The *Drosophila* deoxyhypusine hydroxylase homologue *nero* and its target eIF5A are required for cell growth and the regulation of autophagy

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Hypusination is a unique posttranslational modification by which lysine is transformed into the atypical amino acid hypusine. eIF5A (eukaryotic initiation factor 5A) is the only known protein to contain hypusine. In this study, we describe the identification and characterization of *nero*, the *Drosophila melanogaster* deoxyhypusine hydroxylase (DOHH) homologue. *nero* mutations affect cell and organ size, bromodeoxyuridine incorporation, and autophagy. Knockdown of the hypusination target eIF5A via RNA interference causes phenotypes similar to *nero* mutations. However, loss of *nero* appears to cause milder phenotypes than loss of eIF5A. This is partially explained through a potential compensatory mechanism by which nero mutant cells up-regulate eIF5A levels. The failure of eIF5A up-regulation to rescue *nero* mutant phenotypes suggests that hypusination is required for eIF5A function. Furthermore, expression of enzymatically impaired forms of DOHH fails to rescue *nero* clones, indicating that hypusination activity is important for *nero* function. Our data also indicate that *nero* and eIF5A are required for cell growth and affect autophagy and protein synthesis.

**Introduction**

Hypusination is a unique form of posttranslational modification occurring in eukaryotic organisms that transforms the amino acid lysine into the atypical amino acid hypusine (Park et al., 1982, 1997; Gordon et al., 1987). Thus far, a specific lysine residue on eIF5A (eukaryotic initiation factor 5A) is the only known target for this modification (Cooper et al., 1983; Smit-McBride et al., 1989). The formation of hypusine occurs via a two-step process. Each step of this process is catalyzed by a different enzyme (Park et al., 1982). In the first step, deoxyhypusine synthase (DHS) cleaves spermidine and transfers the 4-amino butyl moiety to a specific lysine residue of eIF5A (Wolff et al., 1990). In the second step, deoxyhypusine hydroxylase (DOHH) hydroxylates deoxyhypusine and irreversibly completes the hypusination process (Abbruzzese et al., 1986; Park et al., 2003). The protein responsible for DOHH function has only recently been identified and characterized. DOHH is an atypical hydroxylase consisting of eight HEAT (named after huntingtin, elongation factor 3, protein phosphatase 2A, and target of rapamycin [Tor]) repeat motifs (Joe et al., 1995; Kang et al., 1995; Park et al., 2006). DOHH is a metalloenzyme that requires iron ions for enzymatic activity (Park et al., 1982; Kim et al., 2006). Four conserved histidine–glutamic acid metal-binding motifs have been identified in DOHH (Park et al., 2006). Mutagenesis of these binding motifs impairs the enzyme’s ability to bind iron and concomitantly abolishes its enzymatic activity (Kang et al., 2007).

Several lines of evidence suggest that eIF5A plays a role in cell proliferation. Inhibition of eIF5A function through drug-mediated hypusination blockage causes proliferation arrest in mammalian cell lines. Spermidine analogues that inhibit DHS activity as well as various metal ion chelators that inhibit DOHH enzymatic function have been shown to affect proliferation in vitro (Park et al., 1982, 1994; Jakus et al., 1993; Hanuske-Abel et al., 1994). Furthermore, inhibiting hypusination through cellular spermidine depletion also blocks cell proliferation in both mammalian cell lines as well as in yeast (Byers et al., 1994;
Chattopadhyay et al., 2003). More direct evidence supporting a role for eIF5A in proliferation comes from genetic studies in yeast. Mutations in TIF51A and TIF51B, which encode eIF5A, or loss of DYS1 (DHS homologue) in yeast cause cell inviability as well as G1–S phase arrest (Schnier et al., 1991; Wöhl et al., 1993; Sasaki et al., 1996; Park et al., 1998; Chatterjee et al., 2006). Interestingly, LIA1 (ligand of eIF5A 1; yeast DOHH) is not essential for viability and has only a mild effect on the proliferative ability of Saccharomyces cerevisiae (Park et al., 2006). Moreover, the function of LIA1 remains unknown as no major phenotypes are associated with its loss.

Lack of evidence supporting a role for yeast DOHH in eIF5A proliferation regulation questions the importance of the second step in the hypusination process. Thus, the only current evidence that argues for a role for DOHH in eIF5A regulation comes from drug studies that block DOHH enzymatic function through metal ion chelators in mammalian cell culture systems (Park et al., 1982; Hanuske-Abel et al., 1994), leading to the argument that the second step in the hypusination process is probably important only in higher eukaryotes. However, the effect of metal ion chelators on proliferation may be nonspecific. Therefore, genetic experiments on DOHH in higher eukaryotic organisms may shed light on the importance of the second step in the hypusination pathway.

This study addresses the function of DOHH in Drosophila melanogaster. Mutations in nero, the Drosophila DOHH homologue, were identified in a genetic screen for genes that regulate bristle number. nero is essential for organismal viability and plays a role in a wide number of important processes such as cell growth, proliferation, and autophagy. These phenotypes are reminiscent of mutations in the Tor pathway, but no clear epistatic relationship was found to exist between these pathways. As eIF5A is the sole known target of hypusination, we analyzed eIF5A function using RNAi. Loss of eIF5A causes phenotypes highly similar to but more severe than nero. The similarities in phenotypes imply that both nero and eIF5A govern the same processes. This also implicates eIF5A in processes besides proliferation, including autophagy and cell growth. Interestingly, we find that eIF5A is highly up-regulated in nero mutants. This up-regulation may partially compensate for the loss of nero and possibly accounts for the differences in the severity of the phenotypes of the loss of either gene. Furthermore, we find that the disruption of DOHH enzymatic activity impairs nero function, suggesting that its hypusination function is critical for its function. Finally, knockdown of either eIF5A or Nermo through RNAi in S2 cells affects translation elongation.

Results

nero mutations in Drosophila DOHH affect sensory organ development

Lyman et al. (1996) performed a P element screen to identify insertions that alter bristle number in fruit flies and identified several insertions within genes previously implicated in bristle development. A similar screen in a genetically well-controlled background confirmed that such screens can be used for the purposes of identifying genes that govern neural development (Norga et al., 2003). One P element strain, P[ArB]K48, which was generated in the initial Lyman et al. (1996) screen, exhibits a subtle bristle loss in homozygous flies. Therefore, we decided to focus on this P element insertion. P[ArB]K48 is inserted in an intron of the CG1910 gene and in close proximity to CG2245, potentially affecting both genes (Fig. 1 A). We identified a second P element, P[laW]l921, associated with this locus and inserted within the 5′ untranslated region of CG2245 (Fig. 1 A; Spradling et al., 1999). We excised both P[ArB]K48 and P[laW]l921 and consequently generated three homozygous lethal mutations. To determine whether these mutations affect bristle development, each of the three mutant alleles was recombined onto a flipase (FLP) recombination target (FRT) chromosome for clonal analysis (Xu and Rubin, 1993). Mutant clones generated on the thorax show bristle loss as well as very small bristles (Fig. 1 B). Therefore, we named the gene nero after a nearly bald Flemish comic book character. Two mutations derived from P[laW]l921, nero2 and nero2, are associated with small molecular deletions in CG2245 that remove 189 bp and 123 bp, respectively, of the gene’s open reading frame (Fig. 1 A). Animals carrying these mutations die as second instar (L2) larvae whether they are homozygous or in heteroallelic combinations (Fig. 1 E). Larvae carrying nero alleles in combination with a deficiency that spans the CG2245 genomic region also die as second instar animals, suggesting that all nero alleles are strong loss of function alleles or null alleles (Fig. 1 E).

To verify that the nero mutations constitute a loss of CG2245, we generated a 1.5-kb genomic rescue construct containing the CG2245 coding region (Fig. 1 A). This construct is sufficient to rescue the lethality of nero mutant larvae to full viability as well as bristle phenotypes associated with nero clones (Fig. 1 C). Ubiquitous overexpression of CG2245 cDNA under upstream activation sequence (UAS) regulation using the actin-GAL4 driver rescues the lethality associated with nero mutations (Brand and Perrimon, 1993). Furthermore, overexpression of CG2245 in nero mutant clones using mosaic analysis with a repressible cell marker system is able to rescue the bristle phenotypes (Fig. 1 D; Lee and Luo, 1999). Altogether, these experiments demonstrate that nero mutations correspond to a loss of CG2245. Interestingly, nero overexpression in a wide variety of contexts using different GAL4 drivers has no phenotypic effects.

CG2245 encodes a 302-aa protein bearing a high level of homology to eukaryotic DOHH proteins (57% identity with Caenorhabditis elegans and mouse homologues and a 59% identity with its human homologue). Thus, nero likely encodes the Drosophila DOHH homologue. Similar to the yeast and vertebrate homologues, the Drosophila DOHH protein consists of two dyads of four HEAT repeat domains (Fig. 1 F; Park et al., 2006). The human DOHH (hDOHH) protein bears four histidine–glutamic acid motifs important for DOHH enzymatic activity. These motifs are also conserved in the fly homologue, suggesting functional conservation.

Nero localizes to the ER

To determine the spatial expression of Nero as well as its subcellular localization, an antibody was raised against the full-length protein. The antibody specifically recognizes the Nero protein in immunohistochemical labeling, as all immunoreactivity is lost in
The inability of the antibody to detect Nero in nero mutants is further evidence that the nero alleles are probably null alleles.

The Nero protein is expressed in larval imaginal disc cells (unpublished data). The subcellular distribution shows that the protein is excluded from the nucleus. The protein distribution nero mutant clones (Fig. 2, A and B). This antibody also specifically recognizes Nero on Western blots as a single ~39-kD band in wild-type L2 protein lysates (Fig. 2 C). This band is not detected in nero mutant lysates. Thus, the ~39-kD band observed in wild-type lysates corresponds to the Nero protein (Fig. 2 C).
within the cell appears punctate, indicating that Nero is most likely associated with an organelle (Fig. 2 D). Double labeling of imaginal discs with the Nero antibody and a monoclonal antibody that recognizes the ER retention signal KDEL shows extensive colocalization, revealing that the Nero protein localizes at least in part to the ER (Fig. 2, D–F; Pelham, 1990). ER localization was also observed in larval garland cells (Fig. 2, G–I). As the Nero protein does not bear a signal peptide or a C-terminal KDEL sequence tag, Nero most likely associates with the external surface of the ER.
nero is cell autonomously required for cell growth and affects the cell cycle.

nero mutations cause both mild bristle loss as well as a severe reduction in bristle size (Figs. 1 B and 3 A). Bristles are composed of two external cells, the socket and the shaft. Socket cells dramatically increase in size within a 5-h period between the time points of their birth at 19 h after puparium formation (APF) and 24 h APF (Audibert et al., 2005). To determine whether the specification and the size of socket cells are affected early during the development of these cells, we labeled pupal thoraxes 24 h APF with the suppressor of Hairless (Su(H)) antibody, which is a marker for socket cells. Su(H) is expressed both in the cytoplasm and the nucleus of the socket cell, allowing visualization of the entire cell (Gho et al., 1996). As shown in Fig. 3 (B and C), nero mutant socket cells at this time point are much smaller than wild type. Thus, nero mutant socket cells are specified but fail to reach their mature size.

The growth defects observed in nero mutant clones are not specific to cells of the bristle lineage. As shown in Fig. 3 D, cells adjacent to nero mutant bristles are smaller in size as indicated by trichome size and spacing. This suggests that nero function is not confined solely to the regulation of bristle size. Indeed, ectodermal cells that lack nero in developing thoraxes 47 h APF are significantly smaller than adjacent wild-type cells (Fig. 3, E and F; Woods and Bryant, 1991). These defects are confined to nero mutant cells, and thus, nero is cell autonomously required for cell growth (Fig. 3, E and F). These nero mutant cells also show a decrease in Discs large (Dlg) expression, which is a basolateral cell membrane marker (Fig. 3, E and F). These effects on cell size are not restricted to the thorax, as rhabdomere size in mutant eye clones is also reduced when compared with adjacent wild-type cells, suggesting a defect in photoreceptor size (unpublished data). Thus, nero is cell autonomously required to achieve correct cell size in many tissues. We further analyzed nero function by generating adult somatic clones using the eyeless-FLP system. nero mutant clones generated using the eyeless-FLP technique are generally irrecoverable (Fig. 4, A and B). Interestingly, nero mutant clones are easily obtained using eyeless-FLP when cell competition is ameliorated through the use of an FRT chromosome bearing a cell lethal mutation that effectively removes the mitotic wild-type, sister twin cells (Fig. 4 D). We observe similar results when competition is alleviated using a Minute. This suggests that nero mutant cells are viable but are at a competitive disadvantage. Indeed, nero mutant clones generated using this technique completely overtake the eye (Fig. 4, C and D). However, the resultant nero mutant eyes and heads are much smaller than wild-type heads and resemble the pinhead phenotype associated

Figure 3. nero is required for cell growth. (A) The shaft and socket cells that comprise the external sensory organ on the thorax of the adult fly are typically shorter and smaller in nero mutant clones (genotype: y w hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; FRT82B nero/FRT82B hsp70-CD2 y tub-Gal80). Mutant bristles are marked by the recessive yellow mutation and appear light brown. (B and C) Socket cells observed 24 h APF labeled with Su(H) are smaller than wild-type (WT) socket cells (genotype: y w hs-FLP/+; UAS-FLP/+; FRT82B nero/Cs684-GAL4 FRT82B Ubi-GFP M[3]). The clone is marked by the absence of GFP. (D) Epidermal cells on the adult thorax in the vicinity of nero mutant bristles are often smaller in size, as indicated by smaller trichome size and closer trichome spacing (genotype: y w hs-FLP/+; FRT82B nero/FRT82B Ubi-GFP). (E and F) nero mutant epidermal cells in the thorax 47 h APF are smaller than adjacent wild-type cells. Thoraxes are labeled with Dlg to reveal cell outlines (genotype: y w hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; FRT82B nero/FRT82B hsp70-CD2 y tub-Gal80). The mutant clone is marked positively with GFP. (B, C, E, and F) White lines mark the clonal boundary.
Figure 4. *nero* regulates organ size, cell number, and proliferation. (A and B) *nero* clones generated in a wild-type background are poorly competitive. Clones are marked with the absence of white and thus appear white. (A) Wild-type clones are easily recovered (genotype: *y w eyeless-FLP GMR-lacZ/+; FRT828+/FRT828 w*). (B) *nero* clones are usually irrecoverable (genotype: *y w eyeless-FLP GMR-lacZ/+; FRT828 nero+/FRT828 w*). The white arrow points to a small *nero* mutant clone at the edge of the eye. (C and D) To provide *nero* mutant cells a competitive advantage, clones were made using an FRT-bearing chromosome carrying a recessive cell lethal that effectively eliminates wild-type twin spots. Clones are marked as in A and B. (C) Wild-type clones proliferate and take over most of the eye (genotype: *y w eyeless-FLP GMR-lacZ/+; FRT828+/FRT828 w*; *3*cl). (D) *nero* clones generated in a cell lethal background can, like wild type, dominate the entire eye (genotype: *y w eyeless-FLP GMR-lacZ/+; FRT828 nero+/FRT828 w*; *3*cl). *nero* mutant eyes and heads are smaller than wild-type control heads, suggesting a defect in organ size regulation. Eyes are also rough. (E) Cell number in wild-type (WT) clones is roughly similar to cell number in their twin spots (genotype: *y w hs-FLP; FRT828+/FRT828 Ubi-GFP*). Black bars correspond to the clone, and gray bars correspond to the twin spot. (F) *nero* mutant clones have fewer cells than wild type (genotype: *y w hs-FLP; FRT828 nero+/FRT828 Ubi-GFP*). Black and gray bars are the same as in E. (G and H) *nero* mutant clones marked positively with GFP incorporate BrdU more poorly than adjacent wild-type cells (genotype: *y w hs-FLP tub-GAL4 UAS-GFP::d2MYC-NLS+/; FRT828 nero+/FRT828 tubGAL80-CD2 y tubGAL80*). White lines mark the clonal boundary.

with mutants in several growth pathways (Fig. 4 D; Oldham et al., 2000). Thus, *nero* mutations affect cell size and organ size, but *nero* is not required for cell viability.

Quantification of the number of cells in *nero* mutant clones versus the number of cells in their associated wild-type twin spot in the context of the wing imaginal disc reveals that *nero* mutant cells are underrepresented. Wild-type clones generated in the wing imaginal disc contain a similar number of cells when compared with the number of cells in their associated twin spot (Fig. 4 E). However, *nero* clones are smaller and contain fewer cells than their associated wild-type twin spots (Fig. 4 F). The differences in cell number between *nero* clones and their wild-type twin spots are less obvious in small clones and clearly increase as clones get larger (Fig. 4 F). This is likely caused by differences in NERD protein perdurance in small clones (Garcia-Bellido and Merriam, 1971). *nero* mutant cells are most likely eliminated because of an inability to compete with wild-type cells.

To determine whether the cell cycle is affected in *nero* mutant cells, we assayed their ability to incorporate BrdU (Gratzner, 1982). *nero* mutant clones show a decreased level of
ner" mutants. ATG8b-GFP fusion proteins have been shown to localize to autophagic structures (Scott et al., 2004). LysoTracker-positive structures observed in "nero" mutant fat body cells colocalize with the ATG8b-GFP fusion proteins, confirming that these structures are indeed autophagosomes (Fig. 5, D–F).

The combination of defects in cell size and autophagy suggests a possible link between "nero" and the Tor pathway (Oldham et al., 2000; Zhang et al., 2000; Rusten et al., 2004; Scott et al., 2004). Tsc1 (tuberous sclerosis 1) mutations cause constitutive activation of the Tor pathway and have the opposite phenotype of "nero" mutations. Tsc1 mutations increase cell size, cause tissue overgrowth, increase cell number in clones when compared with their twin spot, and block the induction of autophagy (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001; Rusten et al., 2004; Scott et al., 2004). Therefore, we generated a Tsc1 "nero" double mutant chromosome to ascertain the possibility of an epistatic relationship between these genes. Unlike either "nero" or Tsc1 clones, Tsc1 "nero" double mutant clones cannot be recovered (Fig. S1). Thus, "nero" and Tsc1 do not exist in a straightforward epistatic relationship. Interestingly, Tsc1 fully suppresses the autophagic defect seen in "nero" mutant animals (Fig. S1). However, this epistatic relationship is reversed in other contexts such as larval growth and developmental timing. Tsc1 mutant larvae appear to undergo precocious development. We find that this precocious development is suppressed in the Tsc1 "nero" double mutant larvae (Fig. S1).
Figure 6. Loss of elf5A causes phenotypes reminiscent of nero. (A) Wing imaginal disc in which elf5A dsRNA has been expressed under the regulation of the dpp-GAL4 driver line and labeled with anti-elf5A antibody. Anti-elf5A antibody fails to detect the elf5A protein in cells expressing elf5A dsRNA. (B) Wing imaginal disc in which elf5A under UAS regulation has been overexpressed using dpp-GAL4 and stained with anti-elf5A antibody. Anti-elf5A antibody detects the overexpressed elf5A protein. (C) Anti-elf5A antibody detects an ∼17-kD band in EGFP dsRNA–treated S2 cells but fails to detect this band in elf5A dsRNA–treated S2 cells. Actin is used as the loading control. Protein standards run along with S2 cell lysates are marked with black bars. Their sizes are recorded in kilodaltons on the left. (D) Control adult wing (genotype: dpp-GAL/+). (E) Adult wing in which elf5A dsRNA has been expressed under the regulation of the dpp-Gal4 driver. The distance between the L3 and L4 vein (double-headed arrow) is drastically reduced (genotype: dpp-Gal4/UAS-elf5A RNAi). (F) Adult wing in which wild-type elf5A under UAS regulation was overexpressed using dpp-Gal4. The distance between the L3 and L4 vein (double-headed arrow) appears similar to the experimental control (D; genotype: dpp-GAL4/UAS-elf5A RNAi). (G) RNAi knockdown of
These results suggest that nero may not play a direct role in Tor signaling and most likely alters cell growth through an independent mechanism.

**nero regulates eIF5A**

eIF5A is generally believed to be the sole protein that undergoes hypusination in eukaryotic organisms (Cooper et al., 1983; Smit-McBride et al., 1989). Having established a genetic model in which loss of the second step of the hypusination process causes severe phenotypes, we decided to ascertain whether loss of eIF5A causes phenotypes similar to loss of nero, as would be predicted if nero regulated eIF5A function. To test this, we obtained an RNAi line purported to knock down eIF5A (Dietzl et al., 2007). We used a monoclonal antibody raised against the human eIF5A protein to test whether this RNAi line is able to effectively down-regulate eIF5A. Expression of eIF5A double-stranded RNA (dsRNA) under the regulation of dpp-GAL4 strongly reduces the expression of eIF5A in a central stripe that runs down the middle of the wing imaginal disc, proving that the RNAi line is effective and that the antibody is able to specifically recognize the Drosophila eIF5A protein in vivo (Fig. 6 A). Additionally, this antibody is able to recognize the wild-type overexpressed eIF5A protein (Fig. 6 B). Unfortunately, it is difficult to detect the eIF5A protein on Western blots of larval lysates using this antibody. However, it recognizes an ~17-kD band on Western blots of S2 cell lysates, which corresponds to the expected molecular mass of eIF5A (Fig. 6 C). Furthermore, this 17-kD band is lost in S2 cell lysates treated with eIF5A dsRNA, suggesting that the band specifically corresponds to eIF5A (Fig. 6 C). Altogether, these data suggest that we can efficiently knock down or overexpress eIF5A using UAS-eIF5A RNAi or UAS-eIF5A constructs, respectively. We used UAS-eIF5A RNAi to test whether loss of eIF5A has any phenotypic consequences. Knockdown of eIF5A in the dpp-GAL4 expression domain causes a narrowing of the region between the L3 and L4 wing veins, a phenotype which is often associated with growth mutants in Drosophila (Fig. 6, D and E). However, overexpression of eIF5A appears to have no phenotypic effect on the distance between L3 and L4 veins, and thus, like nero, overexpression of eIF5A has no overt phenotypic consequences (Fig. 6, D and F).

We decided to test whether loss of eIF5A causes phenotypes similar to those associated with nero. Flies carrying a transgenic UAS-eIF5A RNAi construct were crossed to the thoracic driver eq-GAL4 to determine whether loss of eIF5A affects bristle size (Pi et al., 2001; Dietzl et al., 2007). We find that eIF5A knockdown results in decreased bristle size on the thorax, which is similar to what we observe in nero mutant clones (compare Fig. 6 G with Fig. 1 B). We also identified a homozygous lethal P element, P[?PZ]P01296 (eIF5A P01296), inserted within the first intron of eIF5A (Spradling et al., 1999). Excision of this P element reverts the lethality, indicating that the lethality is caused by the insertion. Homozygous mutant eIF5A P01296 larvae exhibit a severe larval growth defect and are much smaller than wild-type larvae, although they live for 8 d (Fig. 6 H). RNAi knockdown of eIF5A using the ubiquitous driver tub-GAL4 severely affects larval growth, and the effect is greater than the phenotype observed in eIF5A P01296 larvae, suggesting that loss of eIF5A using RNAi perhaps constitutes a stronger loss of function condition than eIF5A P01296 (Fig. 6 H). Thus, eIF5A P01296 is most likely a hypomorphic mutation. Interestingly, eIF5A mutations are more severe than nero in terms of larval growth (Fig. 6 H). Finally, we find that the eIF5A P01296 mutation also affects autophagy induction. Similar to nero, eIF5A mutant larval fat bodies undergo constitutive autophagy under fed conditions (Fig. 6 I). The autophagy defect is specific to the P element insertion, as the revertants do not show a defect in autophagy induction (Fig. 6 K). Driving eIF5A RNAi in larval fat bodies using Adh-GAL4 likewise induces autophagy (Fig. 6 J). Thus, the phenotypes associated with loss of eIF5A are very similar to the phenotypes associated with loss of nero, suggesting that Nermo and eIF5A function in the same pathway in flies.

If the target of nero function is eIF5A, loss of eIF5A ought to be epistatic to nero. Thus, eIF5A: nero double mutant larvae should resemble eIF5A mutant larvae. We find that eIF5A: nero double mutant larvae display a slightly more severe larval growth defect than eIF5A mutants alone (Fig. 7 A). As eIF5A P01296 is probably not a null allele, the more severe phenotype is presumably a consequence of increased inactivation of residual eIF5A function in these animals. Interestingly, the larval growth defect caused by the loss of eIF5A is more severe than loss of nero (Figs. 6 H and 7 A). This may be the result of several causes. One possibility is that eIF5A has both hypusination-dependent and -independent functions. Another possibility is that nero is required only for optimal eIF5A function, and thus, eIF5A maintains some modicum of activity in nero mutants. If eIF5A is still partially active even in the absence of hypusination, overexpression of eIF5A ought to ameliorate nero mutant phenotypes. To test this, we overexpressed eIF5A in nero mutant clones. We found that overexpression of eIF5A is not sufficient to rescue bristle size defects associated with nero mutant clones (Fig. 7 B). Moreover, we were surprised to find that eIF5A is strongly up-regulated in nero mutant clones (Fig. 7, C and D). This strong up-regulation possibly accounts for the weaker phenotype observed in nero mutant clones and may represent an adaptive response of the cells to the inefficiently functioning eIF5A protein. Finally, to directly assess the requirement for DOHH activity, we overexpressed wild-type and enzymatically inactive versions of hDOHH in nero mutant clones. Alanine replacement of histidines 56, 89, 207, and 240 of hDOHH, all of which are conserved in the Drosophila protein, has been shown to abolish hypusine enzymatic function and iron-binding ability without affecting eIF5A substrate binding (Kang et al., 2007). These residues were mutated separately...
demonstrates that *nero* is the DOHH homologue in flies. However, overexpression of the four different enzymatically inactive mutant proteins in *nero* mutant clones fails to rescue defects in bristle size, revealing the importance of the hypusination activity for *nero* function (Fig. 7, F–I).

Figure 7. *nero* regulates elf5A levels in vivo and Nero’s DOHH activity is required for *nero* function. (A) Synchronized larvae 72 h after egg hatching of four different genotypes (from left to right): *yw*, *nero*¹, elf5A⁰¹⁲⁹⁶, and elf5A⁰¹²⁹⁶; *nero*¹. The growth of elf5A⁰¹²⁹⁶; *nero*¹ double mutant larvae appears to be more impaired than either elf5A⁰¹²⁹⁶ or *nero*¹ homozygous mutant larvae. (B) Overexpression of elf5A in *nero* mutant clones fails to rescue bristle growth defects associated with *nero* mutant clones (genotype: *yw* hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; UAS-elf5A/+; FRT82B *nero*¹/FRT82B hsp70-CD2 *yw* tub-GAL80). (C and D) elf5A levels are dramatically up-regulated in *nero* mutant clones in the wing imaginal disc. *nero* mutant clones are marked by the absence of GFP (green; genotype: *yw* hs-FLP; FRT82B *nero*¹/FRT82B Ubi-GFP). White lines mark the clonal boundary. WT, wild type. (E) Overexpression of hDOHH in *nero* mutant clones rescues bristle size defects (genotype: *yw* hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; UAS-hDOHH+/+; FRT82B *nero*¹/FRT82B hsp70-CD2 *yw* tub-GAL80). (F–I) Overexpression of mutated forms of hDOHH in *nero* mutant clones fails to rescue bristle size defects. Genotypes are essentially identical to E except mutated forms of hDOHH are expressed under the UAS regulation. (F) Genotype: *yw* hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; UAS-hDOHH H56A+/+; FRT82B *nero*¹/FRT82B hsp70-CD2 *yw* tub-GAL80. (G) Genotype: *yw* hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; UAS-hDOHH H89A+/+; FRT82B *nero*¹/FRT82B hsp70-CD2 *yw* tub-GAL80. (H) Genotype: *yw* hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; UAS-hDOHH H208A+/+; FRT82B *nero*¹/FRT82B hsp70-CD2 *yw* tub-GAL80. (I) Genotype: *yw* hs-FLP tub-GAL4 UAS-GFP-6xMYC-NLS/+; UAS-hDOHH H240A+/+; FRT82B *nero*¹/FRT82B hsp70-CD2 *yw* tub-GAL80. (B and E–I) Mutant bristles are marked by the recessive yellow mutation and appear light brown.

in the hDOHH cDNA and introduced into flies to determine whether these proteins could complement the loss of *nero*. Overexpression of the wild-type hDOHH protein fully complements defects in bristle size associated with *nero* mutant clones on the thorax and does not cause any other obvious phenotype (Fig. 7 E). This result demonstrates that *nero* is the DOHH homologue in flies. However, overexpression of the four different enzymatically inactive mutant proteins in *nero* mutant clones fails to rescue defects in bristle size, revealing the importance of the hypusination activity for *nero* function (Fig. 7, F–I).
Both Nero and eIF5A regulate protein synthesis.
Recent evidence supports the idea that eIF5A regulates translation elongation. Gregio et al. (2009) have shown that the polysome (number of ribosomes per mRNA) profile of a yeast eIF5A mutant resembles that of a translation elongation mutant.

As eIF5A is thought to be the only target for Nero, we predicted that reduction of either Nero or eIF5A should have a similar effect on protein synthesis. To test this hypothesis, we used polysome profiling. Polysome sedimentation in sucrose gradients is one of the most powerful techniques for studying translational control. In this technique, the mRNAs are separated by ultracentrifugation as a function of the number of ribosomes to which they are associated. Conditions that reduce translation elongation result in the binding of mRNAs to multiple ribosomes, thus causing an increase in sedimentation toward the polysomal fraction. We established conditions to decrease levels of either Nero or eIF5A using dsRNA in Drosophila S2 cells (unpublished data). Similar to the yeast eIF5A mutant, RNAi-mediated knockdown of either Nero or eIF5A increases polysome size, indicating a defect in translation elongation (Fig. 8; Gregio et al., 2009). These data suggest that Nero-mediated eIF5A hypusination regulates translation rates.

Discussion
Hypusination, the transformation of the amino acid lysine into the atypical amino acid hypusine, is a process that has been documented in numerous eukaryotic organisms from yeast to humans (Park et al., 1982, 1997; Gordon et al., 1987). The precise function of eIF5A and the significance of hypusination with regard to eIF5A function remain obscure. This study presents the functional characterization of the Drosophila DOHH homologue nero and looks at nero function in relation to eIF5A.

nero is an essential gene and encodes a highly conserved homologue of hDOHH
nero is required for organismal viability in Drosophila. This is in stark contrast to yeast, in which yeast DOHH (LJA1) is not an essential gene (Park et al., 2006). This has lead to the hypothesis that the second step in the hypusination process may not be essential in regard to eIF5A function in lower eukaryotes (Park et al., 2006). We find that DOHH activity is important in higher eukaryotes. The generation of null mutations of nero and the rescue of these mutants using the Drosophila DOHH genomic rescue constructs formally confirm that DOHH function is required in higher eukaryotic organisms such as Drosophila.

The Nero protein appears to be highly conserved in Drosophila. Nero shares 59% amino acid identity with its hDOHH homologue, and the hDOHH protein can substitute for the Drosophila protein in vivo. Overexpression of hDOHH in nero mutant is able to fully rescue the short bristle defect observed in nero mutant clones. Thus, nero constitutes a true homologue of hDOHH. Analysis of hDOHH function has shown that DOHH is a metalloenzyme that requires iron ions for enzymatic activity (Park et al., 1982; Kim et al., 2006). Four histidine–glutamic acid metal-binding motifs have been identified in hDOHH, and these motifs have been shown to be required for DOHH enzymatic function (Park et al., 2006; Kang et al., 2007). These four motifs are fully conserved in the Drosophila homologue. Interestingly, overexpression of hDOHH carrying mutations in these metal ion–binding motifs fails to complement nero mutations, arguing that these residues are also important for DOHH function in Drosophila. All together, Nero appears to be highly conserved both in terms of amino acid homology as well as important residues critical for its function.

nero is required for cell growth, protein synthesis, and autophagy regulation
Clonal analyses of nero mutations during imaginal disc development implicate nero in cell growth. Loss of nero causes small bristles, epidermal cells, and photoreceptors. Furthermore, removing nero function in the developing Drosophila eye-antennal disc causes decreased head size, suggesting that nero function is required for organ size. Mutations that negatively affect cell growth share an array of common phenotypes such as impaired cell competitive ability and altered cell cycle.

Figure 8. RNA knockdown of either Nero or eIF5A blocks translation elongation in Drosophila S2 cells. Polysome analysis of EGFP, nero, and eIF5A dsRNA–treated S2 cells. Cells were harvested, lysed, and fractionated by centrifugation on a 10–50% sucrose gradient. Polysomes were analyzed as described in Materials and methods. EGFP (left), nero (middle), and eIF5A dsRNA–treated (right) images are shown. The positions of the polysomes and ribosomes are indicated.
Such combined defects in cell growth, impaired competitive ability, and altered cell cycle are often attributed to impaired or reduced translation (Neufeld et al., 1998; Moreno et al., 2002). We find that loss of *nero* also affects both competitive ability and altered cell cycle phasing, as indicated by the fact that *nero* mutant cells are poorly recovered and that they poorly incorporate BrdU. All together, these phenotypes suggest that *nero* plays a role in the regulation of translation.

Consistent with the notion that Nero regulates eIF5A activity, inhibition of Nero or eIF5A by RNAi causes a similar impairment in translation elongation. This finding is consistent with data generated in other experimental systems. eIF5A has been shown to be bound to components of the translational machinery in a hypusine-dependent manner (Zaneli et al., 2006). Furthermore, depletion of yeast eIF5A has been shown to block protein synthesis as well as alter polysome profiles (Kang and Hershey, 1994; Gregio et al., 2009). Our data support a function for DOHH in eIF5A-mediated translational control.

Interestingly, *nero* also regulates the induction of autophagy. Unlike effects on translation, defects in the induction of autophagy are generally not common to all cell growth pathways. In *Drosophila*, defects in autophagy regulation have been associated with mutations that affect the insulin and Tor growth signaling pathways (Rusten et al., 2004; Scott et al., 2004). Amino acid nutrient availability has been proposed as an upstream regulator of Tor activity (Dann and Thomas, 2006). This is in line with the observation that starvation induces autophagy in *Drosophila* (Rusten et al., 2004; Scott et al., 2004). This prompted us to test *nero* for a potential role in the Tor pathway. However, we were unable to generate any conclusive genetic evidence to link the two pathways, allowing us to exclude at the very least an integral role for *nero* in the Tor growth pathway.

### Phenotypes associated with loss of eIF5A resemble *nero*, and *nero* regulates eIF5A levels

Inhibition of eIF5A using RNAi-mediated knockdown causes phenotypes similar to the ones observed in *nero* mutants. Knockdown of eIF5A, the target of hypusination, recapitulates *nero* mutant phenotypes, including decreased bristle size, autophagy, and defects in larval growth/development. These observations suggest that both *nero* and eIF5A regulate similar processes in vivo and argue that *nero* is linked to eIF5A function.

Interestingly, we find that eIF5A is up-regulated in *nero* mutants. This effect on eIF5A levels in DOHH mutants has not previously been reported. It is likely that the up-regulation of eIF5A constitutes an adaptive response by the cell to the loss of *nero*. Because the eIF5A mutant phenotype appears to be more severe than the defect observed in *nero* mutant animals, the up-regulation of eIF5A in *nero* mutants may explain the observed differences in larval growth associated with eIF5A and *nero* double mutants. Thus, incompletely hypusinated forms of eIF5A may still be partially functional and able to mildly ameliorate the *nero* mutant phenotype. This model is also compatible with the idea that the loss of eIF5A causes only a partial loss of eIF5A activity. In conclusion, our work highlights the importance of *nero* DOHH function in *Drosophila*, implicates eIF5A and *nero* in cell growth and autophagy regulation, and provides genetic evidence that links eIF5A function with *nero* DOHH regulation.

### Materials and methods

#### Drosophila stocks and genetics

We used the following stocks: y w [control], Canton-S [control], P[ArB] GF (provided by T.F. MacKay, North Carolina State University, Raleigh, NC; Tapon et al., 2001), hs-AIT16b-GFP (provided by T.P. Neufeld, University of Minnesota, Minneapolis, MN; Scott et al., 2004), eq-GAL4 [P et al., 2001], UAS-CG3186 RNAi (Dietzl et al., 2007), Adh-GAL4/Cyo [Fischer et al., 1988], Ubx-FLP, FRT82B UbI-GFP M(3), y w hs-FLP tub-GAL1640-GFP-oxMYC-NLS; FRT82B hsp70-CD2 y' tub-GAL4/TM6 [provided by G. Struhl, Columbia University, New York, NY]; y w; D/TM3 Sc-GAL4 UAS-GFP, and FRT82B UbI-GFP (provided by G. Halder, The University of Texas M.D. Anderson Cancer Center, Houston, TX). y w; Act-GAL4/Cyo, D(f3)[R]D0461, y w eyeless-FRM GMR-ac3; FRT82B w + [I]cl, FRT82B w + [P2]; Spradling et al., 1999), tub-GAL4/TM3 [Lee and Luo, 1999], and UAS-lacW1212 [Spradling et al., 1999] were obtained from the Bloomington *Drosophila* Stock Center.

*nero* mutants were generated through imprecise excision of P[ArB]GF and P[UacW1212]. Genomic lesions in *nero* and *nero* alleles were generated from the mobilization of P[UacW1212]. Genomic lesions in *nero* and *nero* mutants were determined using PCR. eIF5A was generated through the excision of P[UacW1212].

#### Transgenic flies

To make a genomic rescue construct for *nero*, *Drosophila* genomic sequences corresponding to the *nero* locus were isolated from the DS00235 P1 clone (Berkeley *Drosophila* Genome Project). The P1 clone was digested with SacI, and this fragment was subcloned into the SacI site of the plasmid vector containing yeast technologies. A 1.5 kb fragment was recovered from this construct by digesting with SmaI and MluI and was subcloned into the Hpal site of P[CaSpI]. To make UAS-*nero*, the CG2245 coding region was isolated from LD0536 (Berkeley *Drosophila* Genome Project *Drosophila* Gene Collection) and cloned into the EcoRI and Xhol sites of the *P[UAST]* vector. To make UAS-eIF5A, the CG3186 coding region was PCR amplified from RE4768 (Berkeley *Drosophila* Genome Project *Drosophila* Gold Collection) with adapters bearing EcoRI and XhoI sites and cloned into these sites of the *P[UAST]* vector. To make UAS-*nero*, the CG3186 coding region was PCR amplified from RE4768 with yeast genomic DNA as a template and cloned into the EcoRI and XhoI sites of the *P[UAST]* vector. To make UAS-*deoHH* and its enzymatically inactive version, human cDNA [National Institutes of Health Mammalian Gene Collection] for DOHH was obtained from Invitrogen, PCR amplified, and cloned in pbuEScript. H65A, H89A, H208A, and H240A point mutations in DOHH were generated using a GeneTailer Site-Directed Mutagenesis system (Invitrogen). The wild-type and mutant versions were subsequently cloned into the *P[UAST]*. All of these constructs were subsequently introduced into flies.

#### Antibody generation, Western blotting, immunohistochemistry, and BrdU assay

A full-length Nero-His tag fusion protein was generated by cloning the full-length Nero cDNA into the EcoRI and Xhol sites of the pEt28a cloning vector (EMD). Coding sequences were amplified from LD0536 using primers that introduced EcoRI and Xhol restriction sites. The protein was induced in BL21 DE3 bacteria using IPTG and purified using HisBind resin (EMD). Purified fusion proteins were injected into guinea pigs for antibody production (Cocalico Biologicals, Inc.).

We used standard protocols for Western blotting. Western Lightning Western Blot Chemiluminescence Reagent (PerkinElmer) was used to detect signal. Western blots were incubated in primary antibody using the following dilutions: 1:2,000 guinea pig anti–Nero GP25, 1:1,500 rabbit anti-eIF5A [EP526Y] (Abcam), and 1:10,000 mouse anti–actin C4 (MP Biomedical). For immunohistochemistry, larvae were dissected in PBS and fixed for 20 min in 4% formaldehyde in PBS. Standard protocols were used to label larval tissues with antibodies. Incubations in primary antibodies were performed overnight using the following dilutions: 1:100 mouse anti–DlG 4F3 (Developmental Studies Hybridoma Bank), 1:400 rabbit anti-eIF5A [EP526Y] (Abcam), 1:400 mouse anti–KDEL (Assay Designs), 1:1,000 guinea pig anti–Nero GP26, and 1:2,000 rat anti–Su(H) (provided by F. Schweisguth, Institut Pasteur, Paris, France; Oro et al., 1996). Fluorescent Cy3- and Alexa Fluor 488–conjugated secondary antibodies (provided by

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Note added in proof. After this manuscript was accepted for publication, a paper appeared in Nature online (Saini et al. 2009. Nature doi:10.1038/nature08534) showing that the depletion or inactivation of elf5A in yeast (S. cerevisiae) results in the accumulation of polyosomes, indicating that elf5A promotes translation elongation. These data are in agreement with our observation that RNAi-mediated knockdown of either Nero or elf5A blocks translation elongation and thus increases the size of polyosomes in Drosophila S2 cells.

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