Non3 is an essential Drosophila gene required for proper nucleolus assembly

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The nucleolus is a dynamic non-membrane-bound nuclear organelle, which plays key roles not only in ribosome biogenesis but also in many other cellular processes. Consistent with its multiple functions, the nucleolus has been implicated in many human diseases, including cancer and degenerative pathologies of the nervous system and heart. Here, we report the characterization of the Drosophila Non3 (Novel nucleolar protein 3) gene, which encodes a protein homologous to the human Brix domain-containing Rpf2 that has been shown to control ribosomal RNA (rRNA) processing. We used imprecise P-element excision to generate four new mutant alleles in the Non3 gene. Complementation and phenotypic analyses showed that these Non3 mutations can be arranged in an allelic series that includes both viable and lethal alleles. The strongest lethal allele (Non3^600) is a genetically null allele that carries a large deletion of the gene and exhibits early lethality when homozygous. Flies heterozygous for Non3^600 occasionally exhibit a mild reduction in the bristle size, but develop normally and are fertile. However, heteroallelic combinations of viable Non3 mutations (Non3^317, Non3^259 and Non3^275) display a Minute-like phenotype, consisting in delayed development and short and thin bristles, suggesting that they are defective in ribosome biogenesis. We also demonstrate that the Non3 protein localizes to the nucleolus of larval brain cells and it is required for proper nucleolar localization of Fibrillarin, a protein important for post-translational modification and processing of rRNAs. In summary, we generated a number of genetic and biochemical tools that were exploited for an initial characterization of Non3, and will be instrumental for future functional studies on this gene and its protein product.

Key words: Drosophila melanogaster; nucleolus; Minute-like phenotype; Non3; Fibrillarin; Rpf2; Brix domain; ribosome biogenesis.

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Introduction

Ribosomes are highly conserved macromolecular machines that organize and catalyze mRNA translation in all organisms. In higher eukaryotes, mature cytoplasmic ribosomes include 4 ribosomal RNA molecules (rRNAs; 18S, 25S/28S, 5.8S and 5S) and 79 ribosomal proteins (RPs) (Marygold et al., 2007; Xue, Barna, 2012; Genth, Barna, 2018). *Drosophila melanogaster* has a 2S rRNA instead of 5S rRNA (Stage, Eickbush, 2007). Coordinated processing of 45S/47S precursor rRNA (pre-rRNA) into the mature 18S, 5.8S and 25S/28S rRNAs is a central process in the highly orchestrated ribosome assembly (Henras et al., 2015). Ribosomal biosynthesis includes progressive association of individual ribosomal proteins with maturing rRNAs, export of pre-ribosome particles from the nucleolus to the cytoplasm and their assembly into mature ribosomes. More than 200 different factors are required for ribosome biosynthesis and quality control of ribosome maturation on the path from the nucleolus to the cytoplasm (Kressler et al., 2017).

Depletion of eukaryotic RPs could be associated with disturbances in pre-rRNA processing and nucleolar organization (Neumüller et al., 2013; Farley-Barnes et al., 2018). In *Drosophila*, mutations in 75% of the genes encoding protein components of mature cytoplasmic ribosomes dominantly cause a *Minute* phenotype. The *Minute* syndrome includes short and thin bristles, delayed development, and reduced viability and fertility (Marygold et al., 2007). In addition, decreased levels of some *Drosophila* RPs result in overgrowth of specific tissues and melanotic tumors (Goudarzi, Lindström, 2016). Mutations in genes encoding RPs and ribosome biogenesis factors lead to a series of congenital human disorders collectively called ribosomopathies, and predispose to cancer (Narla, Ebert, 2010; Henras et al., 2015; Mills, Green, 2017; Núñez Villacís et al., 2018). In some cases, these diseases are also caused by haploinsufficiency for these genes caused by somatic mutations (Narla, Ebert, 2010; Núñez Villacís et al., 2018).

Although ribosomes have been considered for many years as ancient and rather invariable molecular machines, several lines of evidence indicate that they are instead heterogeneous in both RPs and rRNA composition (Genth, Barna, 2018). Indeed, mutations in RP coding genes lead to tissue-specific pleiotropic phenotypes in multicellular organisms (Kongsuwan et al., 1985; Drapchinskaia et al., 1999; Marygold et al., 2005; Gupta, Warner, 2014; Shi, Barna, 2015). Pleiotropic phenotypes could be only partially explained by the fact that several of these genes have paralogues (Xue, Barna, 2012). For example, in humans there are three paralogous RP genes (*RPS4X, RPS4Y1* and *RPSY2*) encoding the RPS4 protein, and one of them (*RPSY2*) is specifically expressed in testis and prostate (Fisher et al., 1990; Lopes et al., 2010). In *Drosophila*, a total of 88 genes encoding 79 different RPs have been identified; nine of these genes are present as duplicates (Marygold et al., 2007). These duplicated genes are expressed at different levels, and in some cases with a tissue-specific pattern. For instance, *Rpl22L, Rps5b, Rps19a, Rpl10.4a* and *Rpl37b* exhibit enhanced expression in the testes compared to their paralogues, suggesting specific composition of testis ribosomes (Marygold et al., 2007; Kearse et al., 2011).

A complex sequence of processing steps, involving several protein factors, is required to gradually release the mature rRNAs from precursor pre-rRNA (Henras et al., 2015). Many of these factors contain putative RNA-binding domains (e.g., GAR, RRM, KH, Brix, S1, dsRBD, and Zinc finger) and/or protein-protein interaction domains (WD40, HEAT, TPR, and HAT), but the enzymatic activities of most proteins required for ribosome biosynthesis have not been determined (Henras et al., 2015). One of the Brix domain-containing proteins is the *Saccharomyces cerevisiae* ribosome assembly factor Rpf2 (Ribosome production factor 2), which in complex with Rrs1 plays a role in the early steps of the 60S ribosome subunit maturation (Zhang et al., 2007; Henras et al., 2015; Kressler et al., 2017). Rpf2 binds the Rpl5 and Rpl11 ribosomal proteins and the Rrs1 protein, forming both the 5S ribonucleoprotein particle necessary for 25S rRNA maturation and the large 60S ribosomal subunit (Tutuncuoglu et al., 2016). Depletion of Rpf2 results in defects in pre-rRNA processing (Wehner, Baserga, 2002).

The Rpf2 proteins are highly conserved. The *Drosophila* Rpf2 orthologous protein is encoded by the *Novel nucleolar protein 3 (Non3)* gene (*CG7993*). Non3, which contains a Brix domain, exhibits 66% similarity and 47% sequence identity with human Rpf2 (Gramates et al., 2017). Thus far, most studies on Non3 were carried in tissue culture cells. Most interestingly, an RNAi-based screen showed that Non3 depletion results in short mitotic spindles. In addition, it has been shown that in interphase cells the GFP-tagged Non3 protein localizes to the nucleolus (Moutinho-Pereira et al., 2013). These phenotypes, and the finding that RNAi-mediated depletion of other nucleolar proteins results in short spindles, suggest that the short-spindle phenotype observed in *Non3* RNAi cells is due to limited translation of tubulin and/or other spindle components. However, it is also possible that Non3 has a direct role in spindle formation (Moutinho-Pereira et al., 2013).

To address the mitotic role of Non3, and to provide a *Drosophila* model for the study of the Rpf2 function in nucleolus assembly and ribosome maturation, we characterized the *Non3* gene. We generated several allelic mutations in the gene and...
showed that these mutations affect viability, fertility and bristle formation, resulting in a Minute-like phenotype. We also show that Non3 localizes to the nucleolus and is required for proper formation of this organelle. We believe that the mutations and the reagents generated in this study will be instrumental to define the role of Non3 in living flies.

Materials and methods

Fly stocks. Flies were raised and crossed on standard cornmeal agar media at 25 °C. The fly stocks used in this study are from the Bloomington Stock Center (Bloomington, IN, USA; flystocks.bio.indiana.edu): #30094 (w1118, P{EP}Non3G4706/TM6C, Sb1) (hereafter Non3G4706) and #4368 (y, w; Ki; P{ry}, Δ2-3)99B) (hereafter Δ2-3). The yw, P{γr, 4717} = nosphiC31int(NLS)X; P{γr, 4717} = CaryP{attP40} fly line was kindly provided by Sergei A. Demakov (Institute of Molecular and Cellular Biology of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia).

Generation of new Non3 alleles. The Non3 mutants were generated by imprecise excision (O’Brochta et al., 1991) of the P{EP} transposon located 48 bp downstream of the predicted transcription start site of Non3 in the Non3G4706 line (Fig. 1). The Δ2-3 strain was used as a source of transposase (Robertston et al., 1988), and the F1 progeny of Non3G4706/Δ2-3 flies was screened for loss of the mini-white (wme) marker carried by the P{EP} transposon. 512 independent excision lines were established and analyzed by PCR using primers Ins-non3-F2 (5ʹ-CGTTTGTTCATACCTCAATC-3ʹ) and Ins-non3-R (5ʹ-CTGTCGCACAAATGG-3ʹ) (primer positions are shown in Fig. 1). We identified one line with a large deletion internal to the Non3 gene, three lines with remnants of the P{EP} transposon, and several lines in which the transposon was most probably precisely excised. Only one of the latter lines was further analyzed and confirmed to be generated by precise excision of the P{EP} transposon; it was designated as Non3li. The PCR products obtained from all new Non3 mutant lines were cloned in a plasmid vector and sequenced (Suppl. Fig. 1).

Construction of rescue plasmid and germ-line transformation. To make a rescue construct, we cloned a 2.76-kb genomic DNA fragment [chr3R:18222722–18225482; the coordinates are from Release 6 of the Drosophila melanogaster genome assembly (Hoskins et al., 2015)] carrying the Non3 gene into the pUASTattB vector (Bischof et al., 2007) by substituting a UAS promoter, a multiple cloning site and a SV40 terminator. Details of plasmid construction are available upon request. The cloned genomic DNA fragment contains the coding sequence was PCR-amplified from the genomic DNA fragment [chr3R:18222722–18225482; the coordinates are from Release 6 of the Drosophila melanogaster genome assembly (Hoskins et al., 2015)] carrying the Non3 gene into the pUASTattB vector (Bischof et al., 2007) by substituting a UAS promoter, a multiple cloning site and a SV40 terminator. Details of plasmid construction are available upon request. The cloned genomic DNA fragment contains the coding sequence to produce pGEX-4T-Non3 construct. Details of plasmid construction are available upon request. The GST-Non3 fusion protein was expressed in Escherichia coli and subsequently purified as described previously (Chalkley, Verrijzer, 2004). The purified protein was used to immunize mice. Polyclonal antibodies were affinity purified from serum as previously described (Chalkley, Verrijzer, 2004).

Double-stranded RNA (dsRNA) production. A 730-bp fragment of the Non3 coding sequence was PCR-amplified with primers CG7993-maF1 (5ʹ-TAATACGACTCACTATAGGGAGGGTTTTACCGAG-3ʹ) and CG7993-maR1 (5ʹ-TAATACGACTCACTATAGGGAGGGTTTTACCGAG-3ʹ) (underlined is the added T7 promoter sequence) from the pGEX-4T-Non3 plasmid. The purified PCR product was used as a template to synthesize dsRNA as described earlier (Somma et al., 2002), with the minor modifications: the phenol/chloroform extraction step was omitted and DNaseI treatment was performed at the end of the procedure.

S2 cell culture and RNA interference (RNAi). S2 cells (for details, see Strunov et al., 2015) were cultured at 25 °C in 39.4 g/L Shields and Sang M3 Insect medium (Sigma, S8398) supplemented with 0.5 g/L LKHCO and 20 % heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, 10270106). RNAi treatments were carried out as described previously (Somma et al., 2008), with the following modifications: 25 μg of dsRNA was added to the cells three times (on the first, the third and the fifth day of incubation), and cells were harvested for analyses after 7 days of RNAi.

Reverse transcription followed by quantitative PCR (RT-qPCR). RNAi efficiency in S2 cells was assessed by RT-qPCR. Total RNA was isolated from control and dsRNA-treated cells using RNAzol® RT reagent (Molecular Research Center, RN 190); genomic DNA was eliminated using the Rapid- Out DNA Removal Kit (Thermo Fisher Scientific, K2981) according to the manufacturer’s instructions. Synthesis of cDNA and PCR were performed as described previously (Ogienko et al., 2018), using the following gene-specific primers: RT-Non3-Fw2 (5ʹ-CGTTTGTTCATACCTCAATC-3ʹ) and RT-Non3-Rev2 (5ʹ-CTTTCCTCGTCCAAATGACG-3ʹ) for Non3 (this study), and RPL32-realtime-F (5ʹ-CTAAGCGTGGCACAATGG-3ʹ) and RPL32-realtime-R (5ʹ-AGGAACCTTCTGATCAGG-3ʹ) for Rpl32 (Yang et al., 2013), which was used as a reference gene.

Western blotting. S2 cells were harvested by centrifugation at 200 g for 5 min at room temperature, washed with phosphate-buffered saline (PBS) and centrifuged again. Pellets were lysed in RIPA buffer (Sigma, R0278) containing 1 × HalTM Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, 1861282), and the lysates were clarified by centrifugation at 15,000 g for 15 min at 4 °C. The samples were normalized to the total amount of protein using DC Protein Assay (Bio-Rad, 5000116). Each normalized sample was mixed with an equal volume of 2× Laemmli buffer and incubated at 95 °C for 5 min prior to loading on a SDS-PAGE gel. Larval tissues were dissected, homogenized in 1× Laemmli buffer with a pestle and incubated at 95 °C for 5 min prior to loading on a SDS-PAGE gel. The following primary
antibodies were used: mouse anti-Lamin Dm0 (1:300; DSHB, ADL67.10), mouse anti-α-Tubulin (1:5000; Sigma, T6199), mouse anti-β-Tubulin [1:800; BX69 (Tavares et al., 1996), kindly provided by Harald Saumweber (Humboldt University Berlin, Institute of Biology, Berlin, Germany)] and mouse anti-Non3 (1:5000; this study). The primary antibodies were detected with HRP-conjugated goat α-mouse IgG (1:3500; Jackson ImmunoResearch, West Grove, PA, USA) and mouse anti-β-Tubulin [1:800; BX69 (Tavares et al., 1996), kindly provided by Harald Saumweber (Humboldt University Berlin, Institute of Biology, Berlin, Germany)] and mouse anti-Non3 (1:5000; this study). The primary antibodies were detected with HRP-conjugated goat α-mouse IgG (1:3500; Jackson ImmunoResearch, West Grove, PA, USA) and mouse anti-β-Tubulin [1:800; BX69 (Tavares et al., 1996), kindly provided by Harald Saumweber (Humboldt University Berlin, Institute of Biology, Berlin, Germany)].

**Immunofluorescence (IF) staining.** Brains from late third instar larvae were dissected and immunostained as described earlier (Bonaccorsi et al., 2000). The following primary antibodies were used: mouse anti-Non3 (1:250; this study) and rabbit anti-Fibrillarin (1:300; Abcam, ab5821). They were detected with goat anti-mouse IgG conjugated to Alexa Fluor 488 (1:500; Invitrogen, A-11001) and goat anti-rabbit IgG conjugated to Alexa Fluor 568 (1:350; Invitrogen, A-11036), respectively. DAPI was used to stain DNA. IF images were acquired with a Zeiss Axio Imager M2 fluorescence microscope equipped with an AxioCam 506 mono (D) camera and a NeoFluar 100×/1.3 Oil objective using the ZEN 2012 software.

### Results and discussion

**Generation and characterization of the novel Non3 mutations**

*D. melanogaster* Non3 gene (CG7993) maps to the 90F8 region of polytene chromosomal, includes two small introns, and has a total size of 1221 bp. It is ubiquitously expressed, with the highest expression levels in embryos (0–12 h), nervous ganglia, ovaries and testes (Gramates et al., 2017). When we began this work, only a *P*-element-induced mutation in the *Non3* gene (Non3<sup>870</sup>) was available. Animals homozygous for this mutation are lethal and die at late pupal stages. We sequenced Non3<sup>G4706</sup> and confirmed that this mutation carries a *P*{EP} transposon inserted 48 bp downstream of the predicted transcription start of the gene (see Fig. 1, Suppl. Fig. 1). We then used precise *P*-element excision to generate a set of additional mutations in *Non3*. We isolated three hypomorphic alleles (Non3<sup>197</sup>, Non3<sup>310</sup> and Non3<sup>259</sup>) and one putative null allele (Non3<sup>600</sup>) (see Material and Methods for details). As a control, we also generated a chromosome bearing a precise excision of the *P*{EP} transposon (Non3<sup>3cs</sup>).

Flies homozygous for Non3<sup>cs</sup> are fully viable and fertile, excluding a possible influence of background mutations on the “starting” chromosome (see Fig. 1). All mutations generated by imprecise excision were sequenced and their precise locations and molecular structures are shown in Figure 1 and Suppl. Fig. 1. The Non3<sup>600</sup> allele carries a deletion of 617 bp of the *Non3* coding region and contains 13 residual bp of 3’ end of the *P*-element. The Non3<sup>197</sup>, Non3<sup>310</sup> and Non3<sup>259</sup> mutations carry differently sized remnants of the 5’ and 3’ *P*-element ends (373, 367 and 453 bp, respectively) that do not disrupt the coding region of the gene. Non3<sup>cs</sup> did not show any sequence variation compared to wild type.

**Non3 mutants exhibit a Minute-like phenotype**

We next performed a phenotypic analysis and a functional categorization of the new mutations. This investigation was possible because the “starting” *Non3<sup>G4706</sup>* chromosome does not carry background mutations, as shown by the charac-
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Complementation analysis of Non3 mutant alleles

|            | Non3ex | Non3397 | Non3310 | Non3259 | Non3G4706 | Non3600 |
|------------|--------|---------|---------|---------|-----------|---------|
| ♂          | V, F, NB | V, F, NB | V, F, NB | V, F, NB | V, F, NB | V, F, NB |
| ♀          | V, F, db  | V, F, db  | V, F, db  | V, ss, DB | PLE, S, DB |         |
| Non3397    |         | V, F, db  | V, F, db  | V, F, db  | V, ss, DB | PL      |
| Non3310    |         | V, F, db  | V, F, db  | V, ss, DB | PL        |         |
| Non3259    |         | V, ss, db | V, S, DB | PL       |           |         |
| Non3G4706  |         | PL       | PL       | PL       |           |         |
| Non3600    |         | EL       |         |          |           |         |

Note: V, viable both sexes; F, fertile both sexes; EL, early lethal (L1/L2); PL, pupal lethal; PLE, pupal lethal with escapers; S, sterile in both sexes; ss, semi sterile; NB, normal bristles; DB, defective bristles; db, slightly defective bristles.

Fig. 2. Non3 mutants exhibit a recessive Minute-like bristle phenotype.

Non3G4706/Non3259 flies have shorter and thinner bristles than Non3ex used as control. This is most clearly seen by comparing the notum bristles (red arrows). Non3600/Non3ex and Non3G4706/Non3ex flies exhibit a very weak dominant bristle phenotype. Addition of a wild-type copy of Non3 (P[rescue]) to Non3G4706/Non3ex flies leads to a complete rescue of the Minute-like phenotype.

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In Drosophila, mutations in 75% of the RP coding genes dominantly produce a Minute phenotype characterized by prolonged development, short and thin bristles, and reduced viability and fertility, often accompanied by additional patterning and growth defects such as roughened eyes, abnormal wings, defective abdominal segmentation, and small body size (Marygold et al., 2005). Bristle production and gametogenesis require maximal protein synthesis and are therefore particularly sensitive to a reduction in the translational capacity of
The cell (Marygold et al., 2005). It is thus generally accepted that the Minute syndrome reflects a reduced protein synthesis resulting from insufficient ribosome function (Morata, Ripoll, 1975; Marygold et al., 2005). Mutations in several Drosophila genes involved in ribosome biosynthesis do not have dominant effects but, when homozygous, cause the same defective traits elicited by the dominant Minute mutations. The phenotype produced by these mutations is commonly designated as Minute-like (see for example, Cui, DiMario, 2007).

Non\textsuperscript{3\textalpha}\textsuperscript{A000}/+ and Non\textsuperscript{3\textalpha}G\textsuperscript{7706}/+ heterozygous flies occasionally exhibit a limited reduction in bristle size but develop normally and are fertile. Thus, Non\textsuperscript{3} mutations have only a minimal dominant effect. However, the combination of the Non\textsuperscript{3\textalpha}G\textsuperscript{7706} allele with any other weaker allele results in a clear Minute-like phenotype: prolonged development, poor viability and fertility, and abnormally short and thin bristles (Table, Fig. 2) (some flies had crumpled wings). To verify that all Non\textsuperscript{3} phenotypes were due to a decrease in the Non\textsuperscript{3} protein level, we performed rescue experiments using a transgene that carries a full genomic copy of Non\textsuperscript{3} and fertility, and abnormally short and thin bristles (Table, Fig. 2). Mutant Non\textsuperscript{3} flies carrying one copy of this rescue construct (P[\text{rescue}]) showed normal viability and bristle length, and restored fertility (Fig. 2 and data not shown). Taken together, these findings indicate that Non\textsuperscript{3} mutations behave like Minute-like mutations (Cui, DiMario, 2007). The finding that mutants in Non\textsuperscript{3} exhibit a Minute-like phenotype is not surprising. The S. cerevisiae Rp\text{f}2 protein is one of the many factors necessary for the assembly of the pre-60S subunits (Beidka et al., 2018) and it is likely that its Drosophila homologue Non\textsuperscript{3} participates in the same processes.

**Non\textsuperscript{3} hypomorphic mutations reduce the Non\textsuperscript{3} protein level**

To further characterize the Non\textsuperscript{3} mutant alleles, we raised a polyclonal anti-Non\textsuperscript{3} antibody in mice (see Materials and Methods for details). This antibody recognizes a band of the expected molecular weight (~37 kDa) in Western blots from S2 cell protein extracts; this band is substantially reduced in the extracts from Non\textsuperscript{3} RNAi cells, confirming the specificity of the antibody (Fig. 3, a, b). It was suggested earlier that the short-spindle phenotype observed in Non\textsuperscript{3} RNAi S2 cells is caused by limited translation of tubulin and/or other spindle components (Moutinho-Pereira et al., 2013). Our finding that Non\textsuperscript{3} RNAi-treated cells exhibit a normal tubulin level (see Fig. 3, b) indicates that this phenotype is not caused by limited tubulin availability.

We next analyzed the levels of the Non\textsuperscript{3} protein in larval brains and salivary glands from the different Non\textsuperscript{3} mutants (see Fig. 3, c, d). In larval brain protein extracts from Non\textsuperscript{3G\textalpha}7706 homozygotes, the Non\textsuperscript{3} protein is drastically reduced, supporting the view that Non\textsuperscript{3G\textalpha}7706 is the strongest among the Non\textsuperscript{3} hypomorphic mutations. Two copies of the rescue construct (2 × P[\text{rescue}]) restored the Non\textsuperscript{3} protein level in both Non\textsuperscript{3G\textalpha}7706/Non\textsuperscript{3G\textalpha}7706 and Non\textsuperscript{3Δ600}/Non\textsuperscript{3Δ600} mutants, approximately up to the level of Non\textsuperscript{3\textalpha} homozygous brains (see Fig. 3, c). We also found that Non\textsuperscript{3} is substantially reduced in salivary glands from Non\textsuperscript{3\textalpha}197, Non\textsuperscript{3\textalpha}310 and Non\textsuperscript{3\textalpha}259 homozygotes. In addition to a reduction of the wild-type Non\textsuperscript{3} protein, the salivary glands of these mutants displayed immunoreactive proteins of ~39–40 kDa (see Fig. 3, d). Because the Non\textsuperscript{3\textalpha}197, Non\textsuperscript{3\textalpha}310 and Non\textsuperscript{3\textalpha}259 mutations are carrying remnants of P-element ends, we believe that the aberrant Non\textsuperscript{3} proteins
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...are transcribed starting from ATG codons located within these P-element fragments (see Fig. 3, d; Suppl. Fig. 1).

**Nucleolar Non3 protein is required for proper Fibrillarin localization**

It has been previously shown that S2 cells transiently transfected with a plasmid encoding a GFP-Non3 fusion protein show a specific localization of the protein in the nucleolus (Moutinho-Pereira et al., 2013). We tried to confirm this Non3 localization by immunostaining S2 cells with our antibody, but were unable to see a clear signal. However, we successfully immunostained the nucleoli of larval brain cells (Fig. 4), possibly because nervous ganglia are one of the tissues with the highest level of Non3 expression (Gramates et al., 2017). In wild-type brain cells, Fibrillarin, the main component of the active nucleolus (Neumüller et al., 2013), co-localizes with Non3 in the nucleolus. In brain cells of homozygous Non3G4706 and Non3310 mutants, we observed a drastic reduction of both Non3 and Fibrillarin in the nucleolus, suggesting that Non3 is required for Fibrillarin localization in nucleoli. The presence of the Non3 rescue transgenes (2 × P(rescue)) restored the normal nucleolar signals of both proteins (see Fig. 4).

These results indicate that Non3 is a nucleolar protein that is required for proper Fibrillarin localization in the nucleolus. Fibrillarin is a major nucleolar protein with methyltransferase activity, playing roles in rRNA biogenesis and function (Rodriguez-Corona et al., 2015). Loss of nucleolar proteins such as Nopp140, Nop56, and Nop5 leads to mislocalization of Fibrillarin, compromises several nucleolar functions (Pederson, 1998; Olson, 2004), and causes developmental abnormalities (Cui, DiMario, 2007). The role of Non3 in Fibrillarin recruitment to the nucleolus is currently unknown and will be addressed in future studies.

**Conclusions**

We have generated several mutant alleles of the *Non3* gene and shown that viable combinations of these alleles exhibit a *Minute*-like phenotype, suggesting a role of Non3 in ribosome biogenesis. We have also shown that the Non3 protein localizes to the nucleolus and, most importantly, it is required for Fibrillarin recruitment to this organelle. Fibrillarin is a multifunctional protein that mediates methylation of several RNA species and plays roles in tumorigenesis and stem cell differentiation (Rodriguez-Corona et al., 2015; van Nues, Watkins, 2017). We believe that the Non3-related tools generated in our study will be instrumental to define the role of Non3 in RNA metabolism and to elucidate how it affects mitotic spindle formation. These tools will also help to investigate the mechanisms and biological significance of the interaction between Non3 and Fibrillarin.

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![Fig. 4. Non3 localizes to the nucleoli of larval brain cells.](image_url)

In wild-type (Non3\textsuperscript{ex}) brain cells, Non3 largely co-localizes with the nucleolus marker Fibrillarin. Notably, brain cells from Non3\textsuperscript{G4706} and Non3\textsuperscript{310} homozygous larvae not only show a strong decrease of Non3 signals but also of Fibrillarin signals. Scale bar is 10 µm.
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