Misregulation of the Nucleoporins 98 and 96 lead to defects in protein synthesis that promote hallmarks of tumorigenesis

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Summary Statement:
Compromising Nups 98 and 96 leads to defects in protein synthesis and stress signaling via JNK that triggers compensatory and apoptosis-induced proliferation.

Highlights:
- Compromising Nups 98 and 96 triggers cell death and compensatory proliferation via JNK signaling that becomes tumorigenic when apoptosis is blocked.
- Reducing Nup 98 and 96 function limits nuclear export of the ribosome L1 stalk component RpL10A, leading to defects in protein synthesis which cause stress signaling via JNK.
- Reduced protein synthesis coupled with increased JNK signaling, paradoxically leads to more proliferation with a gene expression signature that resembles a chronic wounding response.
- Overexpression of Nup98, which occurs in oncogenic fusions, also leads to defects in protein synthesis and JNK activation.

Abstract

The Nucleoporin 98KD (Nup98) is a promiscuous translocation partner in hematological malignancies. Most disease models of Nup98 translocations involve ectopic expression of the fusion protein under study, leaving the endogenous Nup98 loci unperturbed. Overlooked in these approaches is the loss of one copy of normal Nup98 in addition to the loss of Nup96 – a second Nucleoporin encoded within the same mRNA and reading frame as Nup98, in translocations. Nup98 and 96 are also mutated in a number of other cancers, suggesting their disruption is not
limited to blood cancers. We found that reducing Nup98-96 function in *Drosophila melanogaster* (where the Nup98-96 shared mRNA and reading frame is conserved) de-regulates the cell cycle. We find evidence of over-proliferation in tissues with reduced Nup98-96, counteracted by elevated apoptosis and aberrant signaling associated with chronic wounding. Reducing Nup98-96 function leads to defects in protein synthesis that trigger JNK signaling and contributes to hallmarks of tumorigenesis when apoptosis is inhibited. We suggest partial loss of Nup98-96 function in translocations could de-regulate protein synthesis leading to signaling that cooperates with other mutations to promote tumorigenesis.

**Introduction**

Communication between the nucleus and cytoplasm occurs through nuclear pore complexes (NPCs), which are composed of highly conserved proteins termed Nucleoporins (Nups). Mutations in several Nups are associated with cancer, including loss-of-function mutations and translocations (Simon and Rout, 2014). Of the Nups associated with translocations, Nup98 is the most promiscuous (Lam and Aplan, 2001; Simon and Rout, 2014).

Nup98 function has been difficult to examine because the gene locus for Nup98 encodes for two essential Nucleoporins, Nup98 and Nup96, which derive from an autocatalytic cleavage of a larger Nup98-96 polypeptide with Nup98 located at the amino terminus (Fontoura et al., 1999; Rosenblum and Blobel, 1999). However, a shorter Nup98 only transcript is also produced by the locus via alternative splicing (Fontoura et al., 1999). Nup98 is a peripheral Nup, found both in nuclear pores and in the nucleoplasm (Griffis et al., 2002). It contains FG (Phenylalanine-Glycine) and GLFG repeats in its N-terminal region that allow Nup98 to interact with different nuclear transport receptors (Bachi et al., 2000; Moroianu et al., 1995) during nucleocytoplasmic shuttling, and it has a role in regulating gene transcription (Capelson et al., 2010; Kalverda et al., 2010). In contrast, Nup96 is a core scaffold protein; it is stably localized at NPC and is part of the core Nup107-160 complex (Walther et al., 2003).

All Nup98 chromosomal translocations that have been observed have a breakpoint in the 3’ end of the Nup98 portion, disrupting the Nup98 coding region located upstream of Nup96 (Xu and Powers, 2009). Thus, Nup98 translocations result in fusions of the N-terminal region of Nup98 with the C-terminal region of a partner gene, which varies (Simon and Rout, 2014). This almost certainly disrupts the expression of Nup96 as well, which requires Nup98-dependent autocatalytic processing from the Nup98-96 precursor protein to be properly localized and functional (Fontoura et al., 1999; Rosenblum and Blobel, 1999).

While most of the attention on Nup98 translocations in cancer has focused on overexpressing the fusion partners, there is increasing evidence that the disruption of endogenous Nup98 and/or Nup96 may contribute to enhanced proliferation that could cooperate with other oncogenic mutations. Mice carrying a stop codon knocked into the 3’ end of the Nup98 portion of the shared Nup98-96 transcript, have been used to examine loss of Nup96 function in the presence of intact Nup98 protein (Faria et al., 2006). Loss of one copy of Nup96 in the mouse leads to mildly enhanced proliferation of T-cells, supporting a potential role for Nup96 as a haplo-insufficient tumor suppressor (Chakraborty et al., 2008), but Nup96+/− mice do not appear to exhibit cell cycle deregulation in other tissues, nor develop cancer (Faria et al., 2006). Conversely, an engineered allele generating loss of one copy of Nup98 in the mouse, but with Nup96 protein expression remaining intact, cooperates with loss of the nuclear export cofactor
Rae1 to increase aneuploidy (Jeganathan et al., 2005), but Nup98+- mice have not been reported to develop cancer, nor to exhibit cell cycle de-regulation on their own (Wu et al., 2001). Studies of Nup98 and Nup96 homozygous mutants have been severely limited by the very early embryonic lethality caused by loss of each Nup (Faria et al., 2006; Wu et al., 2001), and compound mutants have not been reported. Using a small interfering RNA (siRNA) knockdown approach to selectively target Nup98 in human cells, revealed a role for Nup98 in p53-dependent induction of the Cdk inhibitor p21 in response to DNA damage, consistent with a tumor-suppressor function for Nup98 (Singer et al., 2012).

Work in Drosophila revealed an unexpected off-pore role for Nup98 in modulating the expression of several cell cycle genes (Capelson et al., 2010; Kalverda et al., 2010). Loss of Nup98-96 function in Drosophila is lethal and pleiotropic. Flies homozygous for an allele with a stop codon predicted to generate a truncated Nup98 and eliminate Nup96, die prior to metamorphosis (Parrott et al., 2011; Presgraves et al., 2003). A Nup98-96 allele disrupted by a transposon insertion in the fourth exon of Nup98, predicted to disrupt splicing, exhibits germline-specific defects in stem cell proliferation and differentiation (Parrott et al., 2011). Low-level constitutive depletion of Nup98-96 by RNAi in adult flies impacts expression of anti-viral genes (Panda et al., 2014), while acute inhibition of Nup98-96 in imaginal discs leads to misregulation of Hox gene expression (Pascual-Garcia et al., 2014). Consistent with pleiotropic effects, the knockdown of Nup98-96 by RNAi has emerged in a number of screens in Drosophila, revealing roles in nuclear translocation of specific proteins (Dopie et al., 2015; Kristo et al., 2017), and blood progenitor proliferation and differentiation (Mondal et al., 2014).

Human NUP98-96 is located near a known imprinted tumor-suppressor region in the genome (Joyce and Schofield, 1998), which could be significant as loss of heterozygosity via mutation or epigenetic modifications for the remaining Nup98-96 locus may occur in cancers exhibiting translocations. We are not aware of any information reported to date about the expression levels from the non-translocated NUP98-96 gene in these diseases. We simultaneously inhibited Nup98 and 96 in Drosophila using an in vivo RNAi knockdown approach and observed cell cycle de-regulation and cooperation with oncogenic mutations, consistent with a tumor suppressor function for Nup98 and/or 96. Transgenes encoding Nup98 or Nup96 individually do not rescue this phenotype, while expression of a transgene encoding both does – suggesting Nup98 and Nup96 play non-overlapping and potentially synergistic roles in cell cycle regulation.

Here we show that reducing Nup98-96 function via an RNAi approach in Drosophila melanogaster (where the Nup98-96 shared mRNA and reading frame gene structure is conserved) de-regulates the cell cycle. We find evidence of overproliferation in Nup98-96 deficient tissues, counteracted by elevated apoptosis and aberrant JNK signaling associated with wound healing. When the knockdown of Nup98-96 is combined with inhibition of apoptosis, we see synergism leading to overgrowth consistent with a tumor-suppressor function for endogenous Nup98 and/or 96. We suggest that the loss of normal Nup98 and Nup96 function may de-regulate the cell cycle to cooperate with other mutations in cancer.
Results

Loss of Nup98-96 disrupts G1 arrests and causes cell cycle de-regulation

We previously described an RNAi screen to identify genes that promote proper cell cycle exit in the Drosophila eye (Flegel et al., 2016; Sun and Buttitta, 2015). Our initial screen used UAS-RNAi constructs from the Harvard TRiP RNAi collection, driven by the Glass Multimer Repeats (GMR) promoter-Gal4 with an E2F-responsive PCNA-white reporter transgene, which provides adult eye color as a readout of E2F and cell cycle activity (Bandura et al., 2013). This screen successfully identified genes that delay proper cell cycle exit by promoting a delay or bypass of G1 arrest, which directly or indirectly impacts E2F activity (Flegel et al., 2016; Sun and Buttitta, 2015). In this screen, we identified an RNAi line targeting the bi-cistronic Nup98-96 transcript as a potential novel regulator of cell cycle exit in the Drosophila eye.

Cell cycle exit in the eye is normally completed by 24 hours after puparium formation (APF). To confirm whether knockdown of Nup98-96 delayed cell cycle exit in the pupa eye, we performed S-phase labeling via 5-ethynyl-2'-deoxyuridine (EdU) incorporation and examined an E2F transcriptional activity reporter PCNA-GFP in pupal eyes several hours after normal cell cycle exit. We confirmed that knockdown of Nup98-96 delayed proper cell cycle exit in the pupa eye to between 28-36h APF (Supp Fig. 1A). We also confirmed that the RNAi line identified in the screen knocked down endogenous Nup98-96 protein tagged with GFP and that re-expression of both exogenous Nup98 and Nup96 were required to rescue phenotypes due to Nup98-96 bi-cistronic transcript knockdown (Supp Fig. 1 B,C). Neither exogenous Nup98 or Nup96 alone were sufficient to rescue Nup98-96 RNAi phenotypes, suggesting both Nups contribute to the cell cycle exit defect.

We next examined whether knockdown of Nup98-96 in the posterior wing using the driver engrailed-Gal4 (en-Gal4) with a temperature sensitive Gal80 (en-\textsuperscript{TS}) could delay cell cycle exit in the pupal wing, which also completes the final cell cycle by 24h APF. We used Gal80\textsuperscript{TS} to limit expression of the RNAi to pupal stages to avoid developmental delays and lethality and an RNAi to the eye pigment gene white (white\textsuperscript{RNAi}), which has no effect on cell cycle exit served as a negative control (Flegel et al., 2016). Labeling S-phases with EdU incorporation from 26-28h APF and mitoses using anti-phosphorylated Ser10-Histone H3 (PH3) antibody revealed that knockdown of Nup98-96 delayed cell cycle exit in the wing until 28-30h APF (Fig 1A-D).

We have shown that delays in cell cycle exit accompanied with high E2F activity can result from slowing the final cell cycle, or by causing additional cell cycles (Flegel et al., 2016; Sun and Buttitta, 2015). To determine which is the case with knockdown of Nup98-96, we expressed Nup98-96 RNAi in the eye, using a sensitized background with the GMR-Gal4 driver driving the G1-S Cyclin, Cyclin E (CycE) and the apoptosis inhibitor P35 (Hay et al., 1994). This sensitized background causes enlarged eyes and 1-3 extra cell cycles in the pupa eye prior to a robust cell cycle exit (Sun and Buttitta, 2015). The enlarged eyes of this sensitized background are visibly suppressed by factors that delay the cell cycle and enhanced by manipulations that cause extra cell cycles (Sun and Buttitta, 2015). Knockdown of Nup98-96 effectively enhanced the eye overgrowth of this sensitized background and resulted in extra cone cells and extra interommatidial cells in the pupal eye, confirming that the delay of cell cycle exit was caused by additional cell cycles (Fig. 1 E-H).

We next examined proliferating larval wing discs, to determine whether the effects of Nup98-96 knockdown were specific to the pupa or also impacted earlier cell cycles. We used en-Gal4/Gal80\textsuperscript{TS} to express Nup98-96 RNAi in the posterior wing disc, labeled with GFP, for 72h...
prior to dissection and detected mitoses with PH3 or performed 5-10 min of EdU labeling for S-phase immediately prior to fixation. We observed an increase in mitoses when Nup98-96 was knocked down, accompanied by an increase in S-phase labeling (Fig. 1 I-L, Supp. Fig. 1F). Consistent with knockdown of Nup98-96 leading to a bypass of a G1 cell cycle arrest, we also observed abundant S-phases in the posterior zone of non-proliferating cells (ZNC yellow arrowhead, Fig. 1 K-L), which are normally quiescent at this stage (Johnston and Edgar, 1998). Similar effects on larval wing disc proliferation were also observed using two independent Nup98-96 RNAi lines from the VDRC collection (Supp. Fig. 1D).

Increased EdU and PH3 labeling at fixed timepoints can be due to increased proliferation or increased time spent in S and M phases respectively. To examine whether S to M progression is altered when Nup98-96 is knocked down, we performed an EdU pulse/chase assay combined with PH3 labeling in L3 larval wing discs. We fed larvae with food containing EdU for 1 hour followed by a chase without EdU for 7h. At the end of the chase, we fixed larval wing discs and stained for PH3 and scored the number of mitotic cells double positive for EdU and PH3 in the posterior vs. anterior wing pouch for white RNAi vs. Nup98-96 RNAi discs. The posterior to anterior ratio of double-positive cells that transition from S to M-phase in control white RNAi discs is approximately 1, indicating similar cell cycle timing in the posterior and anterior wing disc of late L3 larvae (Mesquita et al., 2010). By contrast, the fraction of EdU-positive mitoses in the posterior compared to the anterior disc was increased when Nup98-96 was knocked down in the posterior, suggesting that more of these cells are progressing from S to M within 7h (Fig. 1 M-O). An increased posterior to anterior ratio could indicate either an increase in proliferation rate in the posterior disc, or a non-autonomous decrease in the anterior (Mesquita et al., 2010). Indeed, the increased ratio of EdU-positive mitoses in the Nup98-96 RNAi domain is in part due to a non-autonomous effect resulting in fewer S-M transitions in 7hr in the anterior compartment with the Nup98-96 knockdown (Supp. Fig 1G). However, when we compare the fraction of EdU-positive mitoses in Nup98-96 RNAi posterior discs to posterior white RNAi wings (an external control), we observe a ~20% average increase in EdU+ mitoses, although it is not statistically significant. Altogether, we conclude that cells with Nup98-96 knocked-down proliferate faster than their neighbors and proliferate at rates similar to or slightly faster than control cells.

Nup98-96 knockdown results in apoptosis and activation of JNK signaling
Despite the increased proliferation and disruption of G1 arrest in the larval and pupal tissues, we noted that the posterior wing expressing Nup98-96 RNAi was consistently smaller than normal suggesting an increase in cell death (Supp. Fig. 1C). Indeed, knockdown of Nup98-96 for 72h dramatically increased apoptosis in the posterior wing disc, as measured by anti-cleaved Caspase 3 and anti-Drosophila Caspase 1 (DCP1) staining (Fig. 2 A-B, Supp. Fig. 2 A-H). The increased apoptosis and reduced size in the posterior disc could be fully rescued by exogenous expression of both Nup98 and 96 in the presence of Nup98-96 RNAi (Supp. Fig. 1C , Supp. Fig 2C,D). Expression of Nup98-96 RNAi in the dorsal wing disc using apterous-Gal4,Gal80TS (apTS) for 72h also induced robust apoptosis, indicating that the effect was not specific to the posterior disc (Supp. Fig. 2E). We knocked down the initiator caspase Dronc or effector caspase Drice in attempt to rescue the apoptotic cells, but neither fully suppressed the apoptotic response to Nup98-96 knockdown (Fig. 2 C,D), nor did co-expression of a dominant negative form of p53 (not shown, Brodsky et al., 2000). We next co-expressed the baculoviral caspase inhibitor P35 with Nup98-96 RNAi, which suppressed apoptosis (Supp Fig 2 F-H) and resulted in dramatic
wing disc overgrowth phenotypes, including folding of the epithelium and occasional duplication of wings (Fig. 2 E,F). The overgrowth and duplication of wing tissues was reminiscent of a phenotype observed during wing damage and regeneration when JNK signaling is activated (Perez-Garijo et al., 2009; Schuster and Smith-Bolton, 2015; Verghese and Su, 2017; Worley et al., 2018). We therefore examined whether Nup98-96 knockdown resulted in activation of JNK signaling by staining for phospho-JNK (Fig. 2G,H) and induction of the JNK signaling transcriptional target puckered (using a puc-LacZ expression reporter, Supp. Fig. 2I). Knockdown of Nup98-96 for 72h led to high levels of compartment-autonomous JNK signaling in the wing disc.

High JNK signaling can paradoxically lead to both proliferation and cell death in Drosophila tissues (Fogarty and Bergmann, 2017). We next tested whether inhibition of JNK signaling via dominant negative form of the Drosophila JNK, Basket (Bsk\textsuperscript{DN}) could suppress the apoptotic and proliferative response to knockdown of Nup98-96. Co-expression of Bsk\textsuperscript{DN} with Nup98-96 RNAi had a complex effect on apoptosis in the wing, enhancing levels of apoptosis in some samples, while suppressing in others (Fig. 2 I-J,M). Unexpectedly, co-expression of Bsk\textsuperscript{DN} with Nup98-96 RNAi did not suppress the increased mitoses observed in posterior wings expressing Nup98-96 RNAi, and even mildly enhanced the differences in mitotic labeling between anterior and posterior compartments (Fig. 2K-L, N). Although, we noted an overall decrease in PH3 labeling across both compartments when Bsk\textsuperscript{DN} was co-expressed in the posterior wing disc (Supp. Fig 2J), suggesting blocking JNK signaling reduced compensatory proliferation both autonomously and non-autonomously. The few adult wings that could be recovered with both Nup98-96 RNAi and Bsk\textsuperscript{DN} expression exhibited a more severely reduced posterior compartment than Nup98-96 RNAi alone (Fig. 2O). This suggests activation of JNK signaling provides compensatory proliferation and may partially increase survival when Nup98-96 is knocked down, consistent with previously described roles in wing damage and regeneration (Bergantinos et al., 2010; Herrera et al., 2013).

**Nup98-96 knockdown leads to mis-patterning and gene expression resembling a wound healing and loser phenotype.**

The JNK signaling and overgrowth phenotypes caused by suppressing apoptosis during Nup98-96 knockdown, are reminiscent of a phenomenon called apoptosis-induced compensatory proliferation (AIP) (Fogarty and Bergmann, 2017), which can impact tissue patterning. As previously described for other JNK-driven Drosophila tumor models, we observed dramatic tissue folding and invasion behaviors at both the A-P and D-V compartment boundaries when Nup98-96 was inhibited in the presence of P35 expression, (Supp. Fig 3A-C) (Muzzopappa et al., 2017). Therefore, we next investigated whether wing disc patterning is disrupted by Nup98-96 knockdown as previously shown in AIP.

We first examined Wg levels in discs expressing Nup98-96 RNAi, since AIP and wing duplications have been associated with ectopic Wg (Baonza et al., 2000; Perez-Garijo et al., 2009; Verghese and Su, 2017; Worley et al., 2018). We found that knockdown of Nup98-96 resulted in ectopic Wg in the dorsal wing hinge and this effect was amplified in in the presence of P35 (Fig. 3 A-D). We also observed ectopic phosphorylation of the transcription factor Mad (Supp. Fig. 3D), consistent with the previously described effect of AIP on Dpp signaling (Perez-Garijo et al., 2009; Pinal et al., 2018).
Both Wg and Notch have been implicated in the G1 arrest in the posterior ZNC (Duman-Scheel et al., 2004; Herranz et al., 2008). We therefore next examined the expression of two targets of Notch and Wg signaling: Cut, which is expressed in G1 arrested cells at the Dorso-Ventral (D-V) boundary, and Vestigial (Vg), which is expressed in a broader domain of the pouch induced by longer-range Wg signaling (de Celis et al., 1996; Kim et al., 1996; Neumann and Cohen, 1997). We found that Cut expression at the D-V boundary was nearly eliminated when Nup98-96 was knocked down, both with and without P35 (Fig. 3 E-G). This suggests Notch signaling at the D-V boundary is compromised when Nup98-96 function is reduced. Vg, an important wing identity and growth regulator (Halder et al., 1998; Williams et al., 1991; Williams et al., 1993; Zecca and Struhl, 2010), was also dramatically reduced in the pouch upon Nup98-96 knockdown (Fig. 3H) suggesting Wg released from the D-V boundary is also compromised. Notch and Wg have been suggested to regulate the ZNC cell cycle arrest via repression of dMyc expression, but we did not observe any effects of Nup98-96 knockdown on dMyc levels in the ZNC (not shown). Interestingly, the downregulation of Vg was also observed in regenerating discs (Smith-Bolton et al., 2009), potentially due to the replacement of dying pouch cells with cells from the neighboring areas of the wing (Zecca and Struhl, 2010). Taken together, these data demonstrate that reduction of Nup98-96 function in the presence of P35 leads to AIP and wing mis-patterning and cell identity changes associated with a chronic wounding and regeneration response.

While high JNK signaling and apoptosis-induced compensatory proliferation can explain many of the phenotypes we observe with Nup98-96 knockdown, this does not reveal the proximal defect caused by loss of Nup98-96 function. To determine additional effects of Nup98-96 knockdown on gene expression in the wing, we performed comparative gene expression analysis via RNAseq to identify mRNAs increased or decreased upon Nup98-96 RNAi compared to the control white RNAi for 72h in late L3 wing discs (Supplemental Table 1). We observed the strong upregulation of many genes directly associated with JNK signaling (e.g. puc, mmp1, Ets21C) (Kulshammer et al., 2015; McEwen and Peifer, 2005; Uhlirova and Bohmann, 2006), Jak/STAT signaling (upd, upd2, Socs36E) (Amoyel et al., 2014) and developmental delays associated with wing damage and regeneration (chinmo, Ilp8) (Colombani et al., 2012; Garelli et al., 2012; Katsuyama et al., 2015; Narbonne-Reveau and Maurange, 2019). Consistent with the wing overgrowth phenotypes, several genes of the genes listed above have been shown to act in combination to promote tumorigenic overgrowth in flies (Toggweiler et al., 2016), and we see a striking overlap of about one third of the genes changed upon Nup98-96 RNAi with gene expression changes observed in a well-established invasive fly tumor model (507 out of 1774 genes, Supplemental Table 1) (Kulshammer et al., 2015).

Consistent with increased proliferation, we also observed the upregulation of several DNA damage and replication genes regulated by E2F activity (Orc1, multiple DNA Polymerases, SpnE, Rnr-L, RfC4) (Buttitta et al., 2010; Dimova et al., 2003). However, we did not observe strong upregulation of other G1-S promoting genes such as dMyc (1.52-fold change), bantam, cycE or cycD. When we compared gene expression signatures globally, we found a strong overlap (2.63-fold more genes than expected by chance) with a wounding and regeneration gene expression signature (Khan et al., 2017, Supplemental Table 2). We also noted upregulation of several genes associated with proteotoxic and oxidative stress (Xrp1, multiple Glutathione S transferases, Aox1, and specific DNA damage response genes) (Baumgartner et al., 2021). We found the strongest overlap of the Nup98-96 knockdown signature with a cell competition “loser” gene expression signature (5.67-fold more genes than expected by chance,
316/443 genes, Supplemental Table 3), which is also known to activate chronic JNK signaling (Kucinski et al., 2017).

**Nup98-96 knockdown leads to defects in proteins synthesis**

The strong overlap of the gene expression changes in Nup98-96 knockdown with the cell competition “loser” signature suggested to us that a proximal effect of Nup98 loss could be on ribosome biogenesis. We further examined a gene expression signature associated with Xrp1, an AT-Hook, bZip transcription factor which mediates signaling downstream of ribosomal protein mutations and proteotoxic stress (Langton et al., 2021; Lee et al., 2018). We found a striking proportion of Xrp1 targets (115 out of 159 overlapping in our dataset, Supplemental Table 4) were upregulated when Nup98-96 was knocked down (Ji et al., 2019). Consistent with a defect in ribosome function, we observed a decrease in protein synthesis when Nup98-96 was knocked down in wings, as measured by a puromycin labeling assay (Deliu et al., 2017), (Fig. 4 A,B). We did not observe downregulation of any ribosomal proteins in our RNaseq dataset, with the exception of a 2-fold decrease in RpS19b, which is a non-minute, duplicated ribosomal protein gene with tissue-specific expression (Marygold et al., 2007). Any effects on RpS19b levels are likely buffered by its paralog RpS19a, which exhibits much stronger expression in larval wings and was unchanged by Nup98-96 knockdown (Brown et al., 2014).

Nups play a key role in the nuclear export of ribosomal subunits in cooperation with the exportin chromosomal region maintenance 1 (CRM1; also known as exportin-1 or XPO1), which binds to nuclear export sequences (NESs) to facilitate export of cargo proteins (Gleizes et al., 2001; Johnson et al., 2002; Moy and Silver, 2002; Oeffinger et al., 2004). We wondered if the proximal defect in Nup98-96 knockdown tissues might be defects in nuclear export of ribosomal complexes. First, we examined whether our partial knockdown of Nup98-96 function by RNAi was sufficient to disrupt nucleo-cytoplasmic localization, since previous work had suggested knockdown of Nup98-96 transcripts in Drosophila S2 cells did not produce such defects (Sabri et al., 2007). We confirmed that by 52h of knockdown with enTS in vivo, we could easily visualize defects in nuclear localization of a ubiquitously expressed RFP with a nuclear localization signal (NLS) and by 72h of knockdown, nuclear localization of NLS-RFP was dramatically reduced (Supp. Fig 4 A). We next confirmed that knockdown of an essential component of the nuclear export machinery for ribosome subunits, Nmd3 (Ma et al., 2017) also effectively reduced protein synthesis (Fig. 4 C). As a positive control we also knocked down CG4364, the fly homolog of the pre-rRNA processing component Pescadillo (Lapik et al., 2004) (Fig. 4 D-E). Inhibition of ribosome export machinery and pre-rRNA processing were both sufficient to induce strong phosphorylation of JNK (Fig. 4 F-G) in the wing disc.

Ribosome large and small complexes are exported from the nucleus separately as assembled pre-ribosomal particles and must associate with cytoplasmic maturation factors to exchange specific components to form mature functional ribosomes (Lo et al., 2010). We screened through collections of endogenously tagged Rp subunits and found RpL10Ab, but not other Rp subunits (RpS20 and RpL5) were mis-localized when Nup98-96 was knocked down (Fig. 4 H-K). Interestingly, the defect in RpL10Ab localization was nuclear retention, the opposite of the effect of Nup98-96 knockdown on NLS-RFP. RpL10Ab (also called L10a or uL1) is required to associate with Nmd3 for efficient pre-60S nuclear export (Musalaonkar et al., 2019). Normally, RpL10Ab is translated in cytoplasm, localized to the nucleolus for assembly into the pre-60S complex and then exported bound to the Nmd3 adaptor. The nuclear retention of RpL10Ab upon Nup98-96 knockdown was initially puzzling as the other RpL
subunits examined did not exhibit similar localization defects. However, recent work has revealed that in mammals RpL10A is associated with a subset of specialized ribosomes and is not found in all 60S complexes (Shi et al., 2017). We suggest that knockdown of Nup98-96 partially compromises protein synthesis by inhibiting proper cytoplasmic translocation of a subset of pre-60S subunits that are RpL10A-associated. Importantly, RpL10Ab is not a Minute gene (Marygold et al., 2007), possibly because it is a sub-stoichiometric ribosome component. Consistent with this, we do not recover significant overlap with the proteasomal stress portion of the “Loser” gene expression signature when Nup98-96 is compromised (Baumgartner et al., 2021), again suggesting protein synthesis is only partially reduced when Nup98-96 function is compromised.

**Nup98-96 knockdown in mammalian cells leads to defects in protein synthesis and JNK activation**

As described in the introduction, there is abundant evidence that loss of Nup98-96 function might contribute to tumorigenesis. We wondered whether inhibition of Nup98-96 in mammalian cells would also impact protein synthesis and JNK signaling as we observe in Drosophila. Of note, a screen for factors involved in ribosome biogenesis in HeLa cells identified several Nups containing FG repeats, including Nup98 as hits involved in pre-60S export, suggesting Nup98 effects on protein synthesis will be broadly conserved (Wild et al., 2010). We used small-interfering RNA (siRNA) to Nup98-96 in MCF7 breast cancer cells and PC3 prostate cancer cells for 72 hours and compared effects on Nup98 protein levels, protein synthesis and pJNK to a control scrambled siRNA (ctrl siRNA). We found that siRNA to Nup98-96 was sufficient to reduce protein synthesis and increase phosphorylation of JNK in both cell types (Fig. 5 A-H, Supp. Fig 5).

**Overexpression of Nup98 leads to defects in protein synthesis and JNK activation**

Most of the attention on Nup98 translocations in cancer has focused on overexpressing Nup98 fusion partners. However, when overexpressed, Nup98 has been shown to behave as a dominant negative and disrupt the nuclear envelope and nuclear transport (Fahrenkrog et al., 2016; Mendes et al., 2020), possibly by forming phase-separated aggregates outside of the nuclear pore (Ahn et al., 2015). We noted that Nup98 overexpression in the posterior wing disc reduced tissue size, and in severe cases disrupted patterning (Fig. 6 A-F). We therefore examined whether Nup98 overexpression in the Drosophila wing disc mimicked aspects of Nup98-96 inhibition, as described for other Drosophila tissues (Pascual-Garcia et al., 2014). Overexpression of a strong UAS-Nup98 cDNA construct (2F) reduced nuclear localization of an NLS-tagged RFP, resulting in increased cytoplasmic accumulation and a reduced nuclear:cytoplasmic ratio (Fig. 6 G-K). Overexpression of a UAS-Nup98 cDNA construct was also sufficient to increase cell death and activate JNK signaling in the posterior wing disc (Fig. 6 I-J), and overexpression of both UAS-Nup98 and 96 or UAS-Nup98 alone (2F) reduced protein synthesis levels (Fig. 6 L-M). We suggest that Nup98-96 acts as a “goldilocks” gene (Braune and Lendahl, 2016), where too much or too little activity leads to chronic stress signaling and increased cellular turnover, potential hallmarks of tumorigenesis. This complication might explain why this locus is particularly prone to mis-regulation by translocations in cancer, which would reduce Nup98-96 normal functions and simultaneously provide additional Nup98-containing fusion proteins.
Discussion

Partial Nup98-96 loss of function leads to paradoxical increases in cell cycling and cell death accompanied by reduced protein synthesis

Protein synthesis and the cell cycle are usually coupled by pathways such as insulin and TOR signaling as well as growth and cell cycle checkpoints, which promote or limit cell cycle progression and protein synthesis coordinately (Grewal, 2009; Lockhead et al., 2020; Romero-Pozuelo et al., 2017; Romero-Pozuelo et al., 2020). Here, we describe a seemingly paradoxical situation where protein synthesis and cell cycle are effectively uncoupled. When Nups 98 and 96 are partially compromised, cells with reduced protein synthesis cycle more and even bypass developmentally induced G1 arrests. This is accompanied by high levels of chronic JNK signaling and induction of apoptosis, along with expression of genes involved in tissue regeneration and compensatory proliferation. When apoptosis is blocked using the caspase inhibitor P35, tissue overgrowth and mis-patterning results, reminiscent of tumorigenesis. We propose that mutations or gene expression changes that reduce Nup98 and Nup96 function, in the presence of apoptosis suppression, can contribute to tumorigenesis. This may help explain contexts of Nup98 and/or Nup96 loss that could pre-dispose for cancer (Franks and Hetzer, 2013; Simon and Rout, 2014; Singer et al., 2012).

The phenotype we describe here for Nup98-96 inhibition is strikingly similar to that recently described for a ribosomal protein mutant, when cell death is blocked (Akai et al., 2021). When we examined the gene expression signature in response to reduced Nup98-96, we observed a strong overlap with conditions of reduced protein synthesis caused by stoichiometric imbalances in ribosomal proteins (Kucinski et al., 2017; Lee et al., 2018). We suggest this effect of Nup98-96 inhibition is due to defects in nucleo-cytoplasmic transport of RpL10A, although we cannot rule out that localization of other ribosomal proteins may also be affected. Because the defect is in RpL10A localization, rather than levels, we were unable to rescue the Nup98-96 knockdown phenotypes with RpL10A overexpression. On the contrary, we observed several stress signaling phenotypes when we overexpressed RpL10A itself even in a wild-type background, suggesting RpL10A levels must also be carefully controlled (Chaichanit et al., 2018; Wonglapasuwnan et al., 2011). This may be of broader consequence to the Drosophila research community since Gal4/UAS-driven overexpression of this ribosomal protein is used for translatome profiling through translating ribosome affinity purification (Thomas et al., 2012). Importantly, localization of 40S and 60S subunits are not globally disrupted in our Nup98-96 knockdown conditions and protein synthesis is only partially reduced. We suggest that this is because RpL10A is a sub-stoichiometric component of ribosomes and that only the subset of ribosomes containing RpL10A are affected. In mammals RpL10A-containing ribosomes have been shown to translate genes required for cell survival and are depleted of those required for cell death (Shi et al., 2017). Whether this is the case for Drosophila RpL10A-containing ribosomes remains to be determined, although increasing RpL10A expression in Drosophila has been shown to affect E-cadherin and InR levels, suggesting components of these pathways could be regulated by RpL10A levels (Chaichanit et al., 2018).

The effects of reducing Nup98-96 expression are likely to be pleiotropic, and we cannot rule out the possibility that Nup98 and 96 mis-regulation may also lead to more direct effects on the cell cycle, independent of JNK signaling and reduced protein synthesis. Indeed, when JNK signaling is blocked by a dominant negative, overall compensatory proliferation is significantly reduced, but Nup98-96 reduced tissue still exhibits a slightly higher mitotic index than tissue
with normal Nup98-96 levels. This could be in part the result of a known Nup98 interaction with the APC/C which leads to aneuploidy when Nup98 levels are reduced (Jeganathan et al., 2006; Jeganathan et al., 2005). This interaction with the APC/C may also explain the disruption of terminal cell cycle arrest caused by reduced Nup98-96, as high APC/C activity promotes proper timing of the final cell cycle (Buttitta et al., 2010; Reber et al., 2006; Ruggiero et al., 2012; Tanaka-Matakatsu et al., 2007). We tested for aneuploidy using flow cytometry on wing discs and did not observe obvious accumulation of aneuploidy when Nup98-96 is knocked down, either with or without apoptosis inhibition. Alternatively, effects on nuclear export of cell cycle factors or their mRNAs may also contribute to the cell cycle phenotypes (Chakraborty et al., 2008), although we did not find obvious changes in protein levels or dynamics of Cyclins A or B. We also examined whether mis-regulation of transcriptional targets of Nup98 regulated through off-pore roles may explain the phenotypes we observe, but we did not find significant overlap of genes altered in our Nup98-96 knockdown with Nup98-bound targets determined by ChIP-seq in larval brains (Pascual-Garcia et al., 2017) or Nup98 regulated genes identified by RNAseq in S2 cells (Kalverda et al., 2010). We found a mild enrichment (1.43-fold over that expected by chance) in the overlap of genes altered in our Nup98-96 knockdown with Nup98-ChIP seq targets in S2 cells. (Pascual-Garcia et al., 2017, Supplemental Table 5). Overall, the previously described wounding/regeneration and “loser” gene expression programs explain nearly half (49.7%) of the gene expression changes we observe in wing discs when Nup98-96 is reduced (Fig. 3) suggesting these may be the main drivers of the phenotypes we observe.

**Potential for AIP in Nup98 cancers**

Blocking apoptosis in cells with inhibited Nup98-96 leads to phenotypes consistent with sustained apoptosis-induced proliferation (AIP), which is thought to contribute to tumorigenesis in epithelia (Fogarty and Bergmann, 2017). Epithelial tumors exhibit wounding phenotypes, chronic inflammation and cell death (Dvorak, 1986; Karin and Clevers, 2016). Chronic AIP leads to sustained proliferation and results in abnormal, hyperplastic overgrowth (Perez-Garijo et al., 2009; Pinal et al., 2018). AIP therefore could contribute to overproliferation in epithelial cancers with disrupted Nup98-96 expression (Perez-Garijo, 2018). AIP has been suggested to occur in colorectal cancer and melanoma (Bordonaro et al., 2014; Donato et al., 2014), both of which have been suggested to exhibit Nucleoporin mis-regulation (Roy and Narayan, 2019). How this might relate to aberrant signaling in hematological malignancies related to Nup98 mis-expression is unclear. It is possible effects of Nup98 mis-regulation impact different tissue types through similar pathways, that impinge on distinct downstream target genes in different tissues. For example, expression of a NUP98-HOXA9 fusion in a Drosophila model with a normal Nup98-96 locus leads to hyperplastic over-proliferation in hematopoietic tissues but minimal effects in epithelial tissues (Baril et al., 2017), while loss of Nup98-96 in larval hematopoietic tissues leads to a loss of progenitors, a phenotype also observed upon inhibition of the ribosomal protein RpS8 (Mondal et al., 2014). NUP98 mutations in leukemias are associated with mutations affecting apoptosis such as BCR-ABL, NRAS, or KRAS and ICSBP (Gabriele et al., 1999; Gough et al., 2011; Gurevich et al., 2006; Hu et al., 2016; Slape et al., 2008). Mouse models with Nup98 protein fusions exhibit increased apoptosis (Choi et al., 2008; Lin et al., 2005), and a zebrafish model of NUP98-HOXA9-driven leukemia upregulates Bcl2 to suppress apoptosis (Forrester et al., 2011). In a mouse model of Nup98-HoxD13-driven leukemia, loss of p300 leads to reduced apoptosis and enhanced activation of Jak/Stat signaling, reminiscent of signaling effects we see in AIP (Cheng et al., 2017). In our Nup98-96 RNAi experiments we
reduce Nup98 protein levels to about 50-70% of the normal level, consistent with other studies using this RNAi approach (Pascual-Garcia et al., 2014). Our data suggests that this locus can behave as a dominant negative when the Nup98 portion is overexpressed through translocations as well as a haplo-insufficient tumor suppressor in some contexts. We propose that disruption of the NUP98-96 locus in cancers with or without NUP98 translocations may contribute to tumorigenesis through aberrant JNK signaling and AIP, in the presence of additional hits that block cell death.

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The authors have no competing interests.

Methods:

Flystocks used:
UAS-Nup98-96 RNAi (TRiP BL28562, VDRC lines, KK100388 and GD6897)
UAS-white RNAi (TRiP BL35573)
UAS-Dronc RNAi (TRiP BL 32963)
UAS-Drice RNAi (TRiP BL 32403)
UAS-Nmd3 RNAi (VDRC105619 and VDRC46166)
UAS-CG4364 RNAi (VDRC27607)
GMR-Gal4, UAS-CycE(I); GMR-P35 from H. Richardson
Nup98-96-GFP (VDRC 318656 FlyFos collection)
UAS-Nup98-96 cDNA(2M), UAS-Nup98 cDNA (3M), UAS-Nup98 cDNA (myc2F), UAS-Nup96 cDNA (myc7M), UAS-Nup96 cDNA (myc8M) all from C. Schulz and M. Capelson.
en^TS is w; en-Gal4,UAS-GFP; tub-Gal80TS/TM6B from (Buttitta et al., 2007)
ap^TS is w; ap-Gal4,UAS-GFP; tub-Gal80TS/TM6B from (Buttitta et al., 2007)
en^TS RFP is w; en-Gal4,UAS-RFP_NLS; tub-Gal80TS/TM6B
UAS-BskDN (on III mutated in kinase domain) and puc^69-LacZ provided by C. Collins.
UAS-P35 on X (BL6298)
RpS20-GFP (Kyoto 109696 w^{118}; PBacRpS20^{KM0175}/TM2)
*RpL5-GFP* (Kyoto 109767 w\textsuperscript{1118}; PBac RpL5\textsuperscript{KM0174} / SM6a and Kyoto 109768 w\textsuperscript{1118}; PBac RpL5\textsuperscript{KM0163})

*RpL10Ab-YFP* (Kyoto 115462 w\textsuperscript{1118}; PBac RpL10Ab\textsuperscript{CPT1003957})

*Ubi-RFP\textsubscript{NLS}*: (derived from BL35496)

*y, w, hs-flp12* (derived from BL1929)

*w; act>stop>Gal4, UAS-GFP\textsubscript{NLS}; UAS-P35* from (Neufeld et al., 1998)

**Immunofluorescence:**

*Drosophila* samples were fixed in 4% paraformaldehyde/1XPBS solution for 20-30 min., rinsed twice in 1X PBS with 0.1% Triton-X-100 detergent (1XPBST). The samples were then incubated in appropriate dilution of antibodies in PAT (1XPBS + 0.1% Triton X-100 + 1% BSA) for 4 h at room temperature or overnight at 4°C. The samples were then washed three times for 10 mins in 1XPBST and incubated in secondary antibody conjugated with required fluorophore for 4 h in PBT-X + 2% normal goat serum (1XPBS + 0.3% Triton X-100 + 0.1% BSA) at room temperature or overnight at 4°C. DAPI or Hoechst 33258 was used as a nuclear counter-stain and samples were mounted on glass slides using 5µl of Vectashield mounting medium (Vector Labs). Slides were imaged using a Leica DMI6000 epifluorescence system with subsequent deconvolution or a Leica SP5 confocal microscope.

For PC3 and MCF-7 cells, fixation and washes were performed as described above, except in 12-well dishes or 8-chamber slides, with just 1h of incubation with primary and secondary antibodies at room temperature. Experiments for each siRNA were performed in triplicate.

Sample sizes are indicated on figures and penetrance, when not 100%, is indicated as the fraction of individuals showing the phenotype (numerator)/ total sample size (denominator). For adult *Drosophila* wings, we mounted only one wing per individual, therefore the sample number represents biological replicates. For larval experiments, we did not keep track of biological replicates vs. technical (e.g. 2 wings per individual), therefore n-values represent both biological and technical replicates (a maximum of two) processed together. Crosses for several of the experiments were repeated multiple times, or at different timepoints or with multiple independent RNAi lines, as indicated in the text.

**EdU labeling and pulse-chase assay:**

Crosses were flipped every day and kept at room temperature (22°C). For EdU labeling in Fig 1K-L (labeling post-dissection), larvae were dissected inverted and incubated in 10μM EdU prior to fixation and labeling. The post-dissection EdU labeling was performed 3 independent times with EdU labeling intervals of 2 min., 5min., and 10min. Data from the 5 min. labeling is shown. For the EdU pulse-chase assay, vials with embryos were transferred to 29°C after 2 days. Larvae at mid- L3, (~66 hrs after the transfer) were removed from the vials by floating in 30% Sucrose/1XPBS solution. The larvae were transferred to a vial with YG food mixed with 100μM EdU and blue food coloring (to track feeding) at 29°C for 1h. Larvae with blue abdomens were then transferred to fresh non-EdU food (chase) for 6-8h at 29°C (equivalent to 7-9h at 25°C). EdU pulsed-chased wandering L3 larvae were collected, dissected, fixed, and antibody stained for EdU, PH3 and GFP (to mark the anterior-posterior compartment boundary). The EdU labeling was performed using a Click it EdU-555 kit (Cat No C10338, Invitrogen) following the manufacturer’s instruction. The slide was then imaged using confocal microscopy and the total number of cells positive for both EdU and PH3 were scored and normalized to the total mitotic.
index. This experiment was replicated 3 independent times for 6h, 7h and 9h pulse-chase intervals, with at least 5 animals per replicate. Data for the 7h replicate is shown.

**Protein synthesis puromycin assay:**
L3 larvae were dissected in Ringer’s solution (Sullivan, 2000) and inverted larvae heads containing wing discs were incubated with 20µm of OPP (O-Propargyl-Puromycin, Invitrogen) in Ringer’s solution for 12 mins. The sample was then fixed with 4% paraformaldehyde/1XPBS solution for 20 min, and labelled using the Click-it OPP kit (Cat No C10457, Invitrogen) following the manufacturer’s instruction.

**Antibodies used:**
Mouse anti-PH3 Cell Signaling 9707 1:1000
Rabbit anti-PH3 Millipore 06-570 1:2000
Rabbit anti-Dcp1 Cell Signaling 9578 1:100
Rabbit anti-pJNK Promega v7931 1:100 (for Drosophila, used slightly younger pre-wandering larvae due to high peripodial signal in later larvae)
Rabbit anti-pSmad Cell Signaling 9516 1:50 (dissection must be performed on ice)
Rabbit anti-GFP Invitrogen A11122 1:1000 (for co-labeling GFP with EdU)
Mouse anti-cut DSHB 2B10 1:100
Mouse anti-lamin Dm0 DSHB ADL67.10 1:100
Mouse anti-Wg DSHB 4D4 1:100
Rabbit anti-Vg (1:200) via G. Schubiger, from S. Carroll
Mouse anti-Patched (1:200) via G. Schubiger, from T. Kornberg
Rabbit anti- Human Nup98 Cell Signaling C39A3 Rabbit used 1/500 for Western and 1/100 for immunofluorescence

**siRNA in mammalian cells:**
MCF7 cells were a gift from S. Merajver lab (U. Michigan). PC3 cells were a stable cell line expressing cell cycle reporters hCdt1-mCherry and p27K-mVenus previously described (Takahashi et al., 2019). The cells were gown to 50-70% confluency in a 12-well plate or 8 well chamber slide. The cells were then transfected with 20nM of Nup98 SiRNA or control siRNA using Lipofectamine RNAi MAX (Invitrogen), following manufacturer’s protocol. The cells were incubated with the indicated siRNA for 72 hrs. The cells were then harvested for fixation and staining or lysed for western blot. siRNAs: Silencer Select Negative Control No. 1 (ThermoFisher) siRNA Catalog number: 4390843. Nup98-96 siRNA#1 – Silencer Pre-designed siRNA Cat no: AM16708 (ThermoFisher), Nup98-96 siRNA#2 – Silencer Select Pre-designed siRNA Cat no: 4392420 (ThermoFisher), Nup98-96 siRNA#3 – Nup98 siRNA (Santa Cruz Biotech, Cat no: sc-43436). Cell lines were tested for mycoplasma routinely, and were negative in June 2021. PC3 cells were authenticated prior to publication (Takahashi et al., 2019).

**Image analysis and quantification:**
Image quantification was performed using FIJI. For quantification of Dcp1, PH3 or pJNK labeling in Figs 1 and 2, regions of similar size (ROIs) in the anterior and posterior wing disc were hand-drawn using the nuclear (Dapi or Hoeschsts 33258) staining to indicate tissue boundaries and GFP labeling for compartment boundaries. Integrated density of labeling was normalized to ROI area for white RNAi and Nup98-96 RNAi under conditions blinded to sample condition.
identity. Area-normalized integrated density with subtraction of background ROIs outside of the tissue, was used for EdU, PH3, Nup98 and puromycin quantification. For ratios in the EdU/PH3 pulse chase assay, double-labeled cells were counted in each compartment and the ratio normalized to total mitotic index across wing discs is shown. Each dot in the scatter plot represents an individual wing disc from a different animal (For Figs 1,2, 4) or individual cells from experiments performed in triplicate (Fig 5).

Mounting and imaging of adult wings:
Adult wings were preserved in Ethanol, washed in Methyl salicylate and mounted in Canada Balsam (Sigma) as described (O'Keefe et al., 2012). Adult wings were photographed under brightfield conditions on a Leitz Orthoplan2 at 5× magnification, using a Nikon DS-Vi1 color camera and Nikon NIS Elements software.

RNAseq:
Experimental animals contained the genotype: UAS-P35/w; ap-Gal4, UAS-GFP/ +; tub-gal80TS/UAS-Nup98-96 RNAi TRiP
Control animals contained the genotype: UAS-P35/w; ap-Gal4, UAS-GFP/ +; tub-gal80TS/UAS-white RNAi TRiP
Crossoes were performed at room temperature and embryos were collected within a 12h window to synchronize developmental staging and shifted to 18°C. Animals were reared in uncrowded conditions (70 larvae per vial). On day 4 animals were transferred to 28°C and 72h later 3rd instar wing discs were dissected in sterile 1X PBS. We followed a Trizol-based RNA preparation protocol with dounce homogenization of 40 wing discs per sample with 3 replicated per genotype, as previously described (Flegel et al., 2016).

Using PolyA selection, the University of Michigan's Sequencing Core generated barcoded libraries for each sample and confirmed the quality via the Bioanalyzer and qPCR. Sequencing was performed with the Illumina HiSeq 2000 platform and high read quality was confirmed using FastQC. Reads were aligned to the BDGP6.82 D. melanogaster genome using Rsubread (v1.21.5), with featureCounts resulting in >77% of the reads being successfully assigned to genes (Liao et al., 2014). Counts per million (cpm) were determined with edgeR (v3.13.4) and transcripts with low expression were identified and removed using the data-based Jaccard similarity index determined with HTSFilter (v1.11.0). The cpm were TMM normalized (calcNormFactors), voom transformed (Law et al., 2014), fit to a linear model (lmFit), then differential gene expression calls were made with eBayes. The full dataset is available on GEO (GSE152679). Differentially expressed genes were defined as having a log₂ fold-change of ± 0.5 (1.42-fold change) and adj. p.value <0.05 (Supplemental Table 1). For significance of overlap in differentially expressed genes with other datasets (Figure 3), hypergeometric probabilities were calculated using the hypergeometric distribution as described (Flegel et al., 2016). For significance of overlap with previously published Nup98 ChIP-seq, our list of differentially expressed genes was compared to lists of genes near Nup98 ChIP-seq peaks and examined for overlap greater than that expected by chance using the hypergeometric distribution.
References


Fig. 1. Inhibition of Nup98-96 leads to G1 bypass and cell cycle de-regulation

(A-D) Using engrailed-Gal4 modified with a temperature sensitive Gal80 (en\textsuperscript{TS}), the indicated UAS-RNAis were expressed in the posterior wing disc from mid L3 to 28h after puparium formation (APF) at 28°C. The dotted line indicates the pupal wing anterior/posterior boundary. Nup98-96 inhibition increased the number of mitoses (indicated by phospho-Ser10 histone H3, PH3) and S-phases indicated by 5-ethynyl-2-deoxyuridine (EdU) labeling in the posterior wing, at stages when the wing is normally post-mitotic. (E) Adult eyes from a heterozygous sensitized
background expressing *UAS-cyclin E (cycE)* under the *GMR-Gal4* promoter and *GMR*-driven *P35* is shown. (F) Adding in *UAS-Nup98-96* RNAi enhanced eye size and folding, (G,H) and increased the number of cone cells and interommatidial cells as shown by staining for the septate junction protein Discs large (Dlg). (I-L) Using *en*<sup>Ts</sup>, the indicated *UAS*-RNAis were expressed in the posterior wing disc for 72h prior to dissection of wandering L3. The dotted line indicates the anterior/posterior boundary. Nup98-96 inhibition increased the number of mitoses and S-phases in the posterior wing disc. The EdU experiment was performed multiple times with 5, 10 or 20 min of EdU labeling. Data and number of replicates from 5 min of EdU labeling is shown. A yellow arrowhead in K’-L’ indicates the posterior zone of non-proliferating cells (ZNC) which is normally G1 arrested, but undergoes S-phases when Nup98-96 is knocked down. (M-O) An EdU pulse for 1 h followed by a 7h chase and PH3 staining was used to label mid L3 wing disc cells that progress from S-M phase in ~8h. This experiment was repeated 3 times, with intervals of 6h,7h, and 8h chase. (N) An example of a PH3 (green)/ EdU (magenta) double labeled disc is shown. (O) Quantification of double labeled cells in the posterior: anterior compartments normalizes for EdU incorporation in each disc and provides an indication of cell cycling speed differences between compartments. RNAi to Nup98-96 increased cycling speed in the posterior wing disc. (P<0.024; t-test with Welch’s correction). Plots of individual biological replicates include mean±s.e.m. Yellow bar = 50 µm
en^TS^>white^{RNAi} + P35  

en^TS^>nup98-96^{RNAi}  

en^TS^>white^{RNAi} + Bsk^{DN}  

en^TS^>nup98-96^{RNAi} + Bsk^{DN}  

Dcp1 density  

PH3 ratio (poutant)  

UAS-White^{RNAi}  

UAS-Nup98-96^{RNAi}  

UAS-Bsk^{DN}  

n=19  
n=18/20  
n=18/19
Fig. 2. Inhibition of Nup98-96 leads to cell death and compensatory proliferation

(A-L) Using en\textsuperscript{TS}, the indicated UAS-RNAis were expressed in the posterior wing disc for 72h prior to dissection of wandering L3 (unless otherwise indicated). The dotted line indicates the anterior/posterior boundary. (A-D) Nup98-96 inhibition increased apoptosis in the posterior disc, as indicated by cleaved Death Caspase-1 (Dcp1). (E-F) Co-expression of UAS-P35 with Nup98-96 RNAi lead to tissue overgrowth (E) and by day 5, wing pouch duplication (F). (G-H) Nup-98-96 knockdown led to activation of JNK signaling as detected by phosphorylated JNK staining (pJNK). (I-N) Co-expression of a dominant negative form of Drosophila JNK, Basket (Bsk\textsuperscript{DN}) had variable effects on Dcp1 staining and increased the ratio of PH3 labeling in posterior:anterior discs, although overall PH3 signal decreased with Bsk\textsuperscript{DN} (Supp. Fig. 2). (Welch’s t-test comparisons, ns= not significant, *P<0.05, ** P<0.01, ***P<0.005.) Plots of individual biological replicates include mean±s.e.m. Yellow bar = 100µm
Fig. 3. Inhibition of Nup98-96 leads to mis-patterning and gene expression changes associated with wounding and a “loser” phenotype

(A-H) Using en\textsuperscript{TS}, the indicated UAS-RNAis were expressed in the posterior wing disc for 72h prior to dissection of wandering L3 (unless otherwise indicated). Discs in C, D, G and H co-express P35 to block apoptosis and allow for tissue overgrowth. Samples in C and D were dissected after 5 days of Nup98-96 RNAi+P35 expression. (A-D) Wg levels are disrupted at the Dorso-Ventral (DV) margin but increased at the dorsal hinge upon Nup98-96 knockdown. The effect on Wg and wing disc overgrowth is enhanced with P35. (E-G) Cut expression at the DV margin is disrupted by Nup98-96 knockdown, independent of P35 expression. (H) Vestigial (Vg) is reduced when Nup98-96 is knocked down. (I-J) RNAseq was performed on dissected late L3 wing discs expressing UAS-Nup98-96 or white RNAi for 72h, driven by apterous-Gal4 with tub-Gal80\textsuperscript{TS} (ap\textsuperscript{TS}). (I) A comparison of the overlap of genes significantly altered by Nup98-96 RNAi (0.5-log\textsubscript{2}fold or more) to previously published "wounding" and "loser" gene expression signatures in wings. The fold-enrichment in the overlap of genes, above that expected by chance is shown. (J) An M-A plot of the RNAseq data with significantly increased expression indicated in red, and significantly decreased expression in blue. Genes in grey are not significantly altered. Yellow bar = 100µm.
Fig. 4. Knockdown of Nup98-96 leads to ribosomal protein mislocalization and compromised protein synthesis

(A-D) Using en_Ts, the indicated UAS-RNAis were expressed in the posterior wing disc for 72h prior to dissection of wandering L3 and labeled for protein synthesis using O-propargyl-puromycin (puro) incorporation. Puro labeling experiments in discs were performed at multiple timepoints (10-20 min), data from one experiment with 12 min. of labeling is shown. (E) The ratio of anterior:posterior puro-labeling is used to normalize for puro incorporation. Nup98-96 and Nmd3 knockdown reduced puro labeling (*P<0.05, unpaired t-test). (F-G) Knockdown of Nmd3 or CG4364 (Pescadillo homolog) for 48h in the posterior wing disc using en_Ts activated...
JNK signaling. (H-K) Using enRFP<sup>TS</sup>, the indicated UAS-RNAis were expressed for 72h in backgrounds expressing GFP or YFP-protein traps for the indicated Rp subunits. (K) RpL10Ab-YFP shows an aberrant nuclear enrichment when Nup98-96 is knocked down. Plots of individual biological replicates include mean±s.e.m.
Fig. 5. Knockdown of Nup98-96 in mammalian cells leads to reduced protein synthesis and JNK signaling

(A-B, D-E, G-H) PC3 cells were treated with small interfering (si) RNAs for 72h and cells were either fixed and stained with anti-Nup98 antibody (A-B), or phospho-JNK (D-E), or labeled with puro for 12 minutes (G-H). Control siRNA (Ctrl) is a scrambled siRNA. Nup98 siRNA reduces Nup98 levels (C) as well as reduced protein synthesis (F) and increases pJNK labeling (I).

Western blot analysis on PC3 cells treated with Ctrl and Nup98 siRNAs (L) shows Nup98 siRNAs reduced the protein level of Nup98 (J) as well as increases phosphorylated JNK (K).

Quantifications of fluorescence were performed on individual cells from three replicates from at least two independent experiments. Plots of individual biological replicates include mean±s.e.m. Quantifications for the western were done in triplicate for 3 different sets of siRNAs****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 by unpaired t-tests, (G) uses Welch’s correction for unequal sample size.
Fig. 6. Overexpression of Nup98 disrupts protein synthesis and activates JNK signaling

(A-F) Using en<sup>TS</sup>, the indicated UAS-cDNA constructs were expressed in the posterior wing from mid-L2 and adult wings were mounted. Overexpression of Nup96 had no effect on the posterior wing while overexpression of Nup98 or Nup98-96 reduced posterior wing size and disrupted vein patterning. (G-H) Using en<sup>TS</sup> a ubiquitous-RFP-NLS was expressed with UAS-Nup98 2F for 24h. The nuclear:cytoplasmic ratio for RFP-NLS was quantified and shown for the anterior wing disc (no Nup98 expression) and posterior wing disc (Nup98 overexpression). Ratios are also provided for Nup98-96 RNAi (from Supp. Fig 4) for comparison. (I-J) Using en<sup>TS</sup>, Nup98-96 cDNA was expressed in the posterior wing disc for 72h prior to dissection of wandering L3 and
labeling with pJNK. UAS-white RNAi serves as a negative control showing endogenous pJNK at this stage is very low. (K-M) Using enTS, the indicated UAS-cDNA or RNAi was expressed for 72h prior to dissection and labeling with puro to measure protein synthesis. Overexpression of Nup98 2F reduced protein synthesis in the posterior disc, while Nup98-96 overexpression had a milder effect. (*P<0.05 by unpaired t-test). Plots of individual biological replicates include mean±s.e.m.
Fig S1.

(A) 28h APF pupal eyes were labeled for 2h with EdU to detect S-phase and stained for GFP to detect PCNA promoter driven GFP. S-phases and PCNA promoter activity is evident when RNAi to Nup98-96 is driven with GMR-Gal4. GMR-Gal4 without any RNAi (in a w^{1118} background) serves as a control. Genotypes are: (top) w; GMR-Gal4+/+; UAS-Nup98-96 RNAi (TRiP)/PCNA prom-GFP (bottom) w; GMR-Gal4+/+; PCNA prom-GFP/+.

(B) Nup98-96-GFP is from the FlyFos collection containing a Fosmid on II with Nup98-96 coding region plus regulatory DNA and a GFP tag (Sarov et al., 2016) We confirmed this line exhibits the expected ubiquitous nuclear envelop labeling by co-staining with Lamin Dm0. (Right) We co-expressed Nup98-96-GFP in a background with en-Gal4 driving RFP alone or RFP in combination with UAS-Nup98-96 RNAi (TRiP), we confirmed effective knockdown of Nup98-96-GFP in the en-Gal4 expressing domain. Genotypes are: (left) w; Nup98-96-GFP/+; (middle) w; Nup98-96-GFP/ enGal4, UAS-RFP; + (right) w; Nup98-96-GFP/ enGal4, UAS-RFP; UAS-Nup98-96 RNAi (TRiP)/+

(C) cDNA rescue constructs providing UAS-Nup98, UAS-Nup96 or UAS-Nup98-96 were tested for the ability to rescue the wing phenotypes caused by Nup98-96 RNAi. Only expression of both Nup98 and Nup96 (middle right) fully rescued posterior wing size. Note that over-expression of both Nup98 and Nup96 without RNAi also led to reduced posterior wing size (see Fig. 6). Genotypes are: (top left) w; en-Gal4, UAS-GFP/+; UAS-white RNAi (TRiP)/+ (top right) w; en-Gal4, UAS-GFP/+; UAS-Nup98-96 RNAi (VDRC GD); UAS-white RNAi (TRiP)/+ (middle left) w; en-Gal4, UAS-GFP/UAS-Nup98-96 RNAi (VDRC GD); UAS-white RNAi (TRiP)/+ (middle right) w; en-Gal4, UAS-GFP/UAS-Nup98-96 RNAi (VDRC GD); UASNup98-96 cDNA/+ (bottom left) w; en-Gal4, UAS-GFP/UAS-Nup98-96 RNAi (VDRC GD); UASNup98-96 cDNA 3M/+ (bottom right) w; en-Gal4, UAS-GFP/UAS-Nup98-96 RNAi (VDRC GD); UASNup98-96 cDNA 7M/+

(D) Two independent RNAi lines from the VDRC gave similar phenotypes to the TRiP RNAi in the larval wing disc. Line GD6897 is shown at wandering L3 after 72h of expression at 28°C with PH3 labeling for mitoses, similar results were obtained with the line KK100388. Genotype: w; en-Gal4, UAS-GFP; UAS-Nup98-96 RNAi GD6897; Tub-Gal80 TS/+.

Quantifications of PH3 for 10 wings of the genotype w; en-Gal4, UAS-GFP; UAS-Nup98-96 RNAi KK100388; Tub-Gal80 TS/+ are shown at right. PH3 signal is significantly increased in the posterior domain. **P<0.01 by a two-tailed paired t-test. Plots of individual biological replicates include mean±s.e.m.

(E) Controls related to Fig 1 E-F. GMR-Gal4 driving Nup98-96 RNAi alone results in a smaller, rough eye, likely due to increased apoptosis, which can be partially rescued by co-expressing GMR>P35.

(F) Quantifications of EdU labeling related to Fig. 1 K-L. Nup98-96 knockdown results in increased EdU labeling in the posterior wing disc compared to the anterior and compared to white RNAi controls. Experiments were repeated independently for 2min, 5 min, and 10 min. of labeling. Genotypes: w; en-Gal4, UAS-GFP/ + ; Tub-Gal80 TS/ UAS-white RNAi or w; en-Gal4, UAS-GFP/ + ; Tub-Gal80 TS/ UAS-Nup98-96 RNAi. Plots of individual biological replicates include mean±s.e.m.

(G) Related to EdU pulse-chase experiment in Fig. 1M-O. Quantification of EdU+ mitoses in wing discs for 7h EdU pulse-chase experiment. Nup98-96 knockdown results in an increased fraction of cells progressing from S-M in 7h in the posterior wing disc (autonomous effect) compared to the anterior (non-autonomous effect), but the increase...
with Nup98-96 knockdown is only ~20% and not statistically significant when compared to an external white RNAi control (mean for Nup98-96 RNAi = 0.425, mean for white RNAi = 0.332). Nup98-96 RNAi anterior to posterior comparison, **P<0.01 by two-tailed paired t-test. white RNAi posterior to Nup98-96 RNAi posterior comparison not significant (n.s.) by unpaired t-test with Welch’s correction for unequal sample size. Plots of individual biological replicates include mean±s.e.m. Genotypes: w; en-Gal4, UAS-GFP/ + ; Tub-Gal80TS/ UAS-white RNAi or w; en-Gal4, UAS-GFP/ + ; Tub-Gal80TS/ UAS-Nup98-96 RNAi.
Fig S2. Nup98-96 knockdown leads to apoptosis which is rescued by co-expression of UAS-Nup98-96 cDNA. The indicated transgenes were driven by en-Gal4, with UAS GFP for 72h prior to dissection using tub-Gal80TS. (A,B) Cleaved caspase 3 labeling indicates apoptosis in the posterior compartment when Nup98-96 is knocked down. (C, D) Co-expression of Nup98-96 cDNA rescues the apoptosis caused by Nup98-96 knock-down, as assessed with Dcp-1. (E) Expression of Nup98-96 RNAi in the dorsal compartment (using ap-Gal4, UAS-GFP; tub-Gal80TS) also leads to apoptosis. (F-H) Expression of Nup98-96 RNAi in clones throughout the wing pouch (using hs-flp with act>stop>-Gal4, UAS-GFP) also leads to apoptosis within clones, while co-expression with P35 (using hs-flp with act>stop>-Gal4, UAS-GFP, UAS-P35) suppresses apoptosis within clones and leads to apoptosis outside of clones expressing Nup98-96 RNAi. (I) Expression of Nup98-96 RNAi in the dorsal compartment (using ap-Gal4, UAS-GFP; tub-Gal80TS) leads to upregulation of puc-LacZ expression (from puc69 allele), a hallmark of JNK signaling. (J) Quantifications of PH3 signal broken down by ant/post compartment. Nup98-96RNAi and Bsk DN are expressed only in the posterior using en-Gal4, UAS-GFP; tub-Gal80TS. Note that overall PH3 labeling is reduced in both compartments when JNK signaling is inhibited with Bsk DN. Plots of individual biological replicates include mean±s.e.m.
Fig S3. Knockdown of Nup98-96 leads to wing disc overgrowth and patterning defects consistent with apoptosis-induced proliferation.

(A) Expression of Nup98-96 RNAi in the dorsal compartment with UAS-P35 for 5d (using ap-Gal4, UAS-GFP/ UAS-P35; tub-Gal80TS) leads to tissue folding, overgrowth and invasion across the D-V boundary as well as ectopic Wg expression.

(B) Expression of Nup98-96 RNAi + P35 in the posterior compartment (with en-Gal4, left) or dorsal compartment (with ap-Gal4, right) abolishes Cut expression at the D-V boundary.

(C) Expression of Nup98-96 RNAi in the posterior compartment with UAS-P35 for 4d (using en-Gal4, UAS-GFP/ UAS-P35; tub-Gal80TS) leads to tissue folding and invasion at the A-P boundary as well as ectopic Ptc expression demonstrating mis-patterning.

(D) Normal pMad staining is shown (top) for en-Gal4, UAS-GFP; tub-Gal80TS driving white RNAi. (bottom) Nup98-96 RNAi expression driven by en-Gal4, UAS-GFP; tub-Gal80TS leads to broad pMad staining in the posterior compartment indicating mis-patterning.
Fig. S4. Knockdown of Nup98-96 disrupts nucleo-cytoplasmic localization and reduces protein synthesis independent of apoptosis.

(A) Nup 98-96 RNAi was expressed in the posterior compartment using en-Gal4, UAS-GFP; tub-Gal80TS in a background expressing Ubiquitin promoter driven-RFP with a nuclear localization signal (Ubi-RFPNLS) at 27°C to minimize cell lethality. By 52h of Nup98-96 knockdown, nuclear localization of RFP is visibly disrupted. By 72h of knockdown nuclear localization of Ubi-RFPNLS is disrupted.

(B) Co-expression of UAS-P35 with Nup 98-96 RNAi did not rescue the reduction in protein synthesis when Nup98-96 is compromised. Protein synthesis was assayed by puro-labeling after 20h (n=4) and 40h (n=6) of Nup98-96 knockdown. The data from 20h is shown. This suggests the reduced proteins synthesis is not a consequence of apoptosis.
Fig S5. Knockdown of Nup98 in MCF7 cells leads to reduced protein synthesis and JNK phosphorylation. (A-B, E-F, H-J) MCF7 cells were treated with small interfering (si) RNAs for 72h and cells were labeled with puro (A’-B’) or fixed and stained with anti-Nup98 antibody (A-B), or phospho-JNK (E-J). Control siRNA (Ctrl) is a scrambled siRNA. Nup98 siRNA reduces Nup98 levels (C) as well as reduced protein synthesis (D) and increases pJNK labeling (G). Quantifications of fluorescence were performed on individual cells from at least two experiments. Three independent siRNAs were individually tested with siRNAs #1 and #2 being most effective on MCF7. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 by unpaired t-tests, (G) uses Welch’s correction for unequal sample size. Plots of individual biological replicates include mean±s.e.m.

Table S1. Excel file containing statistically significant gene expression changes >0.5 log₂-fold change in Nup98-96 knockdown L3 wing discs by RNA seq. Sheet 2 lists genes that overlap between changes in Nup98-96 knockdown and a Ras⁰¹², scrib invasive Drosophila tumor model.

Click here to download Table S1

Table S2. Excel file with the list of genes that overlap in Nup98-96 knockdown L3 wing discs and a wounding/regeneration program (Khan et al., 2017).

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Table S3. Excel file with the list of genes that overlap in Nup98-96 knockdown L3 wing discs and a “loser” gene expression program (Kucinski et al., 2017).

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Table S4. Excel file with the list of genes that overlap in Nup98-96 knockdown L3 wing discs and Xrp1 targets (Lee et al., 2018).

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Table S5. Excel file with the list of genes that overlap in Nup98-96 knockdown L3 wing discs and Nup98 ChIP seq assays (Pascual-Garcia et al., 2017) and Nup98 alterations with RNAseq in S2 cells (Kalverda et al., 2010).

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