Nucleolar stress in *Drosophila melanogaster*
RNAi-mediated depletion of Nopp140

Allison James, Remford Cindass Jr, Dana Mayer, Stephanie Terhoeve, Courtney Mumphrey and Patrick DiMario*

Department of Biological Sciences, Louisiana State University; Baton Rouge, LA USA

**Keywords:** Nopp140, nucleolus, apoptosis, autophagy, *Drosophila*

**Abbreviations:** Nopp140, nucleolar phosphoprotein of 140 kDa; TCS, Treacher Collins-Franceschetti syndrome; RNAi, RNA interference; TEM, transmission electron microscopy; snorRNPs, small nucleolar ribonucleoprotein particles; UAS, upstream activation sequence; JNK, Jun- amino terminal kinase; LC-MS/MS, liquid chromatography-tandem mass spectroscopy

---

**Nucleolar stress results when ribosome biogenesis is disrupted.** An excellent example is the human Treacher Collins syndrome in which the loss of the nucleolar chaperone, Treacle, leads to p53-dependent apoptosis in embryonic neural crest cells and ultimately to craniofacial birth defects. Here, we show that depletion of the related nucleolar phosphoprotein, Nopp140, in *Drosophila melanogaster* led to nucleolar stress and eventual lethality when multiple tissues were depleted of Nopp140. We used TEM, immune-blot analysis and metabolic protein labeling to show the loss of ribosomes. Targeted loss of Nopp140 in larval wing discs caused Caspase-dependent apoptosis which eventually led to defects in the adult wings. These defects were not rescued by a p53 gene deletion, as the craniofacial defects were in the murine model of TCS, thus suggesting that apoptosis caused by nucleolar stress in *Drosophila* is induced by a p53-independent mechanism. Loss of Nopp140 in larval polyplid midgut cells induced premature autophagy as marked by the accumulation of mCherry-ATG8a into autophagic vesicles. We also found elevated phenoloxidase A3 levels in whole larval lysates and within the hemolymph of Nopp140-depleted larvae vs. hemolymph from parental genotype larvae. Phenoloxidase A3 enrichment was coincident with the appearance of melanotic tumors in the Nopp140-depleted larvae. The occurrence of apoptosis, autophagy and phenoloxidase A3 release to the hemolymph upon nucleolar stress correlated well with the demonstrated activation of Jun N-terminal kinase (JNK) in Nopp140-depleted larvae. We propose that JNK is a central stress response effector that is activated by nucleolar stress in *Drosophila* larvae.

---

**Introduction**

The nucleolar phosphoprotein Nopp140\(^{1,2}\) likely functions as a molecular chaperone for small nucleolar ribonucleoprotein particles (snorRNPs), either in their assembly, transport to the nucleolus from Cajal bodies or in their association with pre-rRNA as these snorRNPs mediate site-specific 2'-O-methylation (box C/D snorRNPs) or pseudouridylation (box H/ACA snorRNPs)\(^\text{(reviewed in ref. 4)}\). Previously, *Drosophila melanogaster* expresses two isoforms of Nopp140 by alternative splicing.\(^\text{3}\) The isoforms are identical up to amino acid residue 584, but then differ in their carboxy termini; one (Nopp140-True) is the canonical ortholog of mammalian Nopp140, while the other (Nopp140-RGG) has a Gly and Arg rich domain common to many RNA-associated proteins. Previously, we used RNAi to target the common 5' end of both splice variant transcripts. Nopp140 mRNA levels depleted by ≥ 50% caused larval and pupal lethality, while deletions by only ~30% produced viable adults, but with defective legs, wings, thoracic bristles and abdominal cuticles.\(^4\) These adult structures normally derive from larval imaginal discs and histoblasts which presumably maintain high demands for ribosome biogenesis and thus protein synthesis.

Related to Nopp140 in structure and function is Treacle, a nucleolar protein found thus far in vertebrates along with Nopp140. *TCOF1* is the gene on human chromosome 5 that encodes Treacle. Haplo-insufficiencies in *TCOF1* are closely associated with the Treacher Collins-Franceschetti syndrome (TCS), which is marked by craniofacial malformations during fetus development.\(^5\) TCS arises from the loss of neural crest cells that normally migrate to populate branchial arches I and II on about day 24 of human embryogenesis.\(^6\) Loss of Treacle in these particular neural crest cells results in abnormal ribosome biogenesis (referred to as nucleolar stress) and thus a loss in protein synthesis leading ultimately to cellular stress and p53-dependent apoptosis.\(^7\) Using the murine system, Jones et al.\(^8\) showed that deleting p53 or blocking p53 function prevented apoptosis in these neural crest cells; they were thus able to rescue the craniofacial abnormalities normally associated with the TCS. Malformations in adult flies resulting from the partial loss of Nopp140 in *Drosophila* larval progenitor tissues were reminiscent of the human TCS. The first goal of this study was to determine what cell stress responses resulted from the depletion of Nopp140 by RNAi expression in *Drosophila* larval cells leading either to...
Nucleolar stress caused by the loss of Nopp140. Heterozygous Act5C > Nopp140-RNAi lacked Nopp140 within most, but not all of their imaginal diploid cells (Fig. 1A and B) and polyplloid cells (Fig. 1C and D). Polyplloid cells lacking Nopp140 condense their chromatin (Fig. 1C). These larvae died in the late larval and early pupal stages when reared at 27–28°C. While we used w1118 as our wild type control in Figure 1E–H, homozygous parental larva develop normally (they are viable and fertile) with no detectable loss of Nopp140 in their tissues.

We compared tissues from Act5C > Nopp140-RNAi larva to the same tissues from wild type (w1118) or parental type larva by transmission electron microscopy, immuno blot analysis and metabolic protein labeling to confirm that ribosome synthesis was disrupted due to Nopp140 depletion (i.e., nucleolar stress).

A diploid cell from an Act5C > Nopp140-RNAi larval wing disc (Fig. 2A) contained some rough endoplasmic reticulum, but the number of free ribosomes in the cytosol was greatly reduced compared with that in the wild type disc cell (Fig. 2B). In addition, the nuclei (Nu) in the Act5C > Nopp140-RNAi diploid cells contained numerous virus-like particles (arrow in Fig. 2A). These particles likely arise from copia retrotransposons that can be induced by environmental stress, aging or perturbations in poly(ADP-ribose) polymerase. In preparing tissues for TEM analysis, we noted the small size of the imaginal discs isolated from Act5C > Nopp140-RNAi larvae. We have yet to determine if reduced disc size is due to smaller cells or failure to produce sufficient cell numbers. A polyplloid midgut cell from an Act5C > Nopp140-RNAi larva (Fig. 2C) contained relatively very few ribosomes as compared with the same cell type from a wild type larva (Fig. 2D).

Again, the presence of virus-like bodies the nucleus (arrow in Fig. 2C) indicated these Nopp140-depleted polyplloid cells were under stress. While Act5C > Nopp140-RNAi imaginal...
Disorders of Nopp140 to disrupt ribosome synthesis. Nopp140-RNAi expression cannot effectively deplete enough (see http://flybase.org/reports/FBgn0037137.html) such that larvae depleted for Nopp140. Act5C > Nopp140-RNAi larvae vs. we compared metabolic protein labeling in parental control larval Nopp140 and ribosomes that dwindle as these larvae advance younger larvae likely maintained sufficient quantities of mater-

nals into the third instar stage. Defects such as melanotic tumors (see below) contained near larvae that had yet to show obvious effects such as melanotic tumors (see below) contained near normal levels of ribosomal proteins (lane not shown). These younger larvae likely maintained sufficient quantities of mater-

nals Nopp140 and ribosomes that dwindle as these larvae advance further into the third instar stage.

To verify the effects of ribosome loss in third instar larvae, we compared metabolic protein labeling in parental control larva vs. Act5C > Nopp140-RNAi larvae depleted for Nopp140. The loss of Nopp140, leading to the loss of ribosomes, caused a reduction in protein synthesis to ≤ 40% (Fig. 2F). In this assay, we analyzed two separate samples of Act5C > Nopp140-RNAi. One sample (*) was older with a greater number of melanotic masses compared with the other, yet we saw similar protein labeling profiles in the two samples. We conclude from the various assays in Figure 2 that third instar Act5C > Nopp140-RNAi larvae had a pronounced loss of ribosomes and thus protein synthesis, and this resulted in lethality by the late third larval instar-early pupal stages.

Selective depletion of Nopp140 in larval wing discs. To assess the loss of Nopp140 without inducing lethality, we used the larval wing disc driver, A9-GAL4, on the X chromosome to induce the Nopp140-RNAi gene (UAS-C4.2 on the second chromosome) (A9 > UAS-C4.2). We set up the cross such that all male progeny displayed normal wings. Conversely, female progeny (A9 > Nopp140-RNAi) expressed RNAi in their larval wing discs, which eventually led to malformed adult wings. The wing phenotype in these females varied from a relatively mild wing discs, which eventually led to malformed adult wings. The wing phenotype in these females varied from a relatively mild (vestigial-like) wings (see Table 1). Large fluid-filled blisters were common in the wings of newly eclosed females. Their wings were left badly malformed as the blisters receded over time. Table 1 compares the frequency of the severe wing phenotype to that of the less severe curled wing phenotype. While all the male progeny had normal wings, 47% of the A9 >

![Figure 2](image-url)
Nopp140-RNAi females showed wings with the mild defect, and the remaining 53% showed the severe defect. None of the females had normal wings. We conclude that A9 > Nopp140-RNAi provides a non-lethal phenotype that we can score to access nucleolar stress in diploid imaginal (progenitor) wing disc cells.

TCS arises in mammals when specialized embryonic neural crest cells undergo apoptosis due to the loss of Treacle. To determine if nucleolar stress caused by the loss of Nopp140 in Drosophila wing discs also induces apoptosis, we reversed the original cross for Table 1 such that homozygous A9-GAL4 driver females were crossed to males homozygous for Nopp140-RNAi. All larval progeny from this cross expressed Nopp140-RNAi in their wing discs. We probed these wing discs with anti-cleaved Caspase 3 (Asp175) from Cell Signaling Technology. Although the precise antigen remains unknown, this particular antibody provides a good marker for Caspase-9-like DRONC activity in apoptotic Drosophila cells. A wing disc from an A9 > Nopp140-RNAi larva was heavily labeled by anti-Caspase (Fig. 3A–C). Higher magnification showed that the apoptotic cells contained condensed chromatin within their nuclei (Fig. 3D) and that anti-Caspase 3 labeled the cytoplasm (Fig. 3E) as expected. Wing discs from wild type larvae (w1118) showed minimal anti-Caspase labeling (Fig. 3F–H). Therefore, as with the loss of Treacle in mammalian neural crest cells, selective loss of Nopp140 in Drosophila imaginal wing disc cells induced apoptosis leading to malformed wings in adult flies.

Apoptosis induced by nucleolar stress in Drosophila is p53-independent. Jones et al. showed that apoptosis induced by nucleolar stress in mouse embryonic neural crest cells was p53-dependent. The deletion of the mouse p53 gene or chemical inactivation of p53 suppressed this apoptosis, reducing the severity of phenotypes typically associated with TCS. To test if apoptosis in Drosophila imaginal wing discs resulting from deletion of Nopp140 is p53-dependent, we repeated the original cross described in Table 1 such that only female progeny would display wing defects, but this time the two parental types (the A9-GAL4 driver and the Nopp140-RNAi-expressing lines) were homozygous for the p53 gene deletion (p53Δ/Δ) on the third chromosome (referred to simply as p53Δ). This deletion was originally described by Rong et al. Homozygous deletion flies are viable and fertile, but they display reduced apoptosis in response to DNA damage caused by ionizing radiation.

Genomic PCRs verified that the modified parental lines were in fact deleted for p53 (Fig. 4A). The wild type p53 gene in w1118 flies produced the expected 7.3 kbp PCR product (lane 1), while the modified A9-GAL4 driver (lane 2), the original p53Δ/p53Δ stock from the Bloomington Stock Center (lane 3) and the Nopp140-RNAi line (lane 4) produced the expected 4.0 kbp product indicating homozygous deletion of the p53 gene (see Flybase, http://flybase.org/reports/FBrf0151688.html for descriptions of the expected PCR products). Thus, all A9 > Nopp140-RNAi progeny from the second cross should be homozygous deficient for the p53 gene. While the male progeny from this cross showed normal wings as expected (Fig. 4B, left wing), all female progeny again displayed malformed wings (Fig. 4B, right wing). Table 1 again summarizes the results: of the female progeny obtained, 42% displayed the less severe curled wing edge phenotype, while 58% showed the more severe shriveled wing phenotype (Table 1). This compares well to 47% and 53%, respectively, for those female progeny that were homozygous for the wild type p53 gene. We note that variability in wing phenotype in both crosses (with and without p53) may be caused to slight temperature fluctuations affecting GAL4-mediated induction of UAS-transgenes. To minimize this effect, all crosses were performed at 27–28°C.

We again reversed the cross such that all progeny were A9 > Nopp140-RNAi, p53Δ/p53Δ. These larvae also showed apoptosis in wing discs by anti-Caspase 3 labeling (Fig. 4C and D). We performed these crosses several times to be certain that the

---

Table 1. X and/or Y chromosome, second chromosome and third chromosome designations are separated by semi-colons

| Progeny Phenotypes | Progeny Genotypes | w1118; UAS-C4.2/+; +/+ | A9/wr; UAS-C4.2/+; +/+ | w1118; UAS-C4.2/+; p53Δ/p53 | A9/wr; UAS-C4.2/+; p53Δ/p53 |
|--------------------|--------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Normal wings       | 100%               | 0%                           | 100%                          | 0%                           |
| Mild wing edge curl| 0%                 | 47%                          | 0%                            | 42%                          |
| Severe wing malformation | 0%         | 53%                          | 0%                            | 58%                          |

| Number of Flies Scored | 270 | 172 | 143 | 178 |

Male larva (w1118/Y) lacked the A9-GAL4 driver and therefore did not express RNAi from UAS-C4.2 on their second chromosome. Female larvae (A9/Y) expressed RNAi to deplete Nopp140 in their wing discs. Two crosses were performed, with (+) and without (Δ) the p53 gene on the third chromosome. Abnormal wing phenotypes appeared in only the females as either a mild wing edge curl or a more severe vestigial-like (shriveled) wing. Phenotype frequencies were comparable with or without the p53 gene.
abundance in the midgut of Nopp140-depleted larvae (da-GAL4 > Nopp140-RNAi; UAS-mCherry-ATG8a) (Fig. 5C). Thus, the loss of Nopp140 and nucleolar function induces premature autophagy in the polyploid midgut cells.

Accumulation of phenoloxidase A3 in response to Nopp140 depletion. Coomassie-stained SDS-gels showed an abundant 70 kDa protein in whole lysates of Act5C > Nopp140-RNAi larvae vs. parental control larvae (Fig. 6A). At first, we thought the accumulated protein was a classic heat shock protein (Hsp70) or perhaps a heat shock cognate protein (Hsc70) that was induced upon nucleolar stress. However, antibodies against Hsp70 and Hsc70 failed to show significant accumulations of either protein in the Nopp140-depleted larvae. LC-MS/MS finally identified the protein as phenoloxidase A3 encoded by PO45 (conceptual gene CG8193) in Drosophila melanogaster.

Phenoloxidases in Drosophila are released into the hemolymph from circulating crystal cells, usually as part of an innate immune response to parasitic infection. Upon infection, the phenoloxidases convert phenols to quinones that polymerize to form melanin aggregates that then encapsulate the parasite.24 To test if phenoloxidases were released to the hemolymph of Nopp140-depleted larvae, we isolated larval serum proteins,25 resolved them on native polyacrylamide gels and then either stained the gels presence or absence of p53 had no effect on wing malformations due to Nopp140 depletion. The combined data in Table 1 indicates that there were no appreciable differences in the abnormal wing phenotypes due to Nopp140 depletion when p53 was present or deleted. We conclude, therefore, that larvae selectively depleted for Nopp140 in their wing discs induce apoptosis in these discs, but in a p53-independent manner.

Autophagy in larval polyploid midgut cells depleted of Nopp140. In earlier studies to examine the loss of Nucleostemin I in Drosophila larvae, we observed by TEM premature autophagy in larval polyploid midgut cells.20 Here, we ectopically co-expressed mCherry-ATG8a to determine if excessive autophagy occurred in midgut cells of Nopp140-depleted (da > Nopp140-RNAi; UAS-mCherry-ATG8a) third instar larvae. We first expressed mCherry-ATG8a in the midgut of a second instar larva using the da-GAL4 driver, but in an otherwise wild type background; mCherry-ATG8a distributed diffusely throughout these midgut cells (Fig. 5A). As a positive control for autophagy, we starved similar second instar larvae to induce autophagy.22 As expected, mCherry-ATG8 aggregated into vesicles within the cytoplasm of these midgut polyploid cells (Fig. 5B). We interpret the appearance of these vesicles as autophagosomes and lysosomes.23 Similar vesicles with mCherry-ATG8 appeared prematurely and in great abundance in the midgut of Nopp140-depleted larvae (da-GAL4 > Nopp140-RNAi; UAS-mCherry-ATG8a) (Fig. 5C). Thus, the loss of Nopp140 and nucleolar function induces premature autophagy in the polyploid midgut cells.

Figure 3. Loss of Nopp140 in imaginal wing discs of A9 > UAS-C4.2 larvae resulted in apoptosis. Phase contrast (A) and DAPI staining (B) of a wing disc isolated from an A9 > UAS-C4.2 larva. (C) The wing disc stained heavily with anti-Caspase 3 indicating apoptosis. (D and E) Higher magnification of panels (B and C) showed anti-Caspase 3 staining in the cytoplasm as expected. Cells with copious anti-Caspase 3 labeling showed condensed chromatin by DAPI staining (arrows). (F-H) Wing discs from control (w1118) larvae were probed with anti-Caspase 3 antibody. They showed minimal background labeling.
with Cosmus blue (Fig. 6B) or stained the gels for phenoloxidase activity using tyrosine as a substrate (Fig. 6D). Compared with parental types, Act5C > Nopp140-RNAi larvae showed greater amounts of phenoloxidase in their hemolymph, indicating another phenotype associated with the loss of nucleolar function. In collecting the Act5C > Nopp140-RNAi larvae, we purposely avoided the accumulation of melanotic masses due to the release of phenoloxidase from crystal cells. JNK (also known as the stress-activated protein kinase, SAPK) is a principal cell stress response effector that could link all three phenotypes. Activated Drosophila JNK (also referred to as basket) is known to induce apoptosis by activating Hid and autophagy by inducing d Foxo. Activated JNK also induces the appearance of melanotic masses scattered throughout the larva; for example, Act5C > Nopp140-RNAi larvae showed an elevated level of activated JNK vs. lysates from parental controls. We conclude that activated JNK accumulates in Nopp140-depleted larvae. The anti-pJNK immuno-blots (Fig. 7B) showed two bands in some lysates from both parental types and from the Nopp140-depleted larvae, while we saw only the higher molecular weight band in other separately prepared lysates. At this time we have no clear explanation for the appearance of the bottom band other than partial proteolysis.

In their model of p53-independent apoptosis in Drosophila, McNamee and Brodsky proposed that JNK induces Hid protein expression that then induces apoptosis. To test if Hid was induced, we probed the same lysates used in Figure 7B with a rabbit polyclonal antibody directed against Drosophila Hid. We found Hid levels notably increased in Act5C > Nopp140-RNAi larvae vs. parental controls (Fig. 7A).

**Discussion**

The human TCS results from mutations in the TCOFI gene which encodes the nucleolar phosphoprotein, Treacle. Subsets of embryonic neural crest cells are particularly sensitive to the loss of Treacle, a nuclear chaperone for ribosome biosynthesis. These cells normally migrate to populate embryonic branchial arches I and II that then give rise to adult craniofacial structures. Without Treacle, these neural crest cells fail to meet a relatively high threshold requirement for functional ribosomes. As a result these cells are stressed and undergo p53-dependent apoptosis. Their loss ultimately leads to the craniofacial malformations associated with the TCS. Jones et al. could alleviate the syndrome by blocking p53...
function either by a p53 gene deletion or by a specific p53 protein inhibitor.

Vertebrate Nopp140 and Treacle are related in structure and function. Both proteins are over 100 kDa in size, both contain LidH dimerization motifs in their N-terminal region, and both proteins contain a large central domain consisting of alternating acidic and basic motifs with similar amino acid compositions; the acidic motifs are rich in Glu, Asp and phospho-Ser, while the basic motifs are rich in Lys and Pro. Both proteins are believed to function as chaperones for snoRNP complexes as these complexes direct size-specific methylation and pseudouridylation of pre-rRNA within the dense fibrillar regions of eukaryotic nuclei. While most snoRNAs appear to be non-essential when individually deleted, failure to collectively modify pre-rRNA by the loss of a snoRNP chaperone would likely produce defective or partially functional ribosomes in all cells. Embryonic progenitor cells with high demands for protein synthesis would be most susceptible to the loss of functional ribosomes. Loss of these cells would lead to lethality or developmental defects. Thus Treacle and Nopp140 are required for normal development. Despite their multiple similarities, Nopp140 and Treacle differ in their carboxy termini, suggesting that each protein maintains its own unique interactions and functions. Interestingly, while Drosophila expresses two isoforms of Nopp140 by alternative splicing, Drosophila lacks a Treacle ortholog.

We showed that larval cells depleted for Nopp140 undergo a disruption in ribosome biosynthesis (nucleolar stress). Larval tissues expressing Nopp140-RNAi, both diploid and polyploid, have fewer ribosomes than wild type (Fig. 2A–D). These larvae also have reduced amounts of ribosomal proteins L23 and L34 (Fig. 2E). Metabolic labeling verified these findings, revealing that Nopp140-depleted larvae synthesize significantly reduced amounts of protein (≤40%) compared with parental controls (Fig. 2F). As larval tissues develop, maternally supplied ribosomes and Nopp140 diminish, and cells expressing Nopp140-RNAi are unable to supply either functional ribosomes and/or sufficient protein synthesis. This nucleolar stress thus leads to distinctive cell death pathways. Here we showed that the loss of Drosophila Nopp140 specifically in imaginal wing disc cells lead to apoptosis and impaired wing development, analogous to the loss of Treacle in embryonic neural crest cells. However, while Jones et al.10 rescued the TCS in mice by employing a p53 gene deletion, the similar rescue failed in Drosophila as apoptosis occurred in the absence of the p53 gene (Fig. 3). Induction of apoptosis in the absence of p53 has been well documented in Drosophila.18,33 Besides apoptosis, we documented a strong and premature induction of autophagy in larval midgut polyploid cells that were depleted for Nopp140 (Fig. 5C). As with apoptosis, JNK activation is reportedly linked to autophagy induction22 by way of the downstream activation of dFoxo.20 We note, however, that this stress-induced autophagy is in contrast to a JNK-independent induction of autophagy that normally occurs during development or in response to starvation resulting in the inhibition of Tor signaling.22,23

We also showed that formation of excess melanotic masses in Nopp140-depleted larva was coincident with the accumulation

![Figure 5. Autophagy was prominent in larval polyploid cells depleted for Nopp140.](http://www.landesbioscience.com/Nucleus/129/fig5.png)
Nopp140-depleted larvae are developmentally delayed, we speculate that JNK is a central effector activated in response to perturbations in ribosome biogenesis. Evidence, we propose that JNK is a common link between Hid (apoptosis), dFoxo (autophagy), and the release of phenoloxidases from crystal cells as these cells rupture in JNK-dependent manner. We showed by immuno-blot analysis that Hid expression is upregulated in Nopp140-depleted larvae. While JNK activation has been reported to be delayed in the p53-independent mechanism, once activated, JNK is thought to induce Hid expression and thus apoptosis. While we have yet to test the abundance and activities of dFoxo and dFox in the Nopp140-depleted larvae, others have shown that Hid induction results from JNK activation via two transcription factors. From the combined evidence, we propose that JNK is a central effector activated in response to perturbations in Drosophila ribosome biogenesis. JNK is a member of the MAP kinase superfamily, and while mammals express multiple forms of JNK, Drosophila melanogaster expresses only one version of JNK (basket). Precisely how JNK is activated in response to nucleolar stress in mammals is likely to play out differently than in Drosophila. One possibility supported by Bidla et al. is that apoptosis is followed by secondary necrosis triggers formation of melanotic masses. If so, we would expect to see the formation of multiple melanotic masses at non-hematopoietic sites undergoing heavy apoptosis and secondary necrosis.

Nuclear stress in Drosophila differs from mammals. Our working hypothesis is that depletion of Nopp140 in Drosophila cells disrupts normal ribosome biogenesis, a term now referred to as “nuclear stress.” Studies on nuclear stress in mammalian cells have focused primarily on the nuclear protein ARF (p95/105 in mice, p34/44 in humans), its interactions with MDM2 which is the E3 ubiquitin ligase for p53 and the role that nuclear protein B23 plays in sequestering ARF to the nucleus. Upon stress in mammalian cells, ARF indirectly activates p53 by interacting with MDM2 to suppress its ubiquitination of p53. Under these conditions, p53 accumulates and activates genes necessary for cell cycle arrest, senescence or apoptosis. Interestingly, Drosophila lacks identifiable ARF, MDM2 and B23. Thus nuclear stress in Drosophila likely plays out by an alternative mechanism.

From our work, JNK appears to be a common link between Hid (apoptosis), dFoxo (autophagy), and the release of phenoloxidases from crystal cells as these cells rupture in JNK-dependent manner. We showed by immuno-blot analysis that Hid expression is upregulated in Nopp140-depleted larvae. While JNK activation has been reported to be delayed in the p53-independent mechanism, once activated, JNK is thought to induce Hid expression and thus apoptosis. While we have yet to test the abundance and activities of dFoxo and dFox in the Nopp140-depleted larvae, others have shown that Hid induction results from JNK activation via two transcription factors. From the combined evidence, we propose that JNK is a central effector activated in response to perturbations in Drosophila ribosome biogenesis. JNK is a member of the MAP kinase superfamily, and while mammals express multiple forms of JNK, Drosophila melanogaster expresses only one version of JNK (basket). Precisely how Drosophila JNK is activated in response to nuclear failure remains unknown.

As a clue, Moreno et al. showed that JNK signaling is required for the apoptotic elimination of slowly proliferating Minute cells in larval wing discs where Minute is a dominant, haplo-insufficient mutation in a gene encoding a ribosomal protein. McNamee and Brodsky proposed that gene haplo-insufficiencies in three similar ribosomal protein genes could induce expression of breeder (brk) which encodes a transcription factor in larval wing discs, and that brk is sufficient to activate JNK, thus leading to apoptosis. Also

of phenoloxidase A3 both in whole larval lysates (Fig. 6A) and in the larval hemolymph (Fig. 6B and C). While phenoloxidases are normally synthesized and stored in crystal cells, they are not secreted in a typical secretory pathway; rather, crystal cells rupture in a JNK-dependent manner releasing the phenoloxidases to the hemolymph. How Nopp140-depleted larval lysate synthesis excess amounts of phenoloxidase when nuclear function (ribosome biosynthesis) is impaired remains a perplexing question. Since the Nopp140-depleted larval lysate developmentally delayed, we speculate that phenoloxidase A3 may simply amass in abundance over time despite a slow rate of protein synthesis as compared with wild type larvae. This could explain the excess accumulation of phenoloxidase in whole larval lysates as shown by Coomassie-stained gels (Fig. 6A). The activation of JNK in the Nopp140-depleted larval may then release the phenoloxidase to the hemolymph as shown in the hemolymph enrichments (Fig. 6B and C). Many of the melanotic masses we saw seemed to reside within non-hematopoietic tissues such as the midgut, trachea and salivary glands. One possibility suggested by Bidla et al. is that apoptosis

Figure 6. Phenoloxidase A3 accumulated in Nopp140-depleted larvae. (A) Whole larval lysates from parental control larvae (Act5C-GAL4/CyO, lane 1), homozygous UAS-C4.2/CyO (lane 2) and Nopp140-depleted larvae (UAS-C4.4/CyO/CyO, lane 4). UAS-C4 is the original stock containing UAS-C4.2 on the 3rd chromosome and UAS-C3 on the 2nd chromosome. The prominent 70 kDa protein in the lysate of Nopp140-depleted larvae (arrow) was identified by LC-MS/MS as phenoloxidase A3. (B) Hemolymph proteins were resolved on a native polyacrylamide gel and stained with Coomassie blue. Parental control larvae were homozygous UAS-C4.2/CyO (lane 1) and Act5C-GAL4/CyO (lane 2). Nopp140-RNAi expressing UAS-C4.2/Act5C-GAL4 larvae (lane 3) with only a few melanotic masses were compared with similar UAS-C4.2/Act5C-GAL4 larvae (lane 4) that had excess melanotic masses. (C) A native gel similar to that shown in panel (B) but stained for phenoloxidase activity using tyrosine as substrate. Arrows in (B and C) mark the position of the most prominent phenoloxidase activity. (D) Melanotic masses were found mostly in the midgut of Act5C > UAS-C4.2 larvae. These masses also occurred at other sites within Act5C > UAS-C4.2 larvae. This large mass accumulated at the tips of the dorsal arms of the pyriform scutellum.

©2013 Landes Bioscience. Do not distribute
upstream of Drosophila JNK are at least two JNKKs (Hemipterous and MK4), and several JNKs. Puckered is the Drosophila protein phosphatase that is also induced by JNK to deactivate JNK in a negative feedback loop. How Hemipterous and Puckered are regulated upon nuclear stress in Drosophila remains unknown. Our future efforts in linking nuclear stress to JNK activation will examine the expression levels of hkr, hemipterous, and puckered.

Finally, JNK signaling has been implicated in direct nuclear function. For example, mammalian JNK2 phosphorylates the RNA polymerase I transcription factor, TIF-IA, to downregulate rRNA synthesis.42 JNK activity was also required for the release of B23 and p9676 from mammalian nucleoli upon UV radiation (DNA damage).48 Conversely, Midan et al.49 showed that upon genetic or chemical inhibition of JNK signaling, the mammalian nucleolar RNA helicase, DDX21, is partially redistributed to the nucleoplasm and that rRNA processing is inhibited. Their observation suggests that stresses-induced JNK-activation, JNK signaling maintains homeostasis for the nucleoli in non-stressed cells. Thus the effects of activated JNK on the localization of Drosophila nucleolar components during normal cell growth and upon stress remain largely unknown, but subject for future investigation.

Materials and Methods

Fly lines. Fly lines expressing short hairpin RNA specific for the common 5’ end of mRNAs that encode the two Nopp140 isoforms were P[w[+mC]= UAS-Nopp140 dsRNA]36 and P[w[+mC]= UAS-Nopp140 dsRNA]2,37 as described previously. Here we refer to them simply as UAS-C3 and UAS-C4.2, UAS-C3 resides on the third chromosome. The original UAS-C4 line contained two RNA-expressing transgenes, one on the second chromosome and the other on the third chromosome. The transgene on the third chromosome is UAS-C3. We isolated the second chromosome from UAS-C4 and refer to the resulting line as UAS-C4.2. Both UAS-C3 and UAS-C4.2 lines are homzygous viable and fertile. The P[UAS-mCherry-Agkita] line, D169 (third chromosome), was a kind gift from Thomas Neufeld.38 Four additional lines came from the Bloomington Drosophila Stock Center; stock 6815 is a deletion for the p53 gene (http://www.fruitfly.org/about/methods/inverse.pcr.html). Primers for verifying the p53 gene deletion (p53233-28) were described by K. Golic in a reference report to FlyBase (http://flybase.org/reports/FBf0151688.html). The forward primer is P53-C1: 5'-ACC ACA TAA TGC TTC GCA TTG AAC AAA-3', and the reverse primer is P53-C2: 5'-TCG ATA AAC ATT GCC TAT CAC TAC-3'. Genomic DNA was isolated from parental control larvae (lanes 1–4) and Act5C > Nopp140-RNAi larvae (lanes 5–8) was probed with rabbit anti-p53.34 Lane 1: homozygous Act5C; Lane 2: Act5C-GAL4/ CyO; Lane 3: Act5C-GAL4/+; Lane 4: Act5C-GAL4/+; lanes 5 and 7: UAS-C2-UAS-C4-GAL4; Act5C-GAL4; Lanes 6 and 8: UAS-C4 UAS-C3 GAL4; UAS-C4 is the original transgenic stock that contains UAS-C4 on the 2nd chromosome and UAS-C2 on the third chromosome. (A) A companion blot with the same lysates was probed with rabbit monolocal antibody B1E11 (Cell Signaling #4668) directed against phospho-JNK at 43 kDa. (B) Equal amounts of the same lysates as used in panels A and B were resolved on a Coomassie-stained SDS-gel. Phosphoelastase A3 (PO) is at 70 kDa.

Figure 7. Pro-apoptotic Hid and activated JNK were both upregulated in Nopp140-depleted larvae. (A) An immune-blot containing lysates from parental control larvae (lanes 1–4) and Act5C > Nopp140-RNAi larvae (lanes 5–8) was probed with rabbit anti-Hid.32 Hid at ~30 kDa was substantially increased in the Nopp140-depleted larvae as compared with parental larvae. Lane 1: homozygous UAS-C2; Lane 2; Act5C-GAL4/ CyO; Lane 3: Act5C-GAL4/+; Lane 4; Act5C-GAL4/ CyO; lanes 5 and 7: UAS-C2-UAS-C4-GAL4; Act5C-GAL4; Lanes 6 and 8: UAS-C4-UAS-C3 GAL4; UAS-C4 is the original transgenic stock that contains UAS-C4 on the 2nd chromosome and UAS-C2 on the third chromosome. (B) A companion blot with the same lysates was probed with rabbit monolocal antibody B1E11 (Cell Signaling #4668) directed against phospho-JNK at 43 kDa. (C) Equal amounts of the same lysates as used in panels A and B were resolved on a Coomassie-stained SDS-gel. Phosphoelastase A3 (PO) is at 70 kDa.

Genomic PCR to verify p53Δ. Genomic DNA from adult flies was prepared according to E. Jay Rehm of the Berkeley Drosophila Genome Project (http://www.fruitfly.org/about/methods/inverse.pcr.html). Primers for verifying the p53Δ gene deletion (p53233-28) were described by K. Golic in a reference report to FlyBase (http://flybase.org/reports/FBf0151688.html). The forward primer is P53-C1: 5'-AGC TAA TGT GAC TTC GCA TTG AAC AAA-3', and the reverse primer is P53-C2: 5'-TCG ATA AAC ATT GCC TAT CAC TAC-3'. Genomic PCR to verify p53Δ throughout this report. GAL4 driver lines included P[w[+mC]= Act5C-GAL4/25FO1/G0 (stock 4444, referred to simply as Act5C-GAL4), the homozygous daughters-only-GAL4 line on the third chromosome (stock 8061, referred to as da-GAL4) and the wing disc-specific P[w[+mC]= GAL4/49 on the X chromosome (stock 8760, referred to here as A9-GAL4). A9-GAL4 expresses GAL4 strongly in larval wing and haltere discs.49 We previously used the wing disc line (Bloomington stock 3605) to construct our RNA-expressing transgenic lines, and we use it here as our "wild type" control. All stocks were reared at 22–24°C on standard Drosophila medium. Genetic crosses using GAL4 drivers were kept at 27°C.

www.landesbioscience.com

©2013 Landes Bioscience. Do not distribute
at 1 μg/ml in the final wash buffer and viewed with a Zeiss Axioplan equipped with a SPO T FT/53E CCE1 camera (Diagnostic Instruments).

Transmission electron microscopy of wild type and Nopp140- RNAs-expressing larval tissues was performed as described.32 Diploid tissues (imaginal discs and brains) and polyplody tissues (mid-gut and Malpighian tubules) were hand isolated, fixed and flat-embedded separately so we could section and analyze one known tissue at a time. From Nopp140-RNAs-expressing larval, we captured 16 images of polyploid cells and 20 images of diploid cells. About half as many images were captured from the same wild type tissues. We used a JEOL 1010CX operating at 15 kV and a magnification of 16,000×. Negatives (8 × 10 cm) were scanned at 1200 dpi. Resulting positive images were prepared using Photoshop.

Immunoblot. Third instar larval lysates were prepared by using small plastic pestles designed for Eppendorf tubes. Larvae were homogenized in 267 μl of Laemmli sample buffer to which we added 3 μl of 0.1 M PMSF dissolved in isopropanol, 15 μl of a protease inhibitor cocktail (P-8340, Sigma-Aldrich Corp.) and 15 μl of 2-mercaptoethanol. The homogenate was then sonicated by 5 bursts using a Branson Digital Sonifier; each burst was for 15 μs at 35% maximum power. The resulting lysates were centrifuged for 30 min. The supernatant was boiled for 5 min and re- centrifuged for an additional 30 min. Lysate proteins were resolved by standard SDS-PAGE and blotted to nitrocellulose using a BioRad Trans-Blot semi-dry transfer cell. Blots were blocked with 5% non-fat dry milk in TTBS. We used rabbit antibodies against ribosomal proteins RpL34 (center) and RpL23A (C-terminus) and 100% EtOH. Filters were air-dried and placed separately into 10 ml of Scintiverse (TM) BD Cocktail (Fisher Scientific). CPM was determined using a Beckman LS 6000IC scintillation counter. Ratios of CPM per microgram of proteins were determined for each sample. The entire experiment was repeated three times. We also verified the relative protein concentrations and CPM values for each sample by resolving lysate proteins on SDS-10% poly acrylamide gels. Coomassie blue staining verified the relative protein concentrations. The gels were dried and exposed to Kodak X-OMAT LS film; resulting autoradiograms verified the relative scintillation counts.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Dr Thomas Neufeld (University of Minnesota) for the mCherry-ATG8a transgenic line and Dr Hyung Don Ryoo (New York University Medical School) for the rabbit anti-Hid antibody. We thank Dr Indu Kheterpal and her staff at LSU’s Pennington Biomedical Research Center for performing the mass spectrometry. We thank Dr Yong Xiao for embedding and thin sectioning tissue samples for TEM analysis. We also thank Louisiana State University’s S-STEM Scholars Program for supporting Renford Clinda and the Louisiana Biomedical Research Network (LBRN) for the summer internship awarded to Dana Mayer. This work was supported by the National Science Foundation, award MCB0919709.
References

1. Meier UT. Comparison of the carboxy-terminal protein Nop1p with yeast homologues SRP75: Differential phosphorylation in vivo. J Biol Chem 1996; 271:15387-94. PMID:8703624.

2. Isaac C, Yang Y, Bandyopadhyay P, et al. Targeted mutagenesis by RNAi knockdown of Nop140, a nuclear RNA homologous to the transposable element Copia in Drosophila melanogaster. Mol Biol Cell 2004; 14:239-52. PMID:14711410; http://dx.doi.org/10.1093/gad/986602.

3. Fan Y, Wang MR, Bandyopadhyay P, et al. RNAi knockdown of Nop140, an RNA homologous to the transposable element Copia in Drosophila melanogaster. Mol Biol Cell 2004; 14:239-52. PMID:14711410; http://dx.doi.org/10.1093/gad/986602.

4. Fan Y, Wang MR, Bandyopadhyay P, et al. Knockdown of the RNA homologues Nop140 and SRP40. Differential expression in Drosophila melanogaster. J Biol Chem 2004; 279:44011-8. PMID:15194587; http://dx.doi.org/10.1074/jbc.M406159200.

5. Takebuchi K. Identification of the gene encoding pro-phenoloxidase A(3) in the fruitfly, Drosophila melanogaster. J Biol Chem 2005; 280:19208-15. PMID:15935255; http://dx.doi.org/10.1074/jbc.M410412200.

6. Wolfe J, Akam ME. The developmental interaction of two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila. Development 1998; 125:3977-86. PMID:9735359.

7. Neufeld TP, Pradling AC. alpha-spectrin is a stress sensor: JNK2 inactivates the transcription factor TIF-IA and down-regulates rRNA synthesis. EMBO J 2000; 19:3189-203. PMID:10739703.

8. Jorgensen GM, Fellowes DM, Jungermann K, Grzeschik KH, et al. Prevention of the neurocristopathy Treacher Collins syndrome through inhibition of p53 function. J Cell Sci 2002; 115:3205-15. PMID:12430670; http://dx.doi.org/10.1242/jcs.00483.

9. Tang H. Regulation and function of the melanization factor Nopp140. Mol Biol Cell 2000; 11:567-77; PMID:10739703.

10. 3.1.7747.

11. 25. 2.319.

12. 24. 2.319.

13. 23. 2.319.

14. 22. 2.319.

15. 21. 2.319.

16. 20. 2.319.

17. 19. 2.319.

18. 18. 2.319.

19. 17. 2.319.

20. 16. 2.319.

21. 15. 2.319.

22. 14. 2.319.

23. 13. 2.319.

24. 12. 2.319.

25. 11. 2.319.

26. 10. 2.319.

27. 9. 2.319.

28. 8. 2.319.

29. 7. 2.319.

30. 6. 2.319.

31. 5. 2.319.

32. 4. 2.319.

33. 3. 2.319.

34. 2. 2.319.

35. 1. 2.319.