in *morgue* mutant animals.

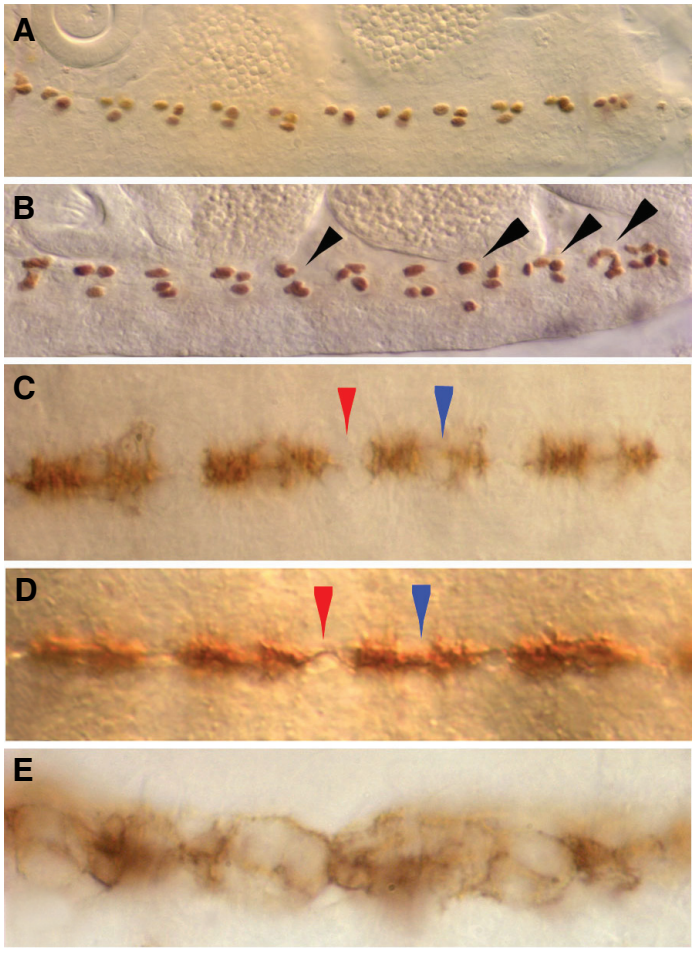

Fig. 3. *Morgue* mutant embryos exhibit altered numbers and morphology of embryonic CNS midline glia. (A,B) *Anti-β-galactosidase immunostaining labels the nuclei of CNS midline glia in stage 16 wild type* **(A)** *and* morgue^{126} *mutant* **(B)** *embryos carrying a P[1.0slit-lacZ] marker. Note that several ganglia of* morgue *mutant embryos contain ectopic and misplaced midline glia (arrowheads).* **(C-E)** *Anti-Wrapper immunostaining labels the membranes of CNS midline glia. Note that in stage 16 wild type embryos* **(C)** *there is clear separation between the anterior and posterior midline glia in each ganglion (red arrowhead) as well as the glia of each ganglion (blue arrowhead). This separation is disrupted in* morgue^{126} *mutant embryos* **(D)** *as glial processes extend aberrantly across (red arrowhead) and within (blue arrowhead) ganglia. In Df(3L)H99 mutant embryos where all midline glial death is blocked, there is a more pronounced disorganization and distortion of these glial processes* **(E)**. *(A,B) sagittal view; (C-E) ventral view.*

***Morgue* mutant embryos exhibit altered numbers, morphology, or positions of specific neurons and glia**

Development of the nervous system requires elaboration of a precise and stereotyped pattern of neurons and glia. This pattern is in part determined by controlled removal of excess or inappropriate cells via programmed cell death. To further characterize the developmental functions of *morgue*, we employed several markers to analyze the differentiation and survival of specific neurons and glia in *morgue* mutants. Significantly, embryos derived from homozygous *morgue*^{457} and *morgue*^{126} mutants were lacking both maternal and zygotic functions of *morgue*.

The CNS midline glia undergo prominent cell death during germ band retraction which decreases their numbers from 9 to 3 per ganglion (Sonnenfeld and Jacobs, 1995; Zhou *et al*., 1995). To determine if midline glial deaths are altered in *morgue* mutants, a P[1.0slit-lacZ] marker was crossed into *morgue* mutant backgrounds and the progeny embryos were analyzed via anti-β-galactosidase immunostaining. In stage 16 *morgue* mutant embryos 1-2 additional midline glia were detected in several, though not all ventral nerve cord ganglia (Fig. 3 A,B). This implies that in the absence of *morgue* the normal level of midline glial cell death was decreased. In addition, anti-Wrapper immunostaining revealed altered morphology along the midline of *morgue* mutants. Compared to wild type embryos, *morgue*^{126} and *morgue*^{457} mutants exhibited aberrant midline glial cytoplasmic processes that were not well separated and often extended across inter-ganglia borders (Fig. 3 C,D). Interestingly, a similar, though more severe, disruption of midline glial processes was observed in Df(3L)H99 mutant embryos which contain a three-fold excess of midline glia (White *et al*. 1994) (Fig. 3E).

The role for *morgue* in cell survival was further analyzed by examining the survival of larval photoreceptor neurons, another cell lineage that undergoes prominent programmed cell death during embryogenesis. In stage 16 wild type embryos these Krüppel-expressing neurons reside in two symmetric clusters of 9 cells each in the dorsal cephalic region while in Df(3L)H99 mutants where cell death is blocked, there are 17 neurons per cluster (Grether *et al*. 1995). Anti-Krüppel immunostaining revealed that in *morgue* mutants the average number of these neurons was unaltered; however compared to wild type embryos there is greatly increased variability in the number of neurons per cluster (average: 8.86 s.d. 1.94 per cluster for the wild type [n=63 clusters] and 8.94 s.d. 5.74 for *morgue*^{126} mutants [n=65 clusters]). The *morgue* mutant embryos exhibited a much greater range of Krüppel-expressing neurons per cluster (Fig. 4 A-D). In addition, unlike wild type or Df(3L)H99 mutants, individual *morgue* mutant embryos also displayed substantial asymmetry in the number of neurons within each of the two clusters.

The defects in cellular number and morphology in *morgue* mutants prompted us to examine whether *morgue* might also influence cell positions within the embryonic nervous system. This was addressed using Anti-Repo immunostaining to analyze peripheral glial cells which migrate large distances during germ band retraction from the dorsal neuroectoderm to dorsal/lateral

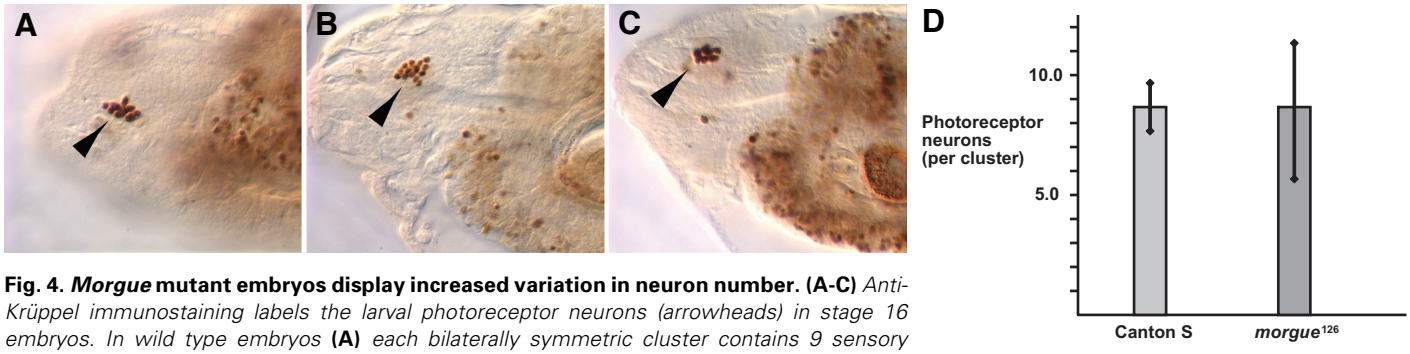

Fig. 4. *Morgue* mutant embryos display increased variation in neuron number. (A-C) *Anti-Krüppel immunostaining labels the larval photoreceptor neurons (arrowheads) in stage 16 embryos. In wild type embryos* **(A)** *each bilaterally symmetric cluster contains 9 sensory neurons.* morgue *mutants exhibit greatly increased variability in the number of these photoreceptor neurons as evidenced by the presence of 14* **(B)** *or 6* **(C)** *neurons in a* morgue^{126} *mutant embryo. All views are sagittal with anterior to left.* **(D)** *Plot of the average number of Krüppel-expressing larval photoreceptor neurons present in clusters from stage 16 wild type (light grey; n=63) and* morgue^{126} *mutant (dark grey; n=65) embryos. The standard deviations are indicated by tipped vertical lines. Note that there is greatly increased variation in neuron numbers observed in the* morgue *mutants.*

positions in the embryo (Sepp *et al.* 2000). In stage 16 *morgue* mutants, altered positions observed for the nuclear positions of both the DBD support glia and PG3 glia. In wild type embryos, these cells are found in an inverted L-shaped configuration where the nucleus of the DBD glia is arrayed horizontally and lies just dorsal and posterior to the nucleus of the PG3 glia which is arrayed vertically (Fig. 5A; Umesono *et al.* 2002). However, in several segments of each *morgue* mutant embryo the DBD and PG3 nuclei were mis-positioned, with the DBD nucleus often located anterior or dorsal to the PG3 nucleus (Fig. 5B). These defects also exhibited variable severity both between individual embryos and within different segments of a single embryo. Interestingly, similar, though more severe disruptions were observed in Df(3L)H99 mutant embryos where the PG3 and DBD glia were often duplicated as well as mis-positioned (data not shown).

A GFP::Morgue fusion protein exhibits widespread localization

The Morgue protein does not contain any identifiable domain or motif that implies a specific site of subcellular localization. To identify the potential subcellular sites of Morgue action we generated a P[UAS-GFP::Morgue] fly strain. Targeted expression of GFP::Morgue exhibited comparable enhancement of R/Grim-induced eye cell death as full length native Morgue (Wing *et al.* 2002 and data not shown), suggesting that it has similar biological activity. In addition, both native Morgue and GFP::Morgue also induced lethal phenotypes when expressed at high levels via a distinct P[Gal4] driver (Y. Zhou and J.R.N. in preparation). Expression of GFP::Morgue was targeted to the larval salivary glands using the P[SaGa52A-Gal4] driver strain (Mukherjee *et al.* 2000). These glands contain large cells with easily distinguished nuclei and cytoplasm. In third instar P[SaGa52A-Gal4]/P[UAS-GFP::Morgue] larvae, GFP::Morgue exhibited widespread localization within salivary gland cells where it was detected in the nucleus and cytoplasm as well as along the plasma membrane (Fig. 6A). To determine if similar distribution is observed in other cell types, a P[repo-Gal4] line was used to target GFP::Morgue expression in glial cells. Analysis of the central nervous system of third instar larvae revealed GFP::Morgue localization in glial nuclei and cytoplasmic processes (Fig. 6B). Morgue protein distribution was further investigated via expression of a Morgue-6xHis protein in cultured insect Sf9 cells. Anti-His immunostaining revealed prominent localization of Morgue-6xHis protein in the cytoplasm and weaker distribution in the nuclei of transfected cells (Fig. 6C). Thus, the tagged Morgue proteins localize in multiple cellular compartments in distinct cell types.

Discussion

Generation of morgue-specific mutants

Initial genetic studies of *morgue* function revealed that a small deletion allele, *morgue*^{19}, exhibits recessive lethality and dominantly suppresses the cell death induced by ectopic Reaper or Hid expression (Wing *et al.* 2002). However, interpretation of loss-of-function *morgue* phenotypes based on *morgue*^{19} is complicated by simultaneous disruption of the adjacent *cg15432* gene. Significantly, the *morgue*^{19} mutant deletes the entire 3'-UTR and last 12

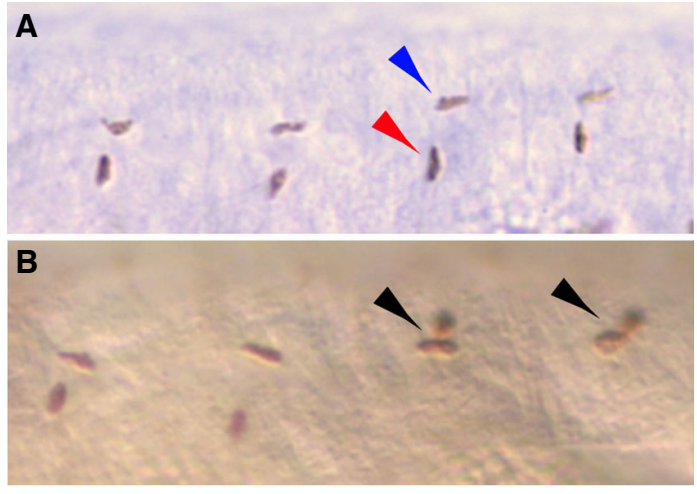

Fig. 5. *Morgue* mutants exhibit mis-positioning of embryonic peripheral glial. (A,B) *Anti-Repo immunostaining labels the nuclei of peripheral glial cells. Note that in stage 16 wild type* **(A)** *embryos the nucleus of the DBD glia (blue arrowhead) resides dorsal and slightly posterior to the nucleus of the PG3 glia (red arrowhead). In stage 16* morgue *mutant embryos* **(B)** *the positions and shapes of these glial nuclei are altered (arrowheads) and the PG3 nucleus often resides directly dorsal to the DBD nucleus. Sagittal views with anterior to left.*

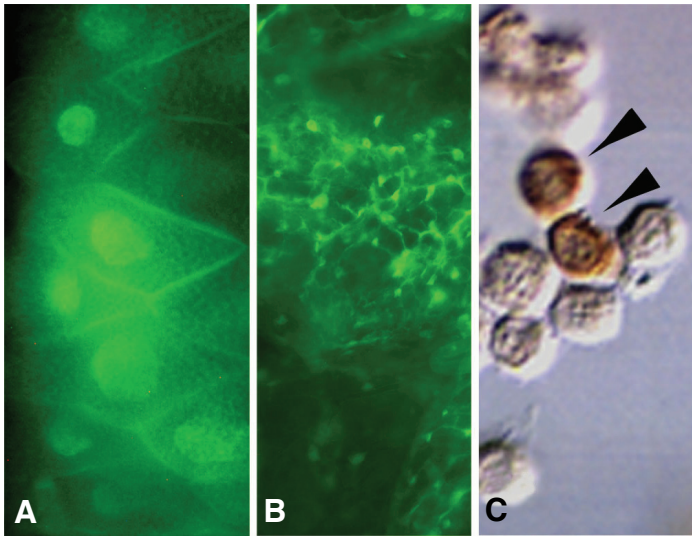

Fig. 6. The MORGUE protein exhibits diverse localization within *Drosophila* cells. (A) *The P[SaGa52A-Gal4] driver strain was used to target expression of GFP::Morgue proteins in third instar larval salivary glands. Note widespread distribution of GFP::Morgue in membrane, cytoplasmic, and nuclear compartments.* **(B)** *Targeted expression of GFP::Morgue via P[repo-Gal4] reveals distribution of GFP::Morgue in the in third instar larval CNS glial nuclei, cytoplasm, and processes.* **(C)** *Detection of a 6xHis-Morgue protein in cultured insect Sf9 cells via anti-His immunostaining reveals cytoplasmic and nuclear localization of 6xHis-Morgue in transfected cells (arrowheads).*

amino acids of the *cg15432* coding region. *cg15432* encodes a DUF1674 domain protein that has a lysine-rich NH_2-region and is a member of the FAM32A protein family. This protein is highly conserved in a wide range of organisms, suggesting important, albeit as yet undetermined functions. We therefore sought to obtain *morgue*-specific mutants to facilitate analysis of Morgue functions. In this study we describe two small *morgue* deletion mutations that remove portions of the *morgue* coding region but do not extend into *cg15432*. Like *morgue*^{19}, the *morgue*-specific alleles also exhibit dominant suppression of Hid-mediated eye cell death, implying that disruption of *morgue* alone is sufficient to modify the actions of *grim-reaper* genes. However, unlike *morgue*^{19}, these new *morgue* alleles are viable. This viability suggests two interpretations for the basis of the lethality of *morgue*^{19}. Either *cg15432* is required for viability and it is the loss of this gene that results in lethality of the *morgue*^{19} allele, or it is the combined loss of *cg15432* and *morgue* that results in the *morgue*^{19} lethal phenotype. While full resolution of this issue awaits analysis of *cg15432*-specific mutants, RNAi experiments suggest that the *C. elegans* homolog K01G5.8, is not an essential gene (http://www.wormbase.org/db/gene/gene?name=WBGene00010479). The close proximity of *morgue* and *cg15432* in other *Drosophila* species suggests that the two genes may share common gene regulatory elements and function in similar pathways, however, the genes are not adjacent in other insects, including the mosquito *Anopheles gambiae* (Schreader and Nambu, 2003), the silkmoth *Bombyx morii*, the honey bee *Apis melifera*, and the red flour beetle *Tribolium castaneum* (data not shown).

Developmental functions of morgue

The isolation of *morgue*-specific loss-of-function mutants facilitated the examination of *morgue* function during development. Within the developing embryonic nervous system, we analyzed the CNS midline glia and larval photoreceptor neurons, two lineages that undergo prominent developmental cell death. *morgue* mutant embryos exhibited ectopic mildine glia in several ganglia. This finding indicates that *morgue* function is not absolutely required for midline glia to die, but it is important for normal patterns of death. In the larval photoreceptor neuron lineage, there was a much greater variability and asymmetry in the number of neurons in the two clusters. This phenotype suggests that *morgue* influences the precision of cell survival patterns, and that in its absence, the patterns become more stochastic. The modest embryonic phenotypes of *morgue* mutants are similar to those for mutations in other cell death regulators, including *reaper* (Pedersen *et al*. 2000), *dbruce* (Vernooy *et al*. 2002) and bcl-2 family members (Sevrioukov *et al*. 2007). As Morgue clearly enhances the cell killing actions of Grim-Reaper proteins (Wing *et al*. 2002), its major role may be to sensitize cells to other pro-death stimuli. Morgue could also have partially redundant functions with other death regulators or be essential for death in a very specific set of cells.

The midline glia exhibited aberrant cytoplasmic extensions in *morgue* mutants and the peripheral glia often failed to exhibit normal localization. Aberrant cytoplasmic processes of *morgue* mutant midline glia could result from the presence of ectopic glia and/or altered morphology of normally surviving glia. Similar, though more severe midline defects are observed in Df(3L)H99 mutants, where all midline glial cell death is blocked. The defects in peripheral glial location could indicate disruption in normal cell migration or differentiation. Overall, these phenotypes suggest that Morgue could also influence cellular processes distinct from apoptosis. In this regard DIAPs and caspases function in a wide range of non-cell death processes, including the innate immune response (Stoven *et al*. 2003; Gesellchen *et al*. 2005; Kleino *et al*., 2005; Leulier *et al*. 2006; Huh *et al*. 2007), actin cytoskeleton dynamics (Oshima *et al*. 2006), spermatid individualization (Arama *et al*. 2003; Huh *et al*. 2004a; Vernooy *et al*. 2004), and germ cell migration (Geisbrecht and Montell 2004). In addition, dying cells can activate a caspase-dependent signaling pathway to induce neighboring cells to proliferate (Ryoo *et al*. 2004; Huh *et al*. 2004b; Perez-Garijo *et al*. 2004; Kondo *et al*. 2006). In these processes caspase activities are modulated in such a manner that they do not result in cell death. Perhaps Morgue is involved in this modulation. Morgue may also function in other physiological pathways, such as those controlling circadian rhythmicity (Murad *et al*. 2007). Tightly regulated ubiquitination and protein turnover are also critical for the molecular oscillations that underlie biological clocks (Chiu *et al*. 2008) and given its similarity to both E2 and E3 ubiquitination enzymes, it will be of interest to determine if Morgue may have similar or distinct biochemical actions in cell death and circadian rhythm processes.

Morgue may function in multiple cellular compartments

Ubiquitination proteins can exhibit diverse sites of action that permit access to disparate types of substrate proteins (e.g. Hamilton *et al*. 2001; Deng and Hochstrasser, 2006). We utilized GFP::Morgue and Morgue-6xHis proteins to investigate the sub-

cellular distribution of Morgue. These proteins exhibited diverse subcellular localization that suggested Morgue could potentially function in the nucleus, cytoplasm, and cell membrane. Interestingly, Morgue does not contain a recognizable nuclear localization signal or any other domain that would suggest specific targeting properties. Therefore, Morgue distribution may be largely governed by the proteins with which it interacts. Thus, Morgue could be delivered into nuclei via interactions with nuclear proteins such as the Histone H4 replacement His4r or the ubiquitin specific protease Ubp64E (Giot *et al*. 2003). Overall, the localization of Morgue in diverse cellular compartments suggests that it may impact a wide range of ubiquitination events and substrate proteins.

Materials and Methods

Generation and analysis of morgue *mutants*

A P element excision screen was performed using the P[SUPor-P]KG02386 strain. Deletion events were identified by PCR using *morgue* gene primers and genomic DNA from homozygous excision mutant adults. PCR products from the *morgue* excision alleles were isolated and gel purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and subjected to DNA sequence analysis (Davis Sequencing, Davis CA) to determine the breakpoint sites.

Drosophila genomic DNA was isolated as described previously (Wing *et al*., 2002) from *morgue* excision mutants and analyzed via PCR using sets of primers that correspond to sites within and flanking the *morgue* gene. The resulting PCR products were analyzed via agarose gel electrophoresis, purified via QIAGEN (Valencia CA) Qiaquick Gel Extraction kit, and subjected to DNA sequence analysis (Davis Sequencing, Davis CA) to map the excision mutations.

Immunocytochemistry

Whole mount embryo immunostaining was performed as described by Patel (1994) using the following primary antibodies: Anti-Repo (Developmental Studies Hybridoma Bank, University of Iowa, Iowa; 1:3 dilution), 22C10 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa; 1:3 dilution), anti-Wrapper (gift of J.N. Noordermeer; 1:3 dilution), anti-Kruppel (gift of J. Reinitz; 1:800 dilution), and anti-β-galactosidase (Promega Corp., Madison WI; 1:1000 dilution). Biotinylated anti-mouse (1:200 dilution) and anti-rabbit (1:200 dilution) secondary antibodies (Vector Laboratories, Burlingame CA) and HRP/DAB histochemistry (Sigma Fast 3.3 Diaminobenzidine Tablet Sets; Sigma, St. Louis, MO) were used to visualize the sites of labeling. Stained embryos were dehydrated in an ethanol series, mounted in methyl salicylate and Permount (Fisher Scientific, Pittsburgh PA), and analyzed via DIC optics using a Nikon Optiphot 2.

Generation and analysis of a P[UAS-GFP::Morgue] transformant strain

A GFP-Morgue DNA construct was generated using the pUAST vector (Brand and Perrimon 1993) by cloning the GFP coding region adjacent to the NH_2-terminus of the Morgue coding region (amino acids 2-491). The fidelity of the construct was verified via DNA sequence analysis. pGFP-Morgue plasmid DNA was purified via a QIAGEN plasmid maxi kit. Transformant flies were generated by the Duke University Model System Genomics Facility (Durham, NC). P[UAS-GFP:Morgue] insertions were mapped and balanced or homozygosed as appropriate to establish strains.

GFP fluorescence in animals carrying P[UAS-GFP:Morgue] and a P[Gal4] driver was analyzed using a Zeiss LSM 510 Meta Confocal System at the University of Massachusetts Central Microscopy Center (http://www.bio.umass.edu/microscopy/).

Generation and analysis of pIZT-Morgue in cultured Sf9 cells

A Morgue::His DNA construct where a 6xHis tag was added to the *morgue* open reading frame was generated via PCR. The Morgue::His DNA was cloned into the pIZT vector (Novagen, Madison, WI) and pMorgue::His plasmid DNA purified. Transient transfections were performed on cultured Lepidopteran Sf9 cells with the pMorgue::His plasmid as described previously (Wing et. al., 2002). Immunostaining of transfected Sf-9 cells was performed using a QIAexpress Anti-His HRP conjugase kit (QIAGEN, Valencia, CA). pIZT-Morgue transfected Sf9 cells were washed with 1xPBS and fixed in acetone:methanol (1:1) for 1 min. The fixed cells were washed with 1xPBS and then incubated with blocking buffer provided with the kit for at least 1 hour at 4°C. The cells were washed with 1xPBS, and incubated with Penta-His HRP conjugate antibody in 1xPBS at 4°C overnight. The cells were washed in 1xPBS and stained via HRP/DAB histochemical reactions, and visualized via DIC optics using a Nikon Optiphot 2.

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