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Defining the role of NatC dependent N-terminal acetylation in *Drosophila* fertility

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Dissertação para obtenção do Grau de Mestre
em Ciências Biomédicas

Trabalho efectuado sob orientação de:

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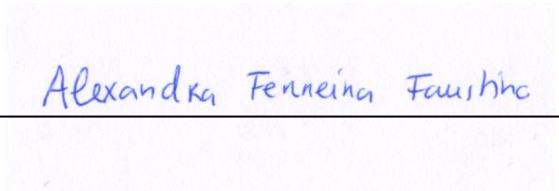
Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e/ou constam da listagem de referências incluída.



Resumo

Acetilação no N-terminal é uma das modificações co-traducionais mais comuns em células eucariotas. Esta reacção química é catalizada por uma família altamente conservada de N-acetiltransferases (NatA - NatF), que possuem subunidades e especificidades para o substrato distintas.

Apesar de a acetilação no N-terminal ter sido descrita há mais de 50 anos atrás, a sua função biológica continua pouco conhecida. Publicações recentes têm demonstrado que poderá ter uma papel na estabilidade, função, localização celular e secreção de proteínas.

Ao nível da regulação do tráfego de proteínas, foi observado em levedura que a acetilação no N-terminal, mediada pelo complexo NatA, inibe a translocação até ao retículo endoplasmático. Pelo contrário, foi demonstrado que a acetilação no N-terminal mediada pelo complexo NatC é importante para que haja a interacção das GTPases Arl3p e Grh1p com a membrana do Golgi, de Arl8a/b com a membrana do lisosoma e da proteína Trm1p-II com a membrana nuclear interna. Contudo, NatC não é um determinante geral da localização celular de proteínas, regulando apenas um grupo específico de substratos.

A função da acetilação no N-terminal durante o desenvolvimento de um organismo multicelular é pouco conhecida. Trabalhos prévios sugerem que o complexo NatC poderá ter um papel na regulação do crescimento e desenvolvimento de uma vasta gama de organismos.

O objectivo desta tese foi identificar e caracterizar fenotipicamente o complexo NatC em *Drosophila melanogaster*. Após identificação da subunidade catalítica (dnaa30), moscas mutantes por deleção foram obtidas (*dnaa30*Δ*74*).

Nós observámos que uma deleção no gene dnaa30 afecta a fertilidade de *Drosophila*: a maioria dos machos revelou ser estéril e uma redução da fertilidade das fêmeas foi observada. A contribuição maternal de *dnaa30*Δ*74* durante a oogénese leva a uma redução da fertilidade das fêmeas devido a problemas de divisão nuclear nos embriões. A contribuição maternal da versão selvagem de dnaa30 é no entanto essencial para a viabilidade dos machos mutantes (mutantes zigóticos).

O efeito observado na fertilidade é um fenótipo específico de dnaa30 pois a expressão ubíqua de dNaa30-Myc em machos mutantes leva à recuperação da fertilidade.

Observámos que a maioria da descendência proveniente do cruzamento de machos mutantes com virgens selvagens parecem ser ovos não fertilizados. Contudo a esterilidade dos machos mutantes não é resultante de problemas na linha germinal, uma vez que os machos mutantes revelaram ter uma espermatogénese e uma motilidade de esperma equivalente ao controlo. Assim, colocámos a hipótese da esterilidade ser devida a problemas de comportamento (ritual de acasalamento).

Apesar de preliminares, os nossos resultados sugerem que machos mutantes para dnaa30 possuem problemas ao nível do ritual de acalamento. Nenhum dos machos mutantes avaliados revelaram ritual de acasalemto.

Estes resultados sugerem que dnaa30 poderá ter uma função neuronal. A função neuronal de dnaa30 foi confirmada e em parte contribui para o fenótipo de esterilidade observado, pois a expressão de dNaa30-Myc nos neurónios de machos mutantes leva à recuperação da fertilidade. O papel de dnaa30 noutros tecidos não pode ser descartada, uma vez que a recuperação da fertilidade foi menor que a observada com a expressão ubíqua de dNaa30-Myc.

Os machos mutantes para dnaa30 revelaram também uma redução na longevidade e na locomoção. Estes fenótipos são independentes da esterilidade observada. Assim sendo, a esterilidade resultante de uma deleção no gene dnaa30 parece ser um fenótipo específico e não uma consequência da redução de mobilidade e longevidade.

Para explicar os fenótipos observados nos machos mutantes para dnaa30, propomos o seguinte modelo: a acetilação de Gie (*Drosophila* Arl8) no N-terminal, mediada por dnaa30, é uma mecanismo que regula a actividade dessa GTPase em diversos processos celulares dependentes de microtúbulos: transporte de proteínas pre-sinápticas, segregação de cromossomas...

Em resumo, o trabalho desta tese revela que naa30 em *Drosophila* tem um papel neuronal que afecta o ritual de acasalamento e a fertilidade. Tanto quanto nós estamos cientes, esta é a primeira vez que uma associação entre acetilação N-terminal e comportamento é observada num organismo multicelular.

Abstract

N-terminal acetylation (N-Ac) is one of the most common co-translational protein modifications in eukaryotic cells. This chemical reaction is catalyzed by a highly conserved family of N-acetyltransferases (NatA – NatF).

The function of N-terminal acetylation during the development of multicellular organisms is poorly known. Previous works suggest that NatC may regulate the growth and development in a wide range of organisms.

The aim of this thesis was to identify and phenotypically characterize the NatC complex in *Drosophila melanogaster*. After identification of its catalytic subunit (dnaa30), deletion mutant flies (*dnaa30Δ74*) were generated and characterized.

We observed that flies with a deletion in dnaa30 have dnaa30-specific fertility defects, which are not caused by problems in the germ-line development. The progeny of mutant males seems like eggs not fertilized or embryos that arrested very early. However, no problems in spermatogenesis were detected. Therefore, we hypothesized that *dnaa30* mutant males have courtship behavior defects.

Although the results are preliminary, we observed no courtship behavior in the evaluated mutant males. This observation suggests that dnaa30 may have a neuronal role in the brain. Indeed, we observed that neuronal expression of dNaa30-Myc is able to rescue the fertility in mutant males.

We also observed that *dnaa30* mutant males have mild locomotor defects and a reduced longevity. Importantly, we observed that these phenotypes and the sterility observed in *dnaa30* mutant males are uncoupled events. So the reduction in courtship behavior/fertility is a specific phenotype and not a consequence of the reduction in locomotion and in longevity.

Collectively, in this thesis we observed that naa30 in *Drosophila* may have a neuronal role which affects courtship behavior and fertility of flies. As far as we are aware, this is the first time that an association between N-terminal acetylation and the behavior of a multicellular organism is made.

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Abbreviations

ACTH	Adrenocorticotropic hormone
CDS	Coding DNA sequences
DA	Dorsal A
DIC	Differential interference contrast
FRT	Flipase recombination targets
GTP	Guanosine-5'-triphosphate
IGF-1	Insulin-like growth factor 1
Ile	Isoleucine
LC-MS-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
Leu	Leucine
Met	Methionine
MSH	α-melanocyte-stimulating hormone
mTORC1	mammalian target of rapamycin complex 1
NAT	N-terminal acetyltransferases
NPF	Neuropeptide F
PCR	Polymerase chain reaction
Phe	Phenylalanine
RNAi	RNA interference
RT	Room temperature
S6K	S6 kinase
STV/AZ	synaptic vesicle protein transport vesicles/active zone
SV	synaptic vesicle
Trp	Tryptophan
UAS	Upstream Activation Sequence
UTR	untranslated region

1. Introduction

N-terminal acetylation (N-Ac), which involves the transfer of an acetyl group from acetyl-CoA to the α-amino group of the first amino acid of a protein, is one of the most common protein modifications in eukaryotic cells [1, 2]. In lower eukaryotes (Saccharomyces cerevisiae), N- Ac was observed in 67.9% of all proteins, whereas in human cells 85% of all proteins are N-acetylated [3].

N-Ac occurs mainly co-translationally, as the nascent polypeptide emerges from the ribosome, and it is catalyzed by a highly conserved family of N-terminal acetyltransferases (NATs). There are several distinct NATs in eukaryotes (NatA to NatF) [3-5], which are composed by different subunits and with distinct substrate specificity, however, substrate redundancy among these enzymes has been shown.

NatA complex acetylates Ser-, Ala-, Thr-, Gly-, Val- and Cys- N-termini after the removal of the first methionine by methionine aminopeptidades. These N-termini amino acids are over-represented in eukaryotes proteome, so NatA is believed to be the major NAT complex in both yeast and human. This way, it was observed that human NatA-associated phenotypes seem to be slightly more severe than the ones observed for human NatB and NatC [6-8]. Aberrant expression of NatA subunits has been observed in different types of cancer cell tissues. Human Naa10 (NatA catalytic subunit) has been proposed as a potential cancer drug target [9].

Human NatC complex is evolutionarily conserved from yeast, being composed by the catalytic subunit Naa30 and the auxiliary subunits Naa35 and Naa38 (Mak3, Mak10 and Mak31 in yeast, respectively). *In vitro*, Naa30 is able to acetylate the first methionine of peptides with N-termini MLALI, MLGTG and MLGTE, being this substrate specificity also conserved from yeast [8]. Furthermore, it was observed that this acetylation is catalyzed by Naa30 independently of its association to the auxiliary subunits of NatC complex [8].

Despite being discovered more than 50 years ago [10], the biological function of N-Ac still mostly unknown. Nevertheless, recent data suggest N-Ac as a regulator of protein's function, stability, interaction with other molecules or cellular localization and secretion [11, 12].

Focusing in the role on N-Ac in protein targeting, it has been observed that yeast and higher eukaryotic proteins, with N-terminal signal sequence, show a bias against the excision of the

N-terminal methionine comparatively to cytosolic proteins. Consistent with this observation, it was shown that NatA-dependent N-Ac, which occurs subsequently to N-terminal methionine excision, inhibits yeast protein translocation into the endoplasmic reticulum [13]. In contrast, NatC-dependent N-Ac was shown to be required for membrane interaction of the GTPases Arl3p and Grh1p with the Golgi membrane and of Arl8a/b with the lysosomal membrane and for the association of the protein Trm1p-II with the inner nuclear membrane [8, 14-17].

The NatC-dependent N-Ac is not a general determinant for substrate subcellular localization since it was recently observed that none of the 13 tested suitable NatC substrates revealed altered localization in the absence of NatC catalytic subunit Naa30 (Mak30), in *S. cerevisiae* [18].

The lysosomal localization of Arl8 requires the N-terminally acetylation of the first methionine, mediated by Naa30, and the hydrophobic residues of its amphipatic helix. If the second amino acid of Arl8b (leucine) is mutated to a small residue (such as alanine), methionine aminopeptidases will remove the N-terminal methionine. This inhibits lysosomal targeting despite the fact that the second amino acid is still N-terminally acetylated by NatA. In contrast, if the leucine is replaced by phenylalanine, the mutant protein is efficiently acetylated without N-terminal methionine excision [16].

Arl8 is highly conserved across phylogeny. It does not exist in yeast, being present only in multicellular organisms. The existing publications suggest that Arl8 is involved in diverse cellular processes related to the microtubule-dependent transport of intracellular organelles. Reduction of Arl8a activity, through expression of dominant-negative mutant or knocking down of *Drosophila* Arl8 (Gie) with RNAi causes abnormalities in chromosome morphology and segregation, a process which requires the coordinated regulation of microtubule dynamics and a number of molecular motors [19]. Furthermore, it has been demonstrated that overexpression of Arl8a/b causes lysosomes to move more frequently and over greater distances, leading to a microtubule-dependent redistribution of lysosomes towards the plasma membrane [16].

The effect of Arl8 in microtubule-dependent processes seems to be not exclusive of proliferating cells since high expression of this small GTPase was detected in non-dividing human tissues such as brain and heart. Furthermore, a more recent work has shown that, in *C. elegans* motoneuron DA9, a loss-of function mutant of arl8 leads to a premature accumulation

of presynaptic cargoes in the proximal axon and concomitantly fail to properly assemble presynapses in distal segments [20, 21]. The authors showed that this phenotype seems to be the result of Arl8 regulating the capture and dissociation of STV/AZ proteins (synaptic vesicle protein transport vesicles/active zone), in part through its association with the anterograde motor protein UNC-104/KIF1A, and this way regulating the proper size, number and location of synapses [21]. The effect of Arl8 on the localization of SVs was showed to be not dependent on lysosomes biogenesis or activity.

If the NatC-dependent N-Ac of Arl8 affects other microtubule-dependent processes regulated by this small GTPase, like chromosome segregation and control of the balance between presynaptic protein transport and assembly, was never tested.

Previous works with functional characterization of NatC suggest that it functions to regulate the growth and development in wide range of organisms. In *S. cerevisiae*, the auxiliary subunit of the NatC complex, Naa35, revealed to be essential for optimal growth on nonfermentable carbon sources [22]. In *Arabidopsis thaliana*, Naa30 mutants are associated with defects in photosynthesis and growth, independently of NatC complex assembly [23]. It was observed that NatC is essential for the embryonic development of *zebrafish* [24], at least in part through the regulation of target of rapamycin (TOR) signaling events and, in *C. elegans*, Naa35 was described as an effector of the insulin/IGF-1 signaling that mediates stress resistance [25]. In human cells, knockdown of NatC subunits causes p53-depedent apoptosis and reduced cell growth [6].

Identification and functional characterization of NatC in *Drosophila melanogaster* was never performed. The aim of this thesis is to identify the catalytic substrate of NatC in *Drosophila* and to determine its functions during the development of this multicellular organism. We hypothesize that NatC may be a regulator of the targeting and function of a subset of proteins during *Drosophila* development.

2. Material and Methods

2.1. Fly work and genetics

Flies were raised under standard procedures. For generation of *Drosophila* naa30 mutant, the Bloomington stock BL# 16976 was used for imprecise excision of the P element P{EPgy2}CG11412EY10202 which is inserted in the 5' UTR of the *Drosophila* naa30 gene (CG11412). Standard protocols were used for performing the excision. Briefly, the Bloomington P element containing line 16976 was crossed to the Δ(2-3)99BSb/TM6B transposase line. F1 male flies were crossed with female virgins Df(1) pn38, w1/FM0 (BL# 7359). Then, F1 female flies, resulting from this second cross, were chosen as candidate deletions (white eye) and single-female crosses with males Df(1) pn38, w1/FM0 were performed for stock balancing. Nearly 100 lines were evaluated. Deletion sizes were determined by PCR and sequencing, using a forward primer 1 kb upstream (CAAGGAAAGTGGAGGAAGTGC) and a reverse primer 1.5 kb downstream (GGTATGTATCCCTCGCCAATG) of the 5' end of CG11412. Two lines with naa30 deletion were obtained: y*naa30*Δ*61*w/FM0 and y*naa30*Δ*74*w/FM0. As control, a line with precise excision of the P element was used: y*naa30*Δ*1*w/FM0.

Recombination of y*naa30*Δ*74*w/FM0 with w+ and of y*naa30*Δ*74*w/FM0 with FRT(w+)101 were performed to generate the stocks y*naa30*Δ*74*/FM0 (in this thesis referred as y*naa30*Δ*74*w+/FM0) and y*naa30*Δ*74*wFRT(w+)101/FM0.

Maternal mutants of naa30 were induced using the FLP/FRTovoD system (Chou and Perrimon, 1992). Germ line clones of y*naa30*Δ*74*w were made by crossing y*naa30*Δ*74*wFRT(w+)101/FM0;+/+ virgins with FRTovoD/Y;FLP22/FLP22 (BL# 1813) males and heat shocking the progeny at 37 °C during second and third larval instar. For control, germ line clones of ywGFP(w+) were made in the same conditions, using the stock ywGFP(w+)FRT(w+)101 (BL# 5153).

To drive the embryonic expression of CG11412-Myc, Nanos-GAL4 homozygous virgins were crossed with UAS-CG11412-6xMyc males. After eclosion, females were selected and crossed with wild-type males. F1 embryos were collected and used for protein extraction.

For complementation assays, the stock y*naa30*Δ*74*w+/FM7;;UAS-Naa30-Myc/TM3Sb was generated. For the single-male fertility assays, y*naa30*Δ74w+/FM7;;UAS-Naa30-

Myc/TM3Sb virgins were crossed with: wild-type, Actin5C-GAL4/TM6B, Nanos-GAL4 and nSyb-GAL4 males and the fertility of F1 males y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/+, y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/Actin5C-GAL4, y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/Nanos-GAL4 and y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/nSyb-GAL4 was evaluated.

To test the effect of Gie downregulation in male fertility, wild-type and nSyb-GAL4 virgins were crossed with Gie RNAi males (VDRC 26085). F1 males were used in the single-male fertility assay. The efficiency of Gie RNAi (VDRC 26085) was tested by crossing Gie RNAi males with Actin5C-GAL4/TM6B virgins.

2.2. Fly genomic DNA extraction

Five flies, both mutant and control, were collected and homogenized in 100 µl of Buffer A (100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS). After incubation at 65ºC for 15 min., 200 µl of 1.5 M KAc solution was added. Incubation on ice for at least 15 min. and centrifugation for 10 min. at RT were performed. Supernatant was collected and then DNA was precipitated using 0.6 times in volume of isopropanol. After centrifugation of 5 min. at RT, pellet was washed with 70% ETOH and dried. Genomic DNA was resuspended in 30 µl of H20 (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Transgenic flies

A PCR product of CG11412 CDS flanked by attB sites was produced. A recombination between the attB-flanked CG11412 CDS and an attP-containing donor vector (pDONR^{TM}221) was catalyzed by the BP Clonase enzyme (Gateway® BP Clonase® II Enzyme mix, Invitrogen, Grand Island, NY, USA). Constructs of Gie CDS (*Drosophila* Arl8), with N-terminal and/or C- terminal STOP codons, in pDONR^{TM}221 were synthetized (GenScript, Piscataway, NJ, USA). The recombination products (attL-substrates) were used for transformation of competent DH5α E. coli cells. The sequence of CG11412 CDS, in the DNA of the picked colonies, was confirmed using the M13 forward and the M13 reverse universal primers. A recombination mediated by the LR Clonase enzyme (Gateway® LR Clonase® II Enzyme mix, Invitrogen, Grand Island, NY, USA) was performed between the attL-containing entry vectors and an attR-containing destination vector. The attL-CG11412 vector

was recombined with a vector containing a UASp promoter and a 6x C-terminal Myc-tag (pPWM). The attL-Gie vector was recombined with: pPW, with a UASp promoter and no tag; pPWM and pPWG, with a UASp promoter and a C-terminal EGFP-tag. To confirm that CG11412 CDS is in frame with the Myc tag and the N-terminal sequence of CG11412 CDS in the expression vector, primers CTCGCCTCGCACGACAATCAGTACG and TGGCCATTGACGACCTCCCCAAGCG were used, respectively. The same was performed for the Gie constructs, using primers GATTCAACATGCGCAAGATC and CCAGCACGAGAACTGGAATG. Constructs were then used to generate transgenic fly stocks by microinjecting into w1118 embryos (BestGene, Chino Hills, CA, USA).

2.4. Protein extraction

Collections of 0–4 hr embryos expressing CG11412 (dNaa30)-Myc, dechorionated with 50% of bleach (commercial solution), were performed. Protein extraction was performed through homogenization of embryos in NB buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.1% NP-40, 1 mM DTT, 10 mM NaF, and EDTA-free protease inhibitor cocktail, Roche, Germany), and centrifugation at 21100xg for 3 min. Supernatant was collected and centrifuged twice. Total protein concentration was determined using Bio-Rad protein assay (BioRad, Hercules, CA, USA) that is based on the Bradford dye-binding method.

In complementation assays, to evaluate dNaa30-Myc expression levels, 5-4 males y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/+, y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/Actin5C-GAL4, y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/Nanos-GAL4 and y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/nSyb-GAL4 were collected and freezed at -80ºC. Protein extraction was performed as described above.

2.5. Co-Immunoprecipitation

Proteins extracts (1.5 mg) of embryos expressing CG11412 (dNaa30)-Myc were incubated with 1 µg of c-Myc Antibody (9E10) (Santa Cruz Biotechnology, Dallas, Texas, USA), during 1 h at 4°C. Subsequently, 0.9 mg of Dynabeads Protein G (Invitrogen, Grand Island, NY, USA) was added to this sample and an incubation of 1 h at 4°C was performed. After washing the beads 3 times with NB buffer, protein elution was performed with 100 µl of 100

mM Glycine pH 3.0 during 1 min. and stopped with 10 µl of 1M Tris Base pH 10.8. Proteins of eluate were precipitated using 5 times in volume of acetone, at -20°C and samples analyzed by liquid chromatography coupled to tandem mass spectrometry (Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Poland).

2.6. Mass spectrometry

Peptides mixtures were analyzed by LC-MS-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using Nano-Acquity (Waters, Milford, MA, USA) LC system and Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA, USA). Prior to analysis, proteins were subjected to standard 'in-solution digestion' procedure, during which proteins were reduced with 100 mM DTT (for 30 min at 56°C), alkylated with 0,5 M iodoacetamide (45 min in darkroom at room temperature), and digested overnight with trypsin (sequencing Grade Modified Trypsin—Promega V5111). The peptide mixture was applied to an RP-18 precolumn (nanoACQUITY Symmetry C18—Waters 186003514) using water containing 0,1% TFA as mobile phase, then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18–Waters 186003545) using an acetonitrile gradient (0%–35% AcN in 180 min) in the presence of 0.05% formic acid with a flow rate of 250 nl/min. The column outlet was directly coupled to the ion source of the spectrometer, operating in the regime of data dependent MS to MS/MS switch. A blank run ensuring no cross contamination from previous samples preceded each analysis.

Raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK, on-site license) against Flybase database. Search parameters for precursor and product ions mass tolerance were 15 ppm and 0.4 Da, respectively, enzyme specificity: trypsin, missed cleavage sites allowed: 0, fixed modification of cysteine by carbamidomethylation, and variable modification of methionine oxidation. Peptides with Mascot Score exceeding the threshold value corresponding to <5% False Positive Rate, calculated by Mascot procedure, and with the Mascot score above 30 were considered to be positively identified.

Human orthologs were determined using DSRC Integrative Ortholog Prediction Tool (DIOPT) (http://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl). Only scores above two were considered such as the best matches when there was more than one match per input.

2.7. Western blotting

To determine the efficiency of dNaa30-Myc immunoprecipitation, proteins of input and of eluate were boiled for 5 min in SDS-PAGE sample buffer, resolved by SDS-PAGE and then transferred onto HybondTM-ECL membrane (GE Healthcare, Little Chalfont, UK). Membrane was blocked with 5% non-fat milk/ phosphate buffered saline (PBS, pH 7.4) containing 0.1% Tween-20 (PBST), during at least 1 hr. Incubation with the anti-Myc rabbit primary antibody (Covance, Princeton, New Jersey, USA) was performed at a dilution of 1:2000 during overnight at 4ºC. Protein was detected using a horseradish peroxidase-conjugated secondary antibody (1:4000) and a luminescence system. In rescue assays, the levels of expression of dNaa30-Myc were determined the same way, using protein extracts of all flies.

In complementation assays, evaluation of dNaa30-Myc expression was performed as described above.

2.8. Immunohistochemistry

0-3 hr embryos, both maternally mutant for *Drosophila* naa30 and control, were collected, dechorionated with 50% of bleach, fixed with 4% of formaldehyde and devitellinized with methanol, vigorously shaking during 1 min. After rehydration, with progressively increased percentage of PBST, DNA staining was performed with DAPI (1:5000).

2.9. Differential interference contrast (DIC) microscopy of live testes

Dissection of testes from newly eclosed y*naa30*Δ*74*w/Y and y*naa30*Δ*1*w/Y males was performed in fresh TB1 buffer: 15 mM potassium phosphate (equimolar dibasic and monobasic) pH 6.7, 80 mM KCl,16 mM NaCl, 5 mM MgCl2 and 1% polyethylene glycol (PEG) 6000. After transfer to a drop of TB1 buffer, testes and seminal vesicles were open up by cutting them with tungsten needles or forceps. A clean cover slip was placed over the testes.

2.10. Embryo staging

Collections of 1-3 hr of embryos/eggs resulting from the cross of 4-8 days old wild-type virgins with 0-3 days old y*naa30*Δ*74*w+/Y males (in a 2:1 proportion) were fixed with 4% of formaldehyde. Staining of DNA was performed with Sytox Green or DAPI (1:5000). As control, yw+/Y (BL# 169) males were also tested, with the same conditions.

The same experiment was performed with y*naa30*Δ*74*w/Y and the control y*naa30*Δ*1*w/Y and w118/Y males. Two replicas were performed.

At least 19 embryos/eggs, per genotype, were classified in: class I, not fertilized- 2 nuclear divisions; class II, 2 nuclear divisions - interphase 14; class III, interphase 14 or class IV- > interphase 14.

2.11. Eclosion of *Drosophila* males

The number of y*naa30*Δ*74*w/Y and FM0/Y males that are eclosing from the cross of y*naa30*Δ*74*w/FM0 virgins with FM0 males was counted every two days, from the 10th day till the 16th day. The eclosion of males was counted in at least two crosses flipped at least two times. As control the number of y*naa30*Δ*1*w/Y and FM0/Y males that are eclosing from the cross of y*naa30*Δ*1*w/FM0 virgins with FM0 males was counted in the same conditions.

2.12. Longevity assay

The longevity of 0-3 days old y*naa30*Δ*74*w+/Y males was tested. Thirty males were distributed by 6 tubes (5 males in each) and the number of males was counted every two days, during 18 days. As control, the longevity of yw+/Y males was also tested, with the same conditions. The same experiment was performed with y*naa30*Δ*74*w/Y and the control y*naa30*Δ*1*w/Y and w118/Y males. Two replicas were performed.

To test the effect of dNaa30-Myc expression in the longevity of mutant males, longevity assay was performed with y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/Actin5C-GAL4, y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/nSyb-GAL4 and y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/Nanos-GAL4 males, using the conditions previously described for the mutant males. As

control, the longevity of y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/+ males was also tested. The assay was performed till the death of all males.

2.13. Single-male fertility

To test the fertility of y*naa30*Δ*74*w+/Y males, 0-3 days old single males were crossed, during at least 3 days, with four wild-type virgins with 3 to 9 days. Tubes were flipped 3 times. The presence or absence of larvae was observed at least 3 days after mating, in the total of 4 tubes. As control, the fertility of single yw+/Y males was also tested, with the same conditions. Two reproducible replicas were performed. At least 26 males were tested, in each replica.

The same experiment was performed with y*naa30*Δ*74*w/Y and the control y*naa30*Δ*1*w/Y and w118/Y males.

To test the effect of dNaa30-Myc expression in the fertility of mutant males, single-male fertility assay was performed with y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/ Actin5C-GAL4 and y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/nSyb-GAL4 and y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/Nanos-GAL4 males, using the conditions previously described for the mutant males. As control, the fertility of single y*naa30*Δ*74*w+/Y;; UAS-Naa30-Myc/+ males was also tested. Two reproducible replicas were performed. At least 31 males were tested, per genotype, in each replica.

To test if Gie dowregulation in the brain phenocopies *dnaa30* mutant, the fertility of +/Gie RNAi and nSyb-GAL4/Gie RNAi males was tested. At least 19 males were tested.

2.14. Egg hatching

Embryos, both maternally mutant for *Drosophila* naa30 and control, were collected on apple juice agar. The number of hatched eggs was counted 2 days after egg laying.

2.15. Male courtship behavior assay

Male courtship behavior was evaluated in 5 couples of 7 days old wild-type females and 1-2 days old *y*naa30Δ*74*w+/Y males. Flies were allowed to recover from anesthesia nearly 18 hr

before female and male were put together. Male courtship behavior was evaluated by the occurrence of the singing step (extension and vibration of one wing) and was performed during 1 hr. As control, yw+/Y males were tested with the same conditions.

2.16. Negative geotaxis assay

Groups of 10 y*naa30*Δ*74*w+/Y males with 0-3 days were sorted and separated. Flies were allowed to recover from anesthesia at least 12 hr before being transferred to a 50 ml graduated cylinder. Flies were acclimatized to the new setting for at least 1 min. and then tap down to the bottom. The number of flies that can climb above a 7.5-cm mark by 10 and 30 sec was measured. For the same group of 10 flies, this assay was repeated at least 10 times, allowing for 1 min. rest period between each trial. The number of flies per group that passed the 7.5-cm mark was recorded as a percentage of total flies. As control, yw+/Y males were tested with the same conditions.

3. Results

All the results presented were obtained by Alexandra Faustino, except the ones of Fig. 3.1.1 D, performed by Thomas Arnesen, Fig. 3.2.1, performed by Bárbara Kellen and Fig. 3.4.1, performed by Paulo Navarro.

3.1. CG11412 is the catalytic subunit of *Drosophila* NatC complex

Arnesen and coworkers presented in a previous work [8], where they described the human NatC complex, the alignments of putative Naa30 and Naa38 candidates from different species and they revealed (Dm) NP_569903 as the putative *Drosophila melanogaster* homologue of yeast Naa30. NP_569903 corresponds to the gene CG11412 isoform C and this record was removed from NCBI database by RefSeq staff.

In order to clarify which is the catalytic subunit of NatC complex in *D. melanogaster*, the sequences of Naa30 protein of *S. cerevisiae* and of *Homo sapiens* were used in a BLASTp search (in NCBI and Flybase) against *D. melanogaster* proteome. As a result of bidirectional BLASTp we obtained CG11412 isoform A and D as the sequence most similar to Naa30 of *S. cerevisiae* and *H. Sapiens*. Isoform D differs from isoform A in the presence of an additional intron and in a shorter N-terminal sequence. By analyzing the RNA-Seq expression data available in modENCODE Transcription Group (www.flybase.org), for several different stages of *Drosophila* development, for both isoforms, we can observe that CG11412 isoform D is infrequent. Therefore, in this thesis we are going to focus on isoform A, referred as CG11412.

Overall, CG11412 is 52.3% (81/155) identical and 65.2% (101/155) similar to yeast Naa30 and 72.7% (109/150) identical and 81.3% (122/150) similar to human Naa30 (Fig. 3.1.1 A and B).

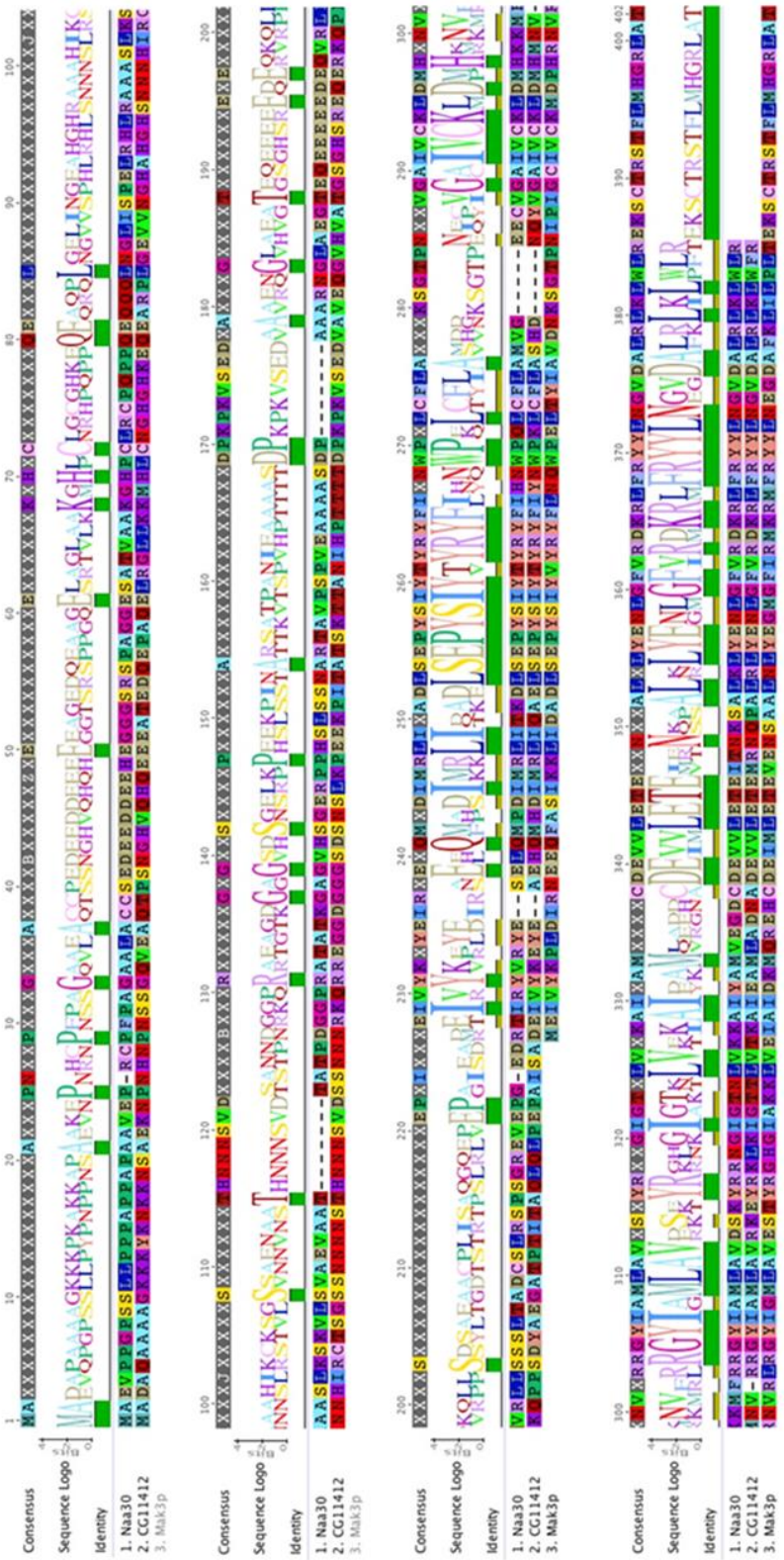
The yeast Naa30 is 201 amino acids shorter than the *Drosophila* ortholog and both share only the C-terminal region of CG11412 (the last 149 amino acids). Interestingly, although CG11412 and hNaa30 have identical sizes, similarity between fly and human orthologs is also only observed in the C-terminal region (the last 148 amino acids) (Fig. 3.1.1 B). The function of the N-terminal domain of Naa30 is not known.

To confirm that CG11412 corresponds to a subunit of NatC complex in *Drosophila*, CG11412-Myc was co-immunoprecipitated from fly embryonic protein extracts (Fig. 3.1.1 C) and the CG11412-interacting proteins were analyzed by LC-MS (Table I). CG11412-Myc protein was efficiently immunoprecipitated (Fig. 3.1.1 C) and a total of 28 interacting proteins were detected by LC-MS (Table I). However, only 7 out of the 28 proteins were detected in both replicas: CG4065, Hsp27, cathD, TER94, alphaTub67C, 26-29-p and CG6509, being CG4065 the *Drosophila* ortholog of hNaa35 (Table I). The co-immunoprecipitation of the ortholog of hNaa35 confirms that our proposed dNaa30 (CG11412) is part of the *Drosophila* NatC complex and that the interaction of Naa30 with Naa35 is conserved from *Drosophila* to humans.

To validate the functional activity of the candidate of *D. melanogaster* orthologue of yeast Naa30, Thomas Arnesen and coworkers investigated whether CG11412 was able to restore the mislocalization of Arl3 observed in yeast naa30Δ. Exogenous expression of CG11412 in a yeast strain lacking naa30 (naa30Δ [dNAA30]) rescues the Arl3-GFP Golgi localization therefore implying the capacity of CG11412 to Nt-acetylate this well characterized NatC substrate (Fig. 3.1.1 D). Thus, our proposed dNaa30 functionally rescues the activity of yeast Naa30, suggesting that the activity of this subunit is conserved from yeast to *Drosophila*.

In resume, we identified CG11412 as the most likely catalytic substrate of *Drosophila* NatC complex and refer to it as dNaa30.

A



(Figure legend is presented in the next page)

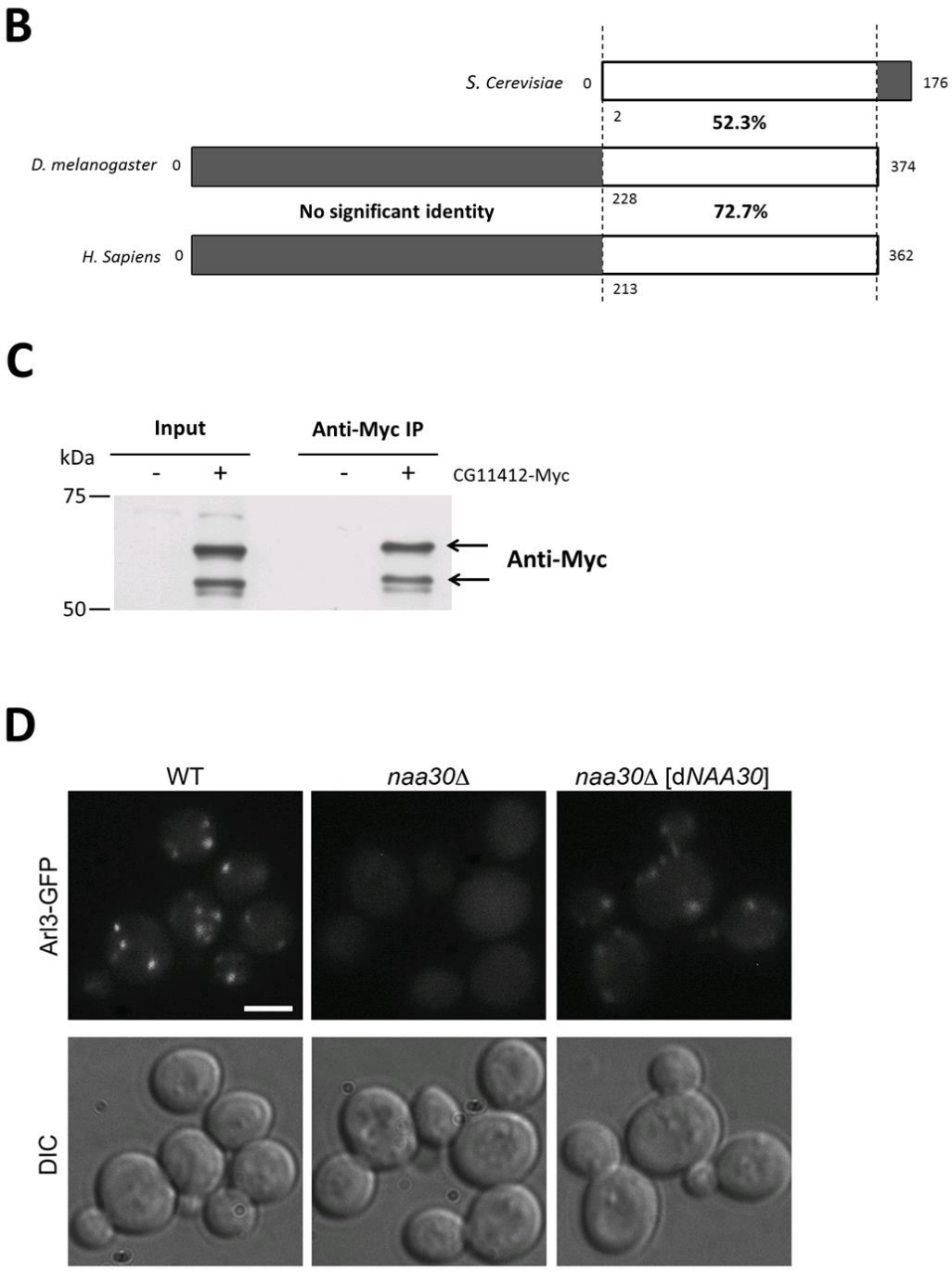


Figure 3.1.1 - CG11412 is the catalytic subunit of *Drosophila* NatC complex. (A) Sequence alignment of the putative *Drosophila* Naa30 (CG11412) with the homologs of *S. Cerevisiae* (Mak3p) and *H. Sapiens* (Naa30). The alignments were performed using the Geneious Aligner. Sequence logo diagram shows sequence heterogeneities. The height of letters indicates the probability of the amino acid at that position. (B) Schematic representation of the identity degree observed between the putative *Drosophila* Naa30 and the homologs of *S. Cerevisiae* and *H. Sapiens*, at the N- and C-terminal protein regions. (C) Co-Immunoprecipitation of CG11412-Myc from protein extracts of fly embryos, using a c-Myc Antibody and Dynabeads Protein G. As control, protein extracts of embryos without (-) expression of CG11412-Myc (wild-type embryos) were used. The efficiency of CG11412-

Myc immunoprecipitation was evaluated by western blot using a c-Myc Antibody. CG11412-Myc is indicated by arrows. (D) Arl3-GFP localization studied by live imaging of yeast cells of different genotype (indicated) (by Thomas Arnesen et al.). The observed mislocalization of Arl3 in naa30Δ cells was reversed by the exogenous expression of the *D. melanogaster* orthologue, dNaa30 (CG11412) (n>100 for each strain). Scale bar equals 2 µm. Bottom panel: DIC (Differential Interference Contrast) microscopy of the cells in the top panel.

Table I. LC-MS analysis of co-immunoprecipitation assays from CG11412-Myc embryos.

Drosophila			**CG11412-Myc embryos**		**Control embryos**		
CG	**Gene**	**Human**	**rep1**	**rep 2**	**rep1**	**rep 2**	**Molecular function**
CG11412	-	NAA30	10	10	-	-	N-acetyltransferase activity
CG4065	-	NAA35	10	7	-	-	-
CG4466	Hsp27	HSPB1	2	2	-	-	Protein binding
CG14792	sta	RPSA	2	-	-	-	Structural constituent of ribosome
CG2207	Df31	-	2	-	-	-	Histone binding
CG11988	neur	NEURL1B	-	1	-	-	DNA binding, ubiquitin-protein ligase activity; phosphatidylinositol phosphate binding; zinc ion binding
CG1548	cathD	CTSD	1	1	-	-	Aspartic-type endopeptidase activity
CG5374	T-cp1	TCP1	1	-	-	-	Hydrogen-exporting ATPase activity, phosphorylative mechanism; unfolded protein binding; ATP binding
CG6692	Cp1	CTSV	2	-	-	-	Cysteine-type endopeptidase activity

CG9091	RpL37a	RPL37	1	-	-	-	Structural constituent of ribosome
CG5525	-	CCT4	1	-	-	-	ATPase activity, coupled; ATP binding; unfolded protein binding
CG2331	TER94	VCP	1	1	-	-	ATPase activity; ATP binding; hydrolase activity; protein binding
CG8308	αTub67C	TUBAL3	1	1	-	-	Structural constituent of cytoskeleton; GTP binding; GTPase activity
CG8863	Droj2	DNAJA4	1	-	-	-	ATP binding; unfolded protein binding; heat shock protein binding
CG7726	RpL11	RPL11	1	-	-	-	Structural constituent of ribosome; protein binding
CG31950	-	LSMD1	-	1	-	-	-
CG8947	26-29-p	CTSF	1	1	-	-	Cysteine-type endopeptidase activity
CG14206	RpS10b	RPS10	2	-	-	-	Structural constituent of ribosome
CG4464	RpS19a	RPS19	2	-	-	-	Structural constituent of ribosome
CG4169	-	UQCRC2	1	-	-	-	Ubiquinol-cytochrome-c reductase activity; metal ion binding
CG3612	blw	ATP5A1	1	-	-	-	Hydrogen-exporting ATPase activity, phosphorylative mechanism; proton-transporting ATPase activity,

							rotational mechanism; ATP binding; proton-transporting ATP synthase activity, rotational mechanism
CG7917	Nlp	NPM3	1	-	-	-	Histone binding; ATPase activity, coupled; histone binding; nucleic acid binding
CG11876	-	PDHB	-	1	-	-	Pyruvate dehydrogenase (acetyl-transferring) activity;
CG6509	-	DLG5	1	1	-	-	-
CG7070	PyK	PKM	-	1	-	-	Pyruvate kinase activity; magnesium ion binding; potassium ion binding
CG1883	RpS7	RPS7	1	-	-	-	Structural constituent of ribosome
CG11154	ATPsyn-beta	ATP5B	1	-	-	-	Hydrogen-exporting ATPase activity, phosphorylative mechanism; ATP binding; proton-transporting ATP synthase activity, rotational mechanism
CG7111	Rack1	GNB2L1	1	-	-	-	Protein kinase C binding
CG1483	Map205	MUC16	1	-	-	-	Microtubule binding

Co-immunoprecipitation was performed using total protein extracts from embryos expressing Myc-tagged CG11412 CDS. Protein extracts from embryos not expressing the Myc-tagged CG11412 were used as negative controls (wild-type embryos). Information about the molecular function of the detected *Drosophila* proteins was obtained in www.flybase.org. Proteins obtained in both replicas are highlighted in orange.

3.2. dnaa30 is required for *Drosophila* fertility.

3.2.1. Generation of a *Drosophila* naa30 mutant by P-element imprecise excision.

dnaa30 gene is located on the X chromosome and is formed by 2665 base pairs (bp). A schematic representation of the genomic organization of dnaa30 is shown in Fig. 3.2.1 A.

To analyze dnaa30 function during *D. melanogaster* development, a *Drosophila* dnaa30 mutant was generated by imprecise excision of the P-element EY10202, which is inserted in the first exon of dnaa30 gene (Fig. 3.2.1 A and B). The isoform A of dnaa30 gene is composed of 3 exons. Exon 1 encodes the 5' untranslated region (represented by grey bar in Fig. 3.2.1 B) and the initiation codon.

The screening of candidates for deletion mutants was done by PCR, using a forward primer 1 kb upstream and a reverse primer 1.5 kb downstream of the 5' end of dnaa30 gene (Fig. 3.2.1 A and B). In control (without deletion), this pair of primers amplifies a band of ~2500 bp, as can be observed in Fig. 3.2.1 C for w118 genomic DNA. Lines with smaller PCR products were chosen as candidates of dnaa30 deletion and deletions sizes were confirmed by sequencing, using the same pair of primers. Two independent excision lines with varying deletion sizes were isolated (Fig. 3.2.1 B and C). Line Δ74 (y*naa30*Δ*74*w/FM0) contains the smallest deletion (1172 bp) that extends from exon 1 (5' untranslated region) into exon 2. Line Δ61 (y*naa30*Δ*61*w/FM0) has a deletion of 1583 bp, starting from nearly 300 bp upstream of the 5' end of dnaa30 gene and extending also into exon 2. For line 1 a PCR band identical to control was observed, suggesting that precise excision of the P-element occurred.

Since its deletion is restricted to dnaa30 gene, line Δ74 was chosen to characterize dnaa30-dependent phenotypes. Line Δ1 was used as a genetic background control.

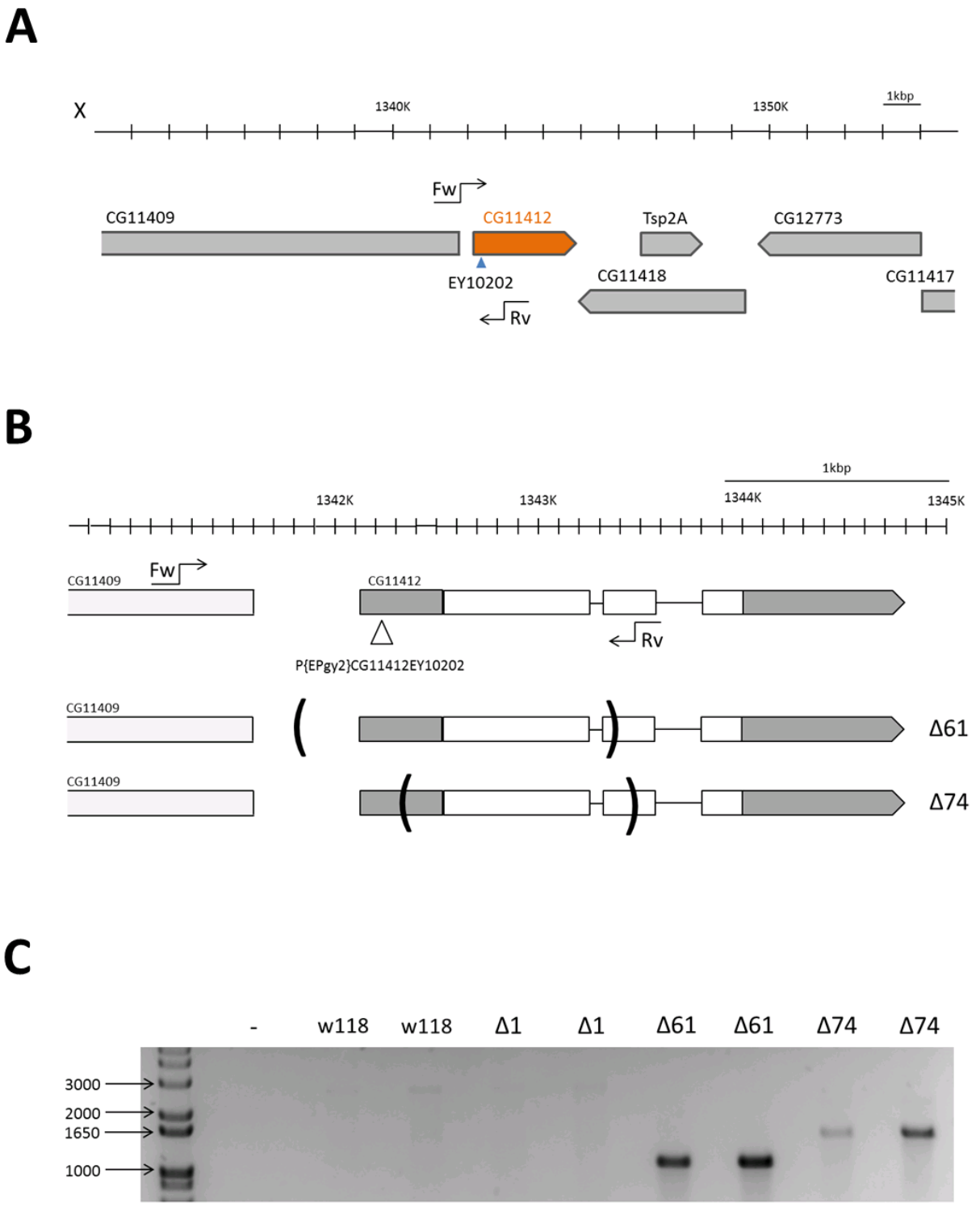


Figure 3.2.1 - Generation of a *Drosophila* naa30 mutant by P-element imprecise excision. (A) Genomic organization of dnaa30. The scheme shows the insertion site of the P-element EY10202 as well as the position of the forward (Fw) and reverse (Rv) primers used to screen deletion candidates by PCR and sequencing. (B) Arrangement of introns and exons of dnaa30 isoform A. The 5' and 3'untranslated regions are represented at grey. The insertion site of the

P-element EY10202 as well as the position of the Fw and Rv primers, used to screen deletion candidates by PCR and sequencing, are represented. Extension of the deletion of dnaa30 in line Δ61 and Δ74 is indicated (between brackets). (C) Screening of possible deletion mutants. Agarose gel picture, with the PCR products obtained with the genomic DNA of w118, line Δ1, Δ61 and Δ74 males, is shown. Amplification was performed using the pair of primers referred in (A) and (B).

3.2.2. Deletion of dnaa30 affects the fertility of male flies.

Characterization of *dnaa30Δ74* mutant line revealed that, in *Drosophila*, naa30 is not essential. Mutant flies look anatomically normal. Interestingly, we observed an absence of females homozygous for the deletion (y*naa30Δ74*w/y*naa30Δ74*w) and a reduction in the expected number of mutant males (y*naa30Δ74*w/Y - expected mendelian frequency is 50%). As dnaa30 is located on the X chromosome, these observations lead us to hypothesize two possible explanations: (1) in homozygosity, dnaa30 deletion affects female flies viability and/or (2) dnaa30 deletion affects the fertility of males. Thus, the fertility of mutant males and of respective controls (y*naa30Δ1*w/Y and w118/Y males) was evaluated. We observed a decreased fertility in mutant males (y*naa30Δ74*w/Y): after three days of mating, almost all mutant males revealed to be sterile (97.5 ± 3.5 %, N=39) while almost all the control males were fertile (100 ± 0.0 %, N=40, for w118 and 90.0 ± 14.1 %, N=40, for Δ1 males) (Fig. 3.2.2 A).

Previous works have implicated the white gene in male courtship control [26-28]. Our original stock, with the P-element inserted in the dnaa30 gene, and the concomitant isolated deletion lines are in white background (w). To validate the fertility defect observed in our mutant, a recombination of *dnaa30Δ74* and wild-type white gene (w+) was generated (confirmed by PCR and sequencing) and single-male fertility assays were performed with the recombinant (y*naa30Δ74*w+/FM0). The fertility defect was confirmed in mutant males with wild-type white gene: after three days of mating, 98.5 ± 2.1 % (N=61) of mutant males (y*naa30Δ74*w+/Y) were sterile while, in control (yellow males), sterility was observed in 30.5 ± 5.0 % (N=65) of males (Fig. 3.2.2 B). This way, we assume that the sterility phenotype observed in *dnaa30Δ74* deletion mutants is specific and independent of the white gene.

3.2.3. Expression of dNaa30-Myc rescues the fertility defects observed in *dnaa30* mutant males.

To determine whether the sterility phenotype observed in our deletion mutant is dnaa30-dependent, rescue experiments were performed. We used the GAL4/UAS system to express dNaa30-Myc in the mutant background and performed single-male fertility assays to evaluate complementation levels. When dNaa30-Myc expression is induced in mutant males (w+ background) by a ubiquitous driver (Actin5C-GAL4), 64.7% ± 22.5% (N=95) of males showed to be fertile while in the control, male flies with UAS-dNaa30-Myc but with no GAL4 driver, only 14.0 ± 7.9 % (N=113) were fertile (Fig. 3.2.2 C). Interestingly, when the Nanos-GAL4 driver was used to induce the expression of dNaa30-Myc in the germ-line, fertility was observed only in 12.3 ± 2.3 % (N=111) of mutant males (Fig. 3.2.2 C).

Fig. 3.2.2 D confirms that the fertility rescue observed with Actin5C-GAL4 driver comparatively to both controls is due to expression of dNaa30-Myc.

These results suggest that the decreased fertility observed in our mutant deletion is dnaa30 specific.

3.2.4. Deletion of dnaa30 affects the fertility of female flies.

To investigate if dnaa30 deletion also affects female fertility, homozygous germ-line *dnaa30*Δ*74* clones were induced in ovaries using the FLP/FRT system. We observed a reduction of nearly 50% in the eclosion of maternal mutant eggs (49.6 ±2.5% of not eclosed eggs, N=6899) (Fig. 3.2.2 E). As control, eclosion of eggs maternally homozygous for GFP was evaluated and almost all eclosed (94.2 ± 0.1%) (Fig. 3.2.2 E, N=3711).

Therefore, dnaa30 seems to be important for the fertility of female and male *Drosophila*.

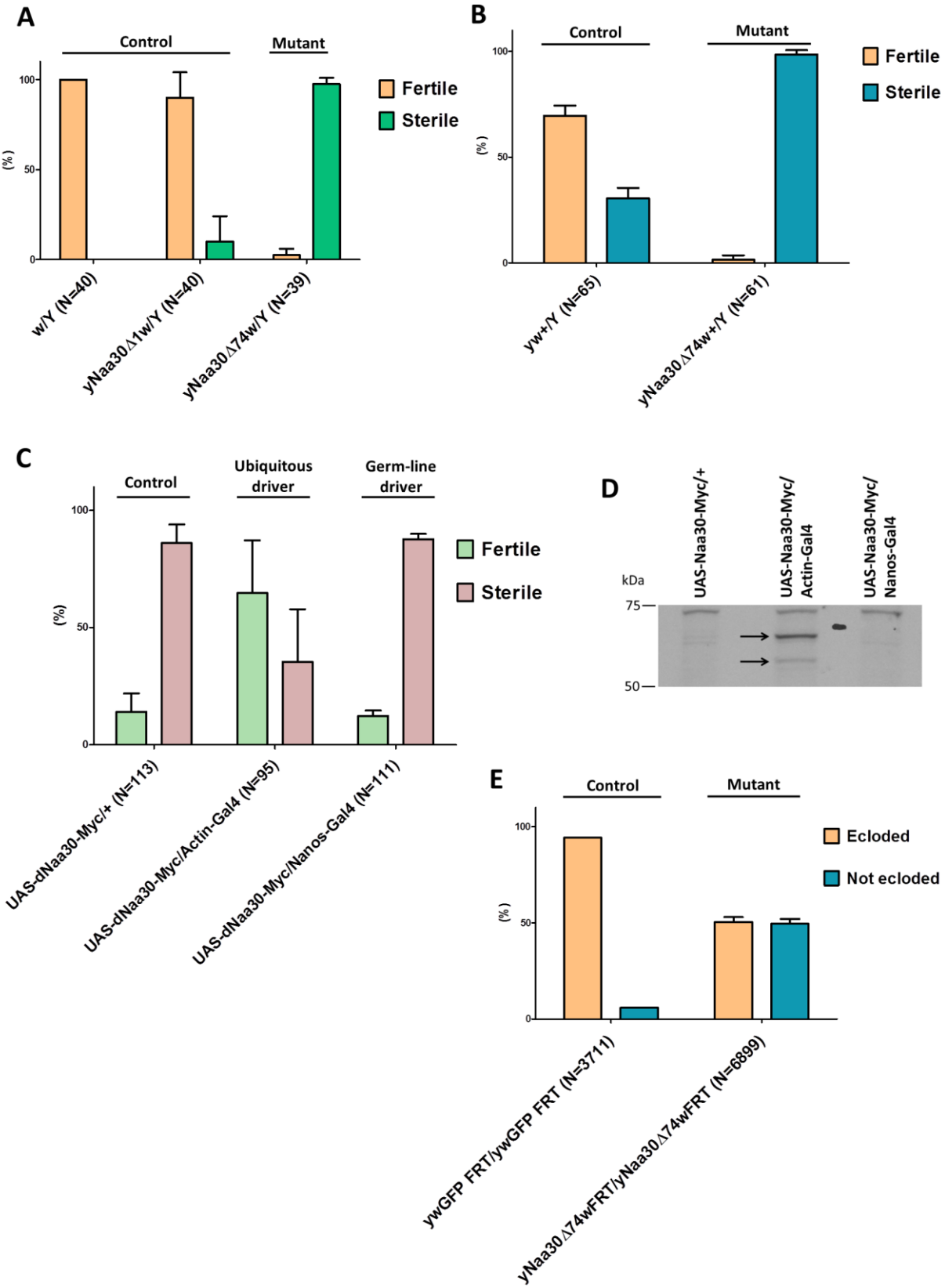


Figure 3.2.2 - Deletion of dnaa30 affects fly fertility. (A) Results of single-male fertility assays performed with mutant males in white background (y*naa30*Δ*74*w/Y) and respective controls (line Δ1 and w118 males), represented in terms of percentage of sterile and fertile males observed. (B) Results of single-male fertility assays performed with mutant males with

wild-type white gene (y*naa30*Δ*74*w+/Y) and respective controls (yellow males, yw+/Y), represented in terms of percentage of sterile and fertile males observed. (C) Results of fertility rescue observed in single-male fertility assays performed with mutant males (with wild-type white gene) with or without induction of dNaa30-Myc expression. The UAS/GAL4 system was used to induce the expression of UAS-dNaa30-Myc with different drivers: the ubiquitous Actin5C-GAL4 and the germ-line Nanos-GAL4 drivers. As control, the fertility of mutant males with UAS-dNaa30-Myc but with no driver was evaluated. In (A), (B) and (C), male fertility was evaluated by the presence or absence of larvae at least 3 days after mating with wild-type virgins. (D) Western blot analysis of dNaa30-Myc expression in protein extracts of whole mutant flies with Actin5C-GAL4, Nanos-GAL4 or no driver. dNaa30-Myc is indicated by arrows. (E) Results of the fertility of females with germ-line homozygous *dnaa30* mutant or GFP clones. Female fertility was evaluated by the percentage of egg eclosion, 2 days after laying. In (A), (B), (C) and (E), at least two independent replicas were performed.

3.3. dnaa30 deletion affects the longevity of mutant males.

Upon characterization of *dnaa30*Δ*74* mutant line we observed no homozygous mutant females and a reduction in the number of expected mutant males.

As dnaa30 is located on the X chromosome we can postulate that the absence of homozygous mutant females may be consequence of the sterility phenotype observed in the mutant deletion males.

To explain the decreased number of mutant males we propose two hypotheses: (1) mutant males eclose in a lower number and/or (2) longevity of mutant males is reduced.

The number of both males with dnaa30 deletion (y*naa30*Δ*74*w/Y) and with the balancer (FM0/Y) was evaluated every two days, from 10th till 16th day. No reduction in the total eclosion of mutant males was observed, since we counted similar total numbers of both type of males (Fig. 3.3.1 A), with a slightly lower number of mutant males than control (1.3 times and 1.1 times lower, replica I and replica II, respectively, Fig. 3.3.1 A).

When we evaluated the number of males per day it was observed that mutant flies seem to have a delayed eclosion relatively to control males with the balancer. This observation is particularly evident at day 10, where a mean of 14.5±3.5% (N=788) control males was observed while only 2.5±0.7% (N=702) of mutant males has eclosed at this day (Fig. 3.3.1 B).

When we compared the eclosion of mutant males with the one of line Δ1 males, we observed that the eclosion of mutant males still seem delayed (Fig. 3.3.1 C).

When we evaluated the number of males resulting from the cross of wild-type males with females carrying homozygous germ-line clones of *dnaa30*Δ*74*, we observed a great reduction in the total eclosion of maternal and zygotic *dnaa30*Δ*74* mutant males (2.9±0.6%, N=6610) (Fig. 3.3.1 D). As control, we counted the number of maternal and zygotic GFP males, that eclosed normally (eclosion of 44.8±1.9%, N=10623, the expected mendelian frequency is 50%). These results suggest that the normal eclosion rate of zygotic *dnaa30*Δ*74* mutant males is due to the maternal contribution of dnaa30.

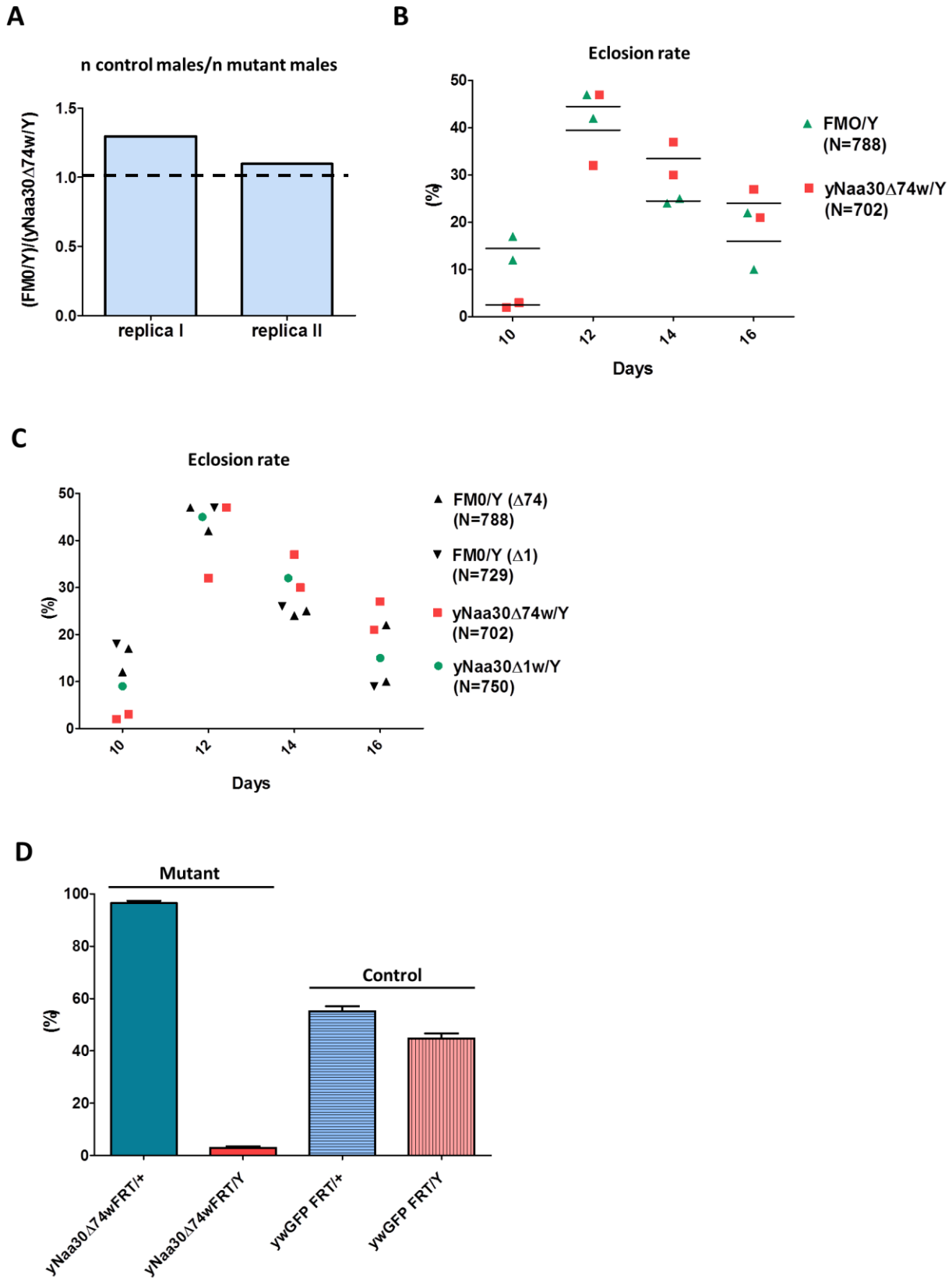


Figure 3.3.1 - *dnaa30Δ74* have no significant effect in the eclosion of male flies. (A) Total number of mutant (y*naa30Δ74*w/Y) and FM0/Y males which eclosed from 10th till 16th day, in two independent replicas. Values are represented as a ratio of the numbers obtained for both type of males (FM0/Y males/mutant males). (B) Eclosion rate of both males with

deletion mutation (y*naa30*Δ*74*w/Y) and with the balancer (FM0/Y). The number of males was counted every two days, from 10th till 16th day. For each day and each type of males the value of two replicas and the respective mean are represented. Results are represented as a percentage of eclosed males. (C) Eclosion rate of line Δ74 (y*naa30*Δ*74*w/Y) and line Δ1 (y*naa30*Δ*1*w/Y) males and of their respective controls (FM0/Y). The number of males was counted every two days, from 10th till 16th day. Results are represented as a percentage of eclosed males. (D) Eclosion of maternal and zygotic mutant flies. The total number of maternal and zygotic mutant females and males (y*naa30*Δ*74*wFRT/+ and y*naa30*Δ*74*wFRT/Y), resulting from the cross of wild-type males with females carrying homozygous germ-line clones of *dnaa30*Δ*74*, was evaluated. As control, the number of maternal and zygotic GFP flies was counted (ywGFP FRT/+ and ywGFP FRT/Y). Results are represented as a percentage of counted flies. In (A), (B) and (D), two independent replicas were performed.

Thus, we can conclude that the lower number of zygotic mutant males observed in *dnaa30*Δ*74* line may not be explained by a reduction in eclosion rate (hypothesis 1). Consequently, we tested if it may be a consequence of a reduction in the longevity of mutant males (hypothesis 2). Indeed we observed that there is decrease in the longevity of mutant males (y*naa30*Δ*74*w/Y) comparatively to control males (w118/Y and y*naa30*Δ*1*w/Y). At day 18, a mean of 41.3 ± 18.9% (N=90) of mutant males were living (63.0%, 33.0% and 28.0% in replica I, II and III, respectively) vs 97.7±2.1% (N=85) of white males (97.0%, 100.0% and 96.0% in replica I, II and III, respectively) and 97.3± 2.3% (N=90) of line Δ1 males (96.0%, 100.0% and 96.0%, in replica I, II and III, respectively) (Fig. 3.3.2 A and A').

This reduction in longevity was also observed in the w+ background. At day 18, a mean of 17.0 ± 14.1% (N=60) of mutant males were living (7.0% in replica I and 27% in replica II) vs 98.0± 14.1% (N=60) of control yellow males (96.0% in replica I and 100.0% in replica II) (Fig. 3.3.2 B and B').

To determine whether the reduction in longevity observed in our deletion mutant is dnaa30-dependent, the longevity of mutant males with or without induction of dNaa30-Myc expression was evaluated. No evident differences were observed between the longevity of mutant males without the Gal4 driver but with the dNaa30-Myc construct (UAS-dNaa30-Myc/+) comparatively to the ones expressing it ubiquitously (Actin5C-GAL4) or in the germ-line (Nanos-GAL4). However a small increase in longevity was observed with the ubiquitous expression of dNaa30-Myc (Fig. 3.3.2 C).

Leaky expression of dNaa30-Myc, with no GAL4 driver, rescues the decrease in longevity observed in mutant males. At day 14, 97.0% (N=30) of mutant males with UAS-dNaa30-Myc and with no driver were living vs 25.0± 21.2% (N=60) of mutant males without this construct

(10.0% in replica I and 40.0% in replica II, Fig. 3.3.2 B'). These results suggest that the sterility and longevity phenotypes observed in the *dnaa30* mutant males are mostly independent of each other. Although a rescue of the longevity phenotype is observed in mutant males with UAS-dNaa30-Myc and no driver, the majority of these males are nevertheless still sterile (86.0±7.9%, Fig. 3.2.2 C).

In resume, we observed that dnaa30 has a possible role in *Drosophila* longevity, which may explain the reduction in the number of mutant deletion males observed in line *dnaa30Δ74*. Yet, the sterility phenotype observed in the *dnaa30Δ74* deletion mutant males is mostly independent of reduced longevity.

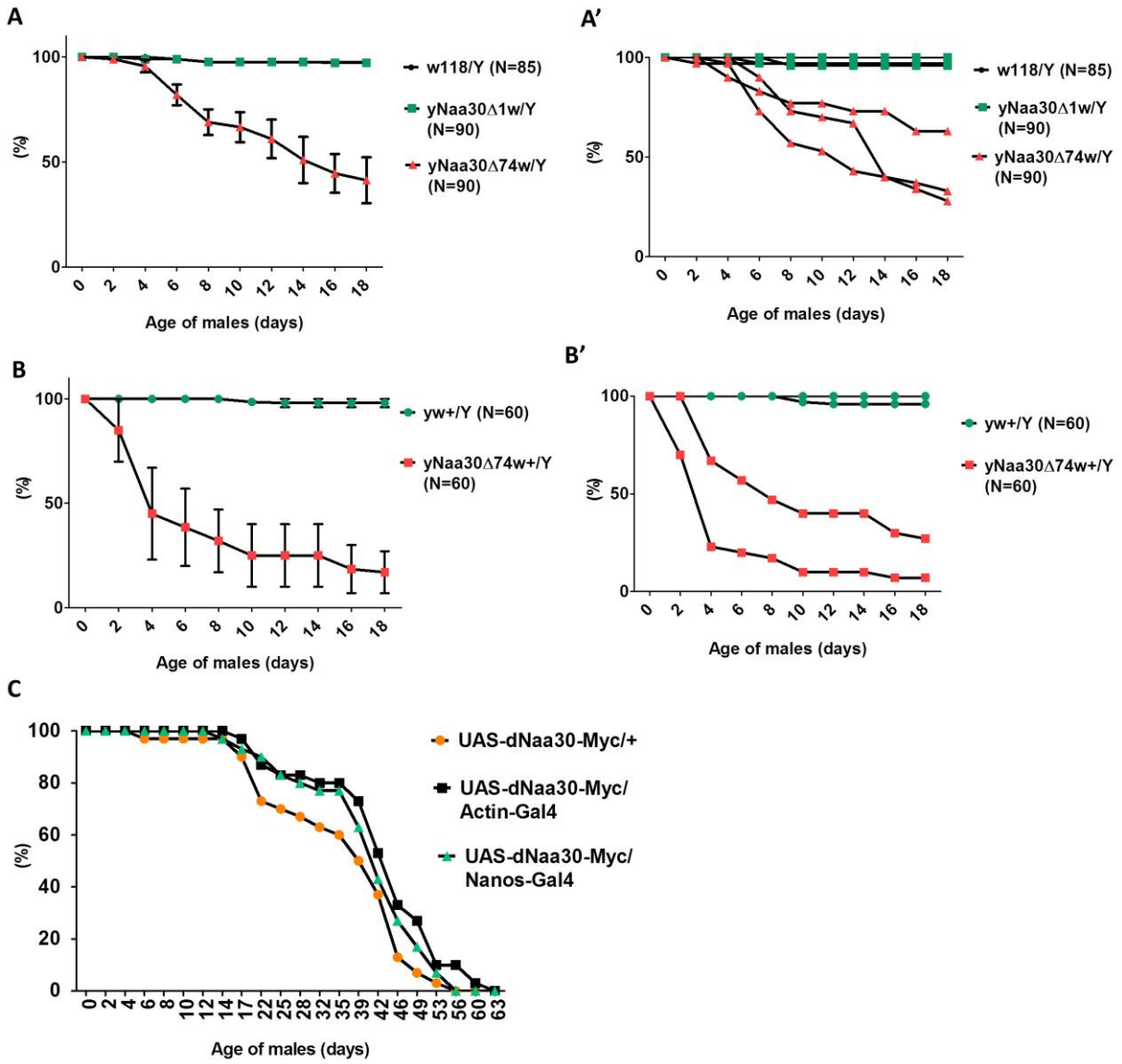


Figure 3.3.2 - dnaa30 deletion affects the longevity of mutant males. (A) Mean results obtained in the longevity assay performed with mutant males in white background

(y*naa30*Δ*74*w/Y) and respective controls (line Δ1 and w118 males). The number of males was counted every two days, during 18 days. (A') Results obtained in two independent replicas for the longevity assay performed with mutant males in white background and respective controls. (B) and (B') Mean results and values obtained in two independent replicas for the longevity assay performed with mutant males with wild-type white gene (y*naa30*Δ*74*w+/Y) and respective controls (yw+/Y). (C) Results of longevity rescue assays performed with mutant males (with wild-type white gene), with or without induction of dNaa30-Myc expression. The UAS/GAL4 system was used to induce the expression of UAS-dNaa30-Myc with different drivers. Longevity was evaluated in mutant males with the ubiquitous Actin5C-GAL4, the germ-line Nanos-GAL4 or no driver. In (A), (A'), (B), (B') and (C), results are represented in terms of percentage of males counted in each day. In (A), (A'), (B) and (B'), two independent replicas were performed.

3.4. *dnaa30* mutant males have normal spermatogenesis but the F1 embryos arrest early.

Previous studies have shown that the most common causes for male sterility are defects in spermatogenesis and sperm maturation [29, 30]. Due to the sterility phenotype observed in our deletion mutant, we evaluated whether *dnaa30Δ74* may have defects in spermatogenesis. To test this hypothesis, we evaluated testes morphology of freshly eclosed mutant (y*naa30Δ74*w/Y) and control (y*naa30Δ1*w/Y) males by Differential Interference Contrast (DIC) microscopy.

In squashed preparations, all the stages of spermatogenesis may be observed by DIC. During spermatogenesis a spatial- temporal progression occurs from the apical region of testis, where germ-line stem cells reside, till the distal end with nearly mature sperm, leading into the seminal vesicle [31].

Whole testis squash revealed that in mutant males no evident morphological defects are observed (Fig. 3.4.1 A). When we analyzed the apical tip of mutant testis (Fig. 3.4.1 B) we observed that the multistep differentiation program characteristic of spermatogenesis seems to occur normally, at least differentiation of germ-line stem cells into primary spermatocytes.

Finally, we performed a movie of the sperm produced in mutant and control testis in order to evaluate its motility. Fig. 3.4.1 C represents snapshots of the movies performed for both males (full movies are presented in the folder “Movies”). We observed that in mutant males, similarly to control, individual and motile sperm was formed.

Therefore, the sterility observed in the *dnaa30* mutant males was not likely to be explained by defects in spermatogenesis or sperm motility. These results are in accordance with the previous observation of no fertility rescue when dNaa30-Myc was expressed in the germ-line of mutant males (Fig. 3.2.2 C).

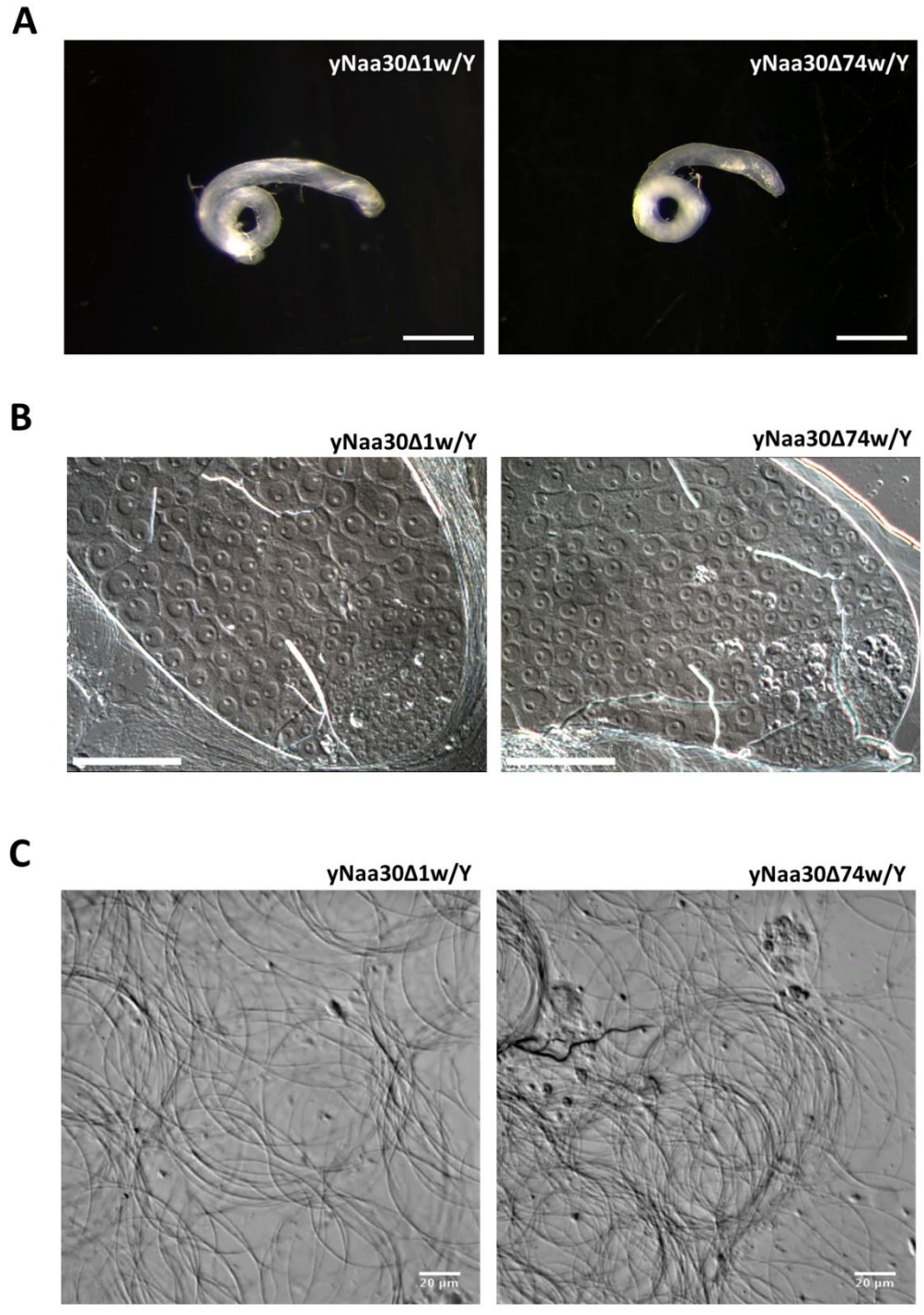


Figure 3.4.1 - *dnaa30* mutant males have normal spermatogenesis. (A) Images of whole testis of mutant (y*naa30*Δ*74*w/Y) and control (y*naa30*Δ*1*w/Y) males. (B) Differential interference contrast (DIC) images of the apical tip of slightly squashed testis of mutant (y*naa30*Δ*74*w/Y) and control (y*naa30*Δ*1*w/Y) males. (C) Snapshots of movies performed to evaluate the motility of the sperm produced in mutant (y*naa30*Δ*74*w/Y) and control (y*naa30*Δ*1*w/Y) testis. Scale bars: 500 µm in A; 20 µm in B.

After egg fertilization, the early embryogenesis of *D. melanogaster* is characterized by a rapid sequence of 13 nuclear divisions in a common syncytium, without cytokinesis [32]. The first 9 nuclear divisions take place in the interior of the embryo. After cycle 9, nuclei migrate to the cortex of the embryo where they divide more 3 times (10 to 13). The cellularization process starts in the interphase of cycle 14 and lasts for ~65 to 70 min. Completion of blastoderm cellularization occurs ~180 min. after the egg fertilization.

To clarify the sterility phenotype observed in our deletion mutant we stained the DNA of 1-3 hr F1 embryos, resulting from the cross of wild-type virgins with mutant males, and classified them in: class I, not fertilized - 2 nuclear divisions; class II, 2 nuclear divisions - interphase 14; class III, interphase 14 and class IV, > interphase 14. As control we used line Δ1 males. We observed that the majority of the F1 generation resulting from mutant males seem like eggs not fertilized or embryos that arrested very early (97.5± 0.7%, N=122, Fig. 3.4.2), while the ones resulting from control males were mainly in interphase 14 (50.5 ± 17.7%, N=83) or older (24.5±23.3%, N=83).

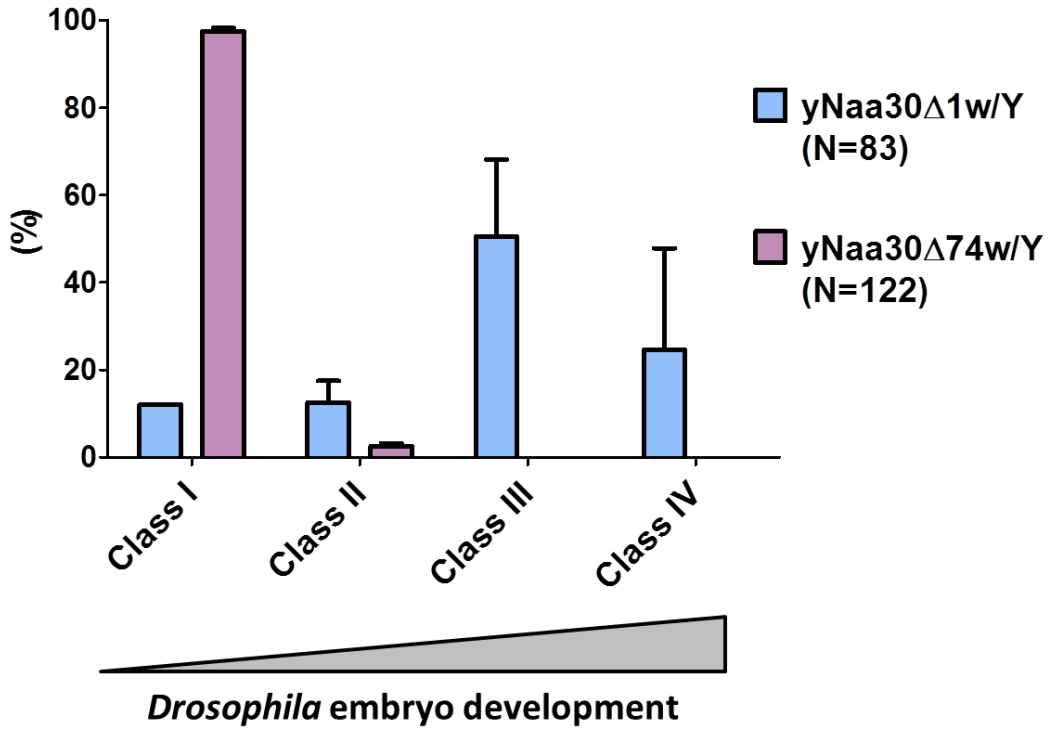


Figure 3.4.2 – The majority of eggs resulting from *dnaa30* mutant males are potentially unfertilized. Mutant males (y*naa30*Δ*74*w/Y) were crossed with wild-type virgins. Collections

of 1-3 hr of the resulting embryos/eggs and subsequent DNA staining were performed. Four developmental classifications were performed: class I, not fertilized- 2 nuclear divisions; class II, 2 nuclear divisions - interphase 14; class III, interphase 14 or class IV- > interphase 14. As control, the same experiments were executed with control males (y*naa30*Δ*1*w/Y). Results are represented in terms of percentage of eggs/embryos observed in each developmental stage. Two independent replicas were performed.

3.5. *dnaa30* mutant males have behavior defects

3.5.1. *dnaa30* mutant males have courtship behavior defects.

In resume, we observed that males with a deletion in dnaa30 are sterile and that this phenotype is probably due to defects in the fertilization of wild-type eggs. However, no problems in spermatogenesis were detected. Therefore, we hypothesize that *dnaa30* mutant males have courtship behavior defects.

The courtship behavior of *D. melanogaster* is characterized by many distinct steps [33, 34]. When a male fly notices a female, firstly he orients his body axis toward her and then he starts to chase her (orientation and following, step 1). During the following, male fly usually tap female's abdomen with his foreleg and extend and vibrate one of its wings (tapping and singing, step 2 and 3, respectively). While approaching, male fly licks the genitalia of female (licking, step 4) and, in order to copulate, tries to mount her back while curling his abdomen (attempted copulation, step 5).

The extension and vibration of one wing is an obligatory step in fly courtship, thus, we focused in this step to evaluate the courtship behavior of our mutant males (y*naa30*Δ*74*w+/Y) and respective control (yellow males). Our preliminary results reveal that none of the 5 mutant males evaluated performed the courtship song in the presence of a wild-type virgin, during 1 hr. On the other hand, we observed the singing step in all 5 tested control males, after 15 min. In Fig. 3.5.1 we can observe two snapshots representative of the movies obtained, showing the singing step for the control and the absence of courtship behavior in mutant males (full movies are presented in the folder "Movies").

To confirm that the sterility of *dnaa30* mutant males is due to courtship behavior defects, courtship assays are going to be performed in collaboration with Maria Luisa Vasconcelos and coworkers (Innate Behavior group of the Champalimaud Neuroscience Programme).

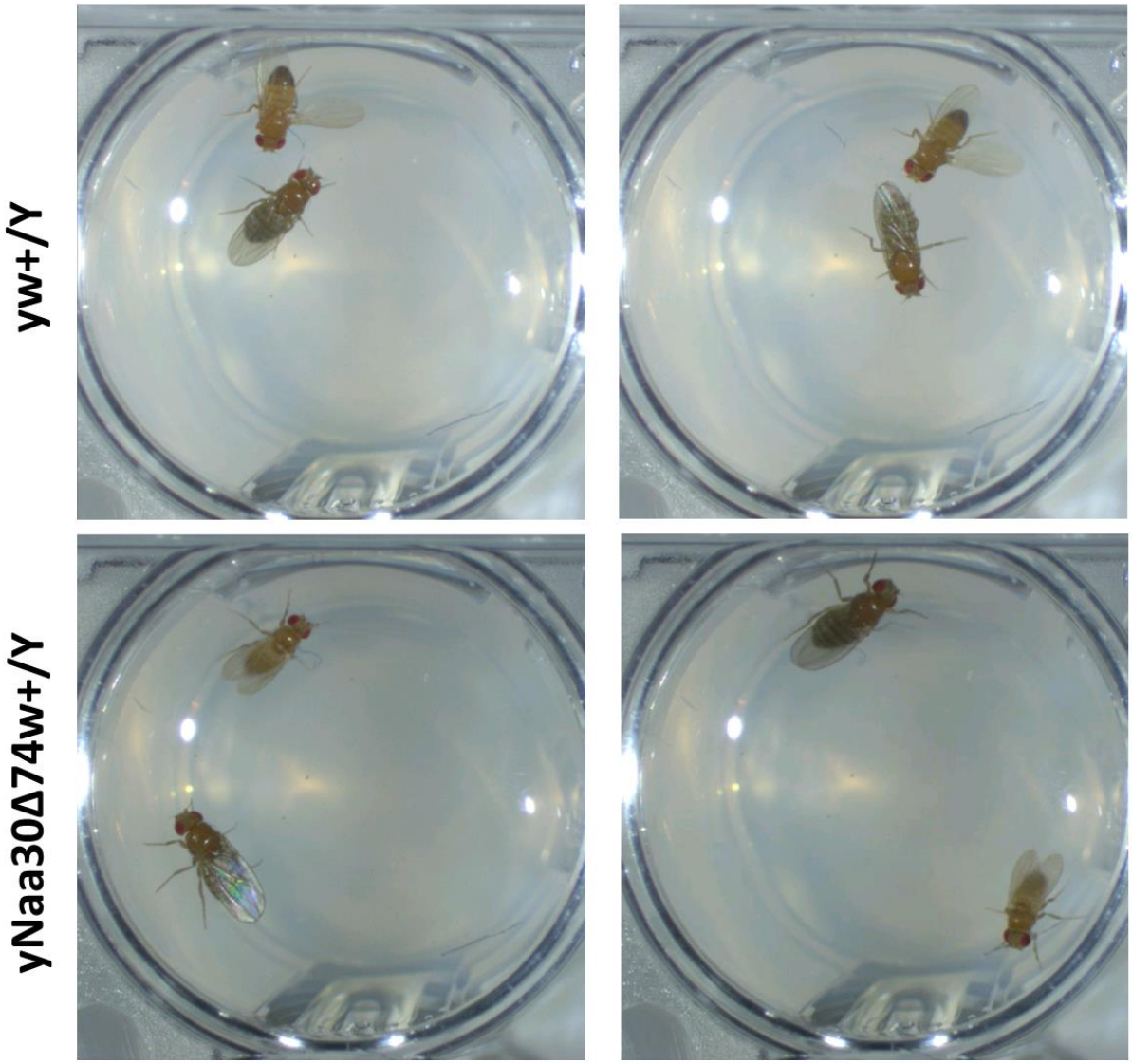


Figure 3.5.1 - *dnaa30* male mutants have courtship behavior defects. Male courtship behavior was evaluated by the occurrence of the singing step (extension and vibration of one wing) and was performed during 1 hr. Snapshots of the movies performed with mutant (y*naa30*Δ*74*w+/Y) and control (yw+/Y) males are shown and are representative of the courtship behavior observed in 5 evaluated couples.

3.5.2. *dnaa30* mutant males have climbing/locomotor deffects.

Courtship is an adult fly behavior that involves fine motor coordination [34]. To define if the courtship defects observed in *dnaa30* mutant males may be a consequence of locomotor

disability we performed a negative geotaxis assay. Negative geotaxis assay is a traditional fly behavioral assay used to determine alterations in locomotor or learning and memory capacities [34, 35].

We observed that mutant males (y*naa30*Δ*74*w+/Y) take more time than control males (yw+/Y) to cross a 7.5-cm mark (Fig. 3.5.2 A). After taping, 90.8±6.3% (N=49) of yellow males crossed the mark in the first 10 sec. while a lower number of mutant males reached it (62.6±10.1%, N=50). When we gave more time to reach the mark (30 sec.), similar fly numbers were observed: 97.4±1.5% (N=49) of control males and 91.1±5.1% (N=50) of mutant males. Thus, our deletion mutant males respond to gravity upon agitation but with a slower rate, showing that they have some mild locomotor behavior defects.

To determine whether the locomotor behavior defective phenotype observed in our deletion mutant is dnaa30-dependent, the negative geotaxis assay was performed with or without induction of dNaa30-Myc expression, in the mutant background. Similarly to what was observed in the longevity assay, rescue of the locomotor defects of *dnaa30* mutant males was observed due the leaky expression of dNaa30-Myc with no GAL4 driver (93.9%, N=18, of males crossed the mark in 10 sec., Fig. 3.5.2 B). This way, no marked differences were observed between the negative geotaxis response of mutant males without dNaa30-Myc expression induction (UAS-dNaa30-Myc/+) and mutant males with ubiquitous (UAS-dNaa30-Myc/Actin5C-GAL4) or germ-line expression (UAS-dNaa30-Myc/Nanos-GAL4) of this protein (Fig. 3.5.2 B).

These results suggest that the sterility observed in the *dnaa30* mutant males may not be explained by motor defects since a rescue of the locomotor defective phenotype is observed in mutant males with UAS-dNaa30-Myc and no driver males but the majority of these males are still sterile (86.0±7.9%, Fig. 3.2.2 C).

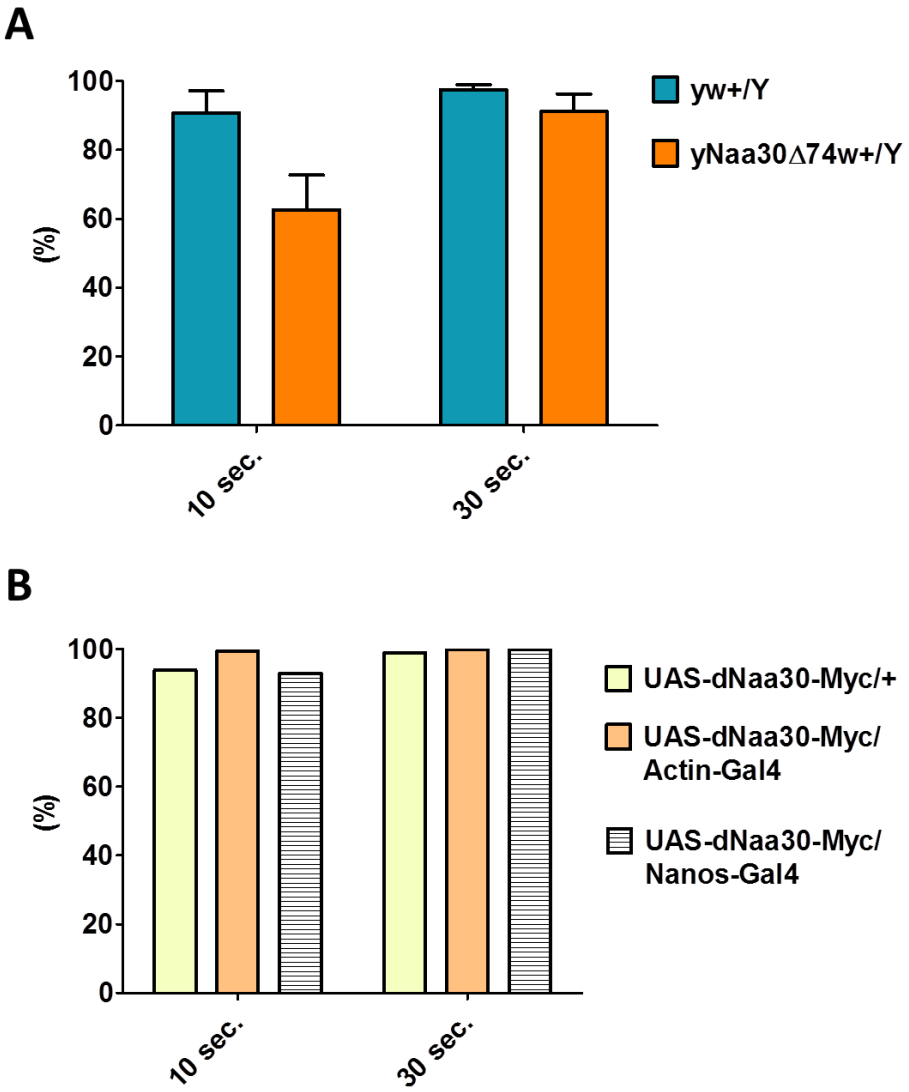


Figure 3.5.2 - *dnaa30* mutant males have climbing/locomotor defects. (A) Results of negative geotaxis assay performed with mutant (y*naa30*Δ*74*w+/Y) and control (yw+/Y) males. Results are represented in terms of percentage of males that can climb above a 7.5-cm mark, in 10 and 30 sec. Three independent replicas were performed. (B) Results of negative geotaxis assay performed with mutant males (with wild-type white gene) with or without induction of dNaa30-Myc expression. The UAS/GAL4 system was used to induce the expression of UAS-dNaa30-Myc with different drivers. The climbing ability in 10 and 30 sec. was evaluated in mutant males with the ubiquitous Actin5C-GAL4, the germ-line Nanos-GAL4 or no driver.

3.5.3. Neuronal expression of dNaa30-Myc rescues the sterility phenotype of *dnaa30* mutant males.

The observation that *dnaa30* deletion mutant males have courtship and locomotor defects suggest that dnaa30 may have a neuronal role, which could explain these phenotypes.

Thus, we evaluated if an expression of dNaa30-Myc induced by the pan-neuronal driver nSyb-GAL4 could rescue dnaa30-associated phenotypes.

Firstly, to evaluate the levels of fertility rescue, single-male fertility assays was performed with mutant males having or not expression of dNaa30-Myc induced by the nSyb-GAL4 driver. We observed that the neuronal expression of dNaa30-Myc restored the fertility of 40.7 ± 12.2% (N=117) males while in male flies with Nanos-GAL4 or no GAL4 driver, fertility was observed in 12.3 ± 2.3% (N=111) and 14.0 ± 7.9% (N=113), respectively (Fig. 3.5.3 A).

These results suggest that the fertility defects observed in *dnaa30* mutant males may at least in part be explained by o role of this protein in the nervous system. The observation of lower levels of rescue than with a ubiquitous driver (40.7 ± 12.2% vs 64.7% ± 22.5% with Actin5C-GAL4 driver) might potentially suggest an additional role of dnaa30 in other tissues.

We also evaluated the rescue of the other dnaa30-associated phenotypes: locomotor defects and longevity reduction. No evident differences were observed between the negative geotaxis response and the longevity of mutant males with neuronal expression of dNaa30-Myc (nSyb-GAL4 driver) and of positive (Actin5C-GAL4 driver) and negative controls (Nanos-GAL4 and no GAL4 drivers) (Fig. 3.5.3 B and 3.5.3 C).

As already mentioned, in humans, Arl8 was defined as a substrate of NatC whose lysosomal localization depends on Naa30-mediated N-terminal acetylation [16]. It has been proposed that Arl8 is involved in diverse cellular processes related to the microtubule-dependent transport of intracellular organelles. A more recent work has shown that, in *C. elegans* motoneuron DA9, Arl8 regulates the balance between presynaptic protein transport and assembly [20, 21].

Having in account the fertility rescue observed with the neuronal expression of dNaa30-Myc and the described role of Arl8 in controlling the capture and dissociation of synaptic vesicle protein transport vesicles/active zone proteins, we propose that the sterility observed in our deletion mutant may be mediated by the neuronal role of Arl8.

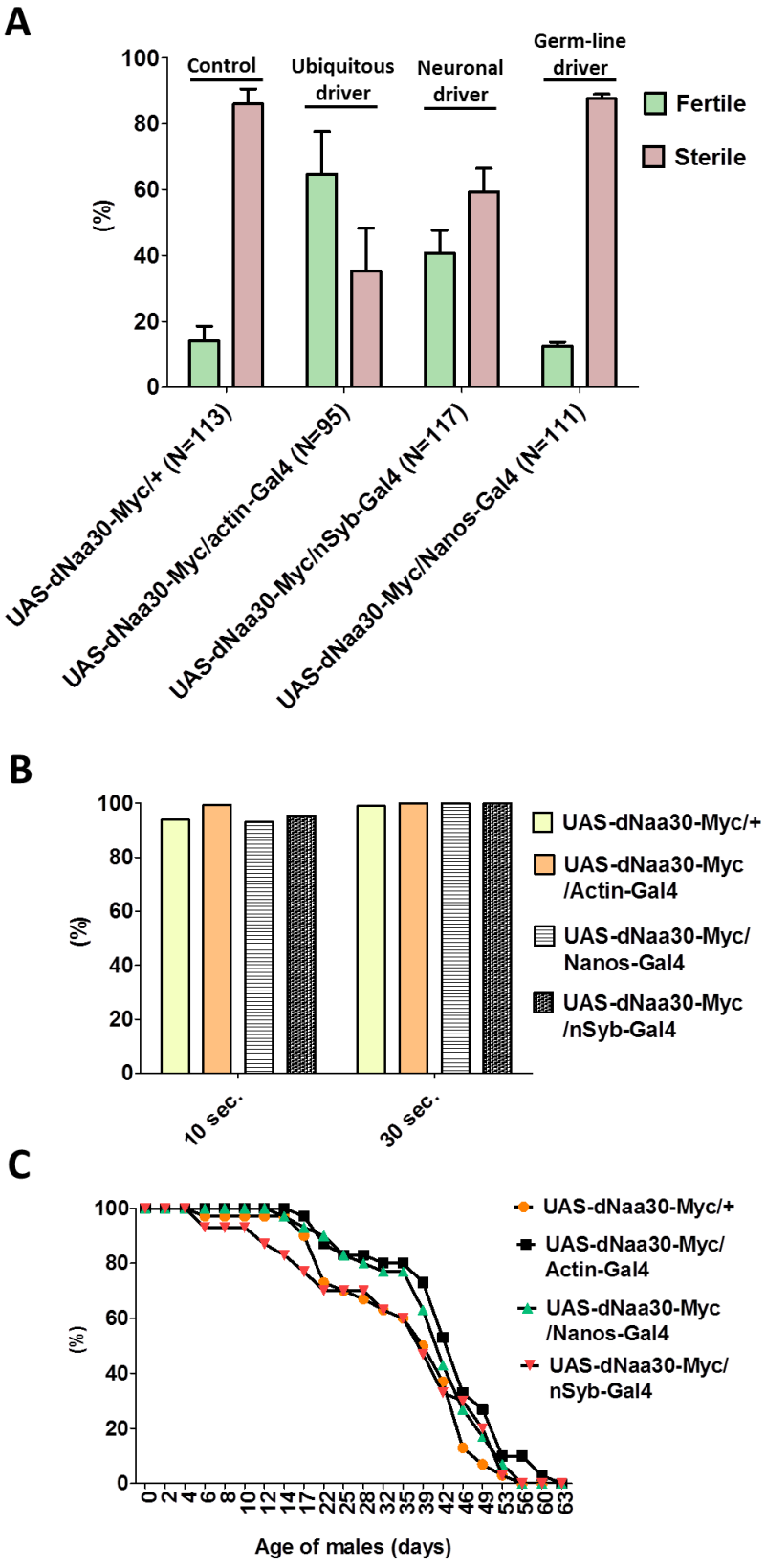


Figure 3.5.3 – Neuronal expression of dNaa30-Myc rescues sterility phenotype of dnaa30 male mutants. Results of (A) single-male fertility, (B) negative geotaxis assay and (C) longevity assay performed with mutant males (y*naa30*Δ*74*w+/Y), with or without induction of dNaa30-Myc expression in the nervous system. The expression of dNaa30-Myc in the

nervous system was induced with the nSyb-GAL4 driver. For the ubiquitous and germ-line expression, Actin5C-GAL4 and Nanos-GAL4 drivers were used, respectively. As control, the fertility of mutant males with UAS-dNaa30-Myc but with no driver was evaluated. In (A), (B) and (C), the results shown with Actin5C-GAL4, Nanos-GAL4 and no driver are a duplication of Fig. 3.2.2 C, Fig. 3.5.2 B and Fig. 3.3.2 C. In (A), three independent replicas were performed.

3.6. Maternal dnaa30 deletion leads to mild mitotic phenotypes

One of the microtubule-dependent phenotypes described for *Drosophila* Arl8 (Gie) is chromosome morphology and segregation abnormalities during mitosis, as a consequence of reduction in Gie activity in *Drosophila* S2 cells [19]. Being Arl8 one of the known substrates of Naa30, we questioned ourselves if a deletion in dnaa30 could also lead to mitotic phenotypes?

Having in account that *Drosophila* early development is characterized by a rapid sequence of 13 nuclear divisions, we took advantage of this model to evaluate if embryos maternally mutant for *dnaa30*Δ*74* (y*naa30*Δ*74*wFRT) reveal mitotic defects. Indeed we observed mild mitotic phenotypes with chromosome bridges in maternal *dnaa30* mutant embryos (Fig. 3.6.1 A). No mitotic defects were observed for control embryos (ywGFP FRT).

When we counted the number of maternal and zygotic mutant flies, resulting from the cross of wild-type males with females carrying homozygous germ-line clones of *dnaa30*Δ*74* (y*naa30*Δ*74*wFRT/y*naa30*Δ*74*wFRT), we observed at low frequency the generation of gynandromorphs (0.3%, N= 6610 vs 0.0%, N=10623, in control). This suggests some degree of chromosome disjunction during mitosis.

A gynandromorph is an organism that contains both male and female characteristics. Fig. 3.6.1 B shows an example of one of the gynandromorphs that we have observed with half of the fly displaying male characteristics (white-eye, yellow wing and body) and the other half of a female (red-eye, wild-type wing and body). These flies can occur when chromosome nondisjunction leads to aneuploidy or trisomy of sexual chromosomes. If the X chromosome is lost (or gain) in a subset of proliferating cells during early embryogenesis, half of the fly will look like a male (X0 or XY) and the other half like a female (XX or XXY), as sex-determination is a cell-autonomous event. If this event occurs after the first embryonic mitotic division, the fly is going to be mosaic gynandromorph with patches that are male or female. If this chromosome nondisjunction happens during meiosis the entire fly will have the same sex.

In resume, the *dnaa30* deletion mutants show mild defects in chromosome segregation, which can lead to chromososme nondisjunction, chromossome bridges, and aneuploidy. These results support the possibility of Gie as a mediator of *dnaa30* mutant phenotypes, since a mitotic effect of Gie was also described.

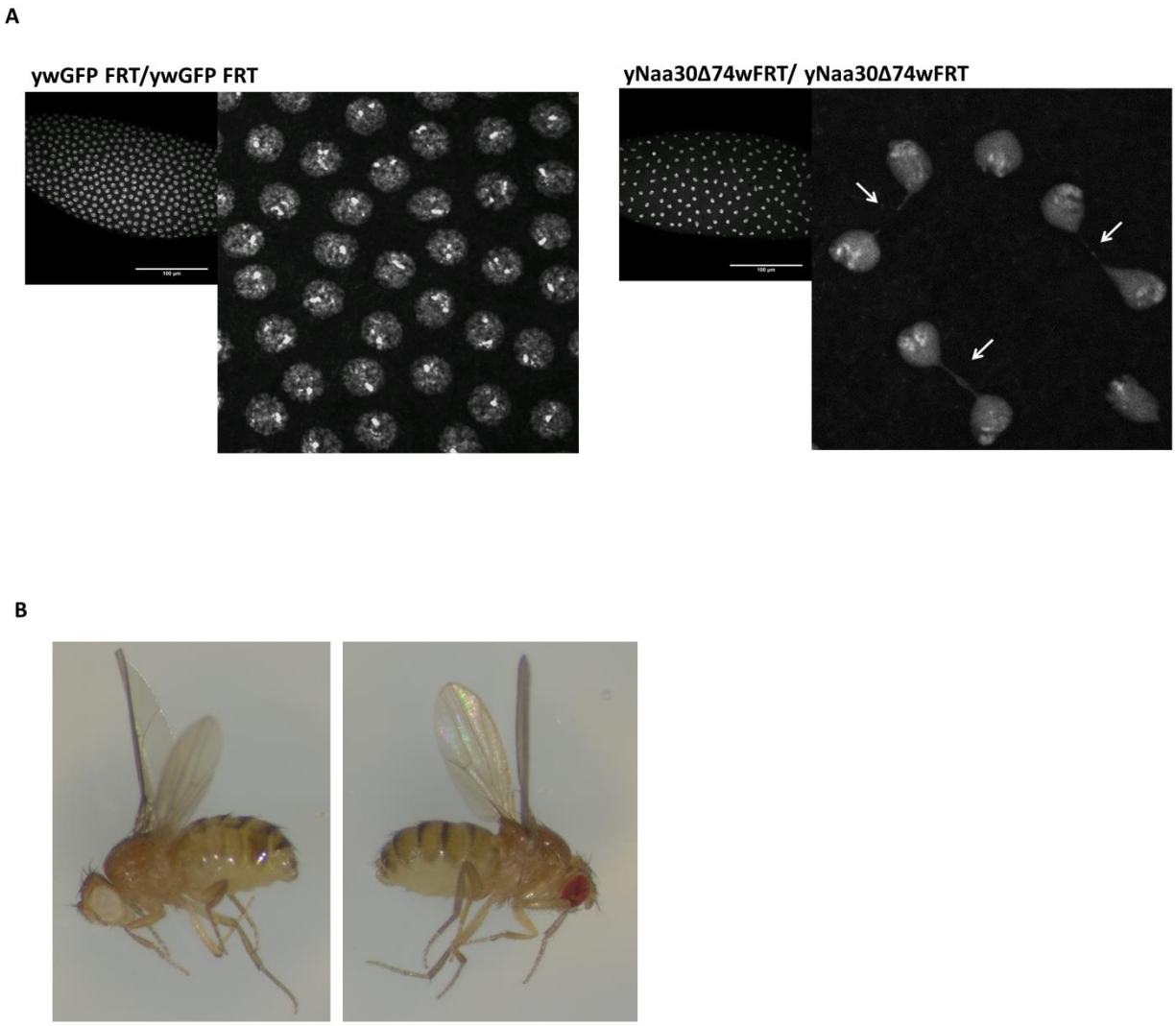


Figure 3.6.1 - Maternal dnaa30 deletion leads to mild mitotic phenotypes. (A) DNA staining of embryos maternally mutant for dnaa30 (y*naa30*Δ*74*wFRT). As control, embryos ywGFP FRT were evaluated. Arrows indicate chromosome bridges. (B) Picture showing the left and right side of a gynandromorph resulting from the cross of wild-type males with females carrying homozygous germ-line clones of *dnaa30*Δ*74* (y*naa30*Δ*74*wFRT/y*naa30*Δ*74*wFRT).

3.7. Neuronal expression of Gie RNAi does not phenocopy the sterility phenotype of *dnaa30* mutant males

To clarify if Gie may mediate the sterility phenotype observed in *dnaa30* mutant males, we performed single-male fertility assays with males having neuronal expression of Gie RNAi. As control we used males with Gie RNAi and no GAL4 driver. All tested males with depletion of Gie expression in the nervous system revealed to be fertile, similarly to control (Fig. 3.7.1). These results, apparently, do not result from low RNAi efficiency: when a ubiquitous driver (Actin5C-GAL4) was used, adult flies viability was strongly impaired and only a few females with wing and bristle phenotypes were observed. At this stage it is unclear if Gie is required within the *Drosophila* nervous system for male fertility. In the future, we are going to evaluate if overexpression of Gie in *dnaa30* mutant background can rescue dnaa30-associated phenotypes.

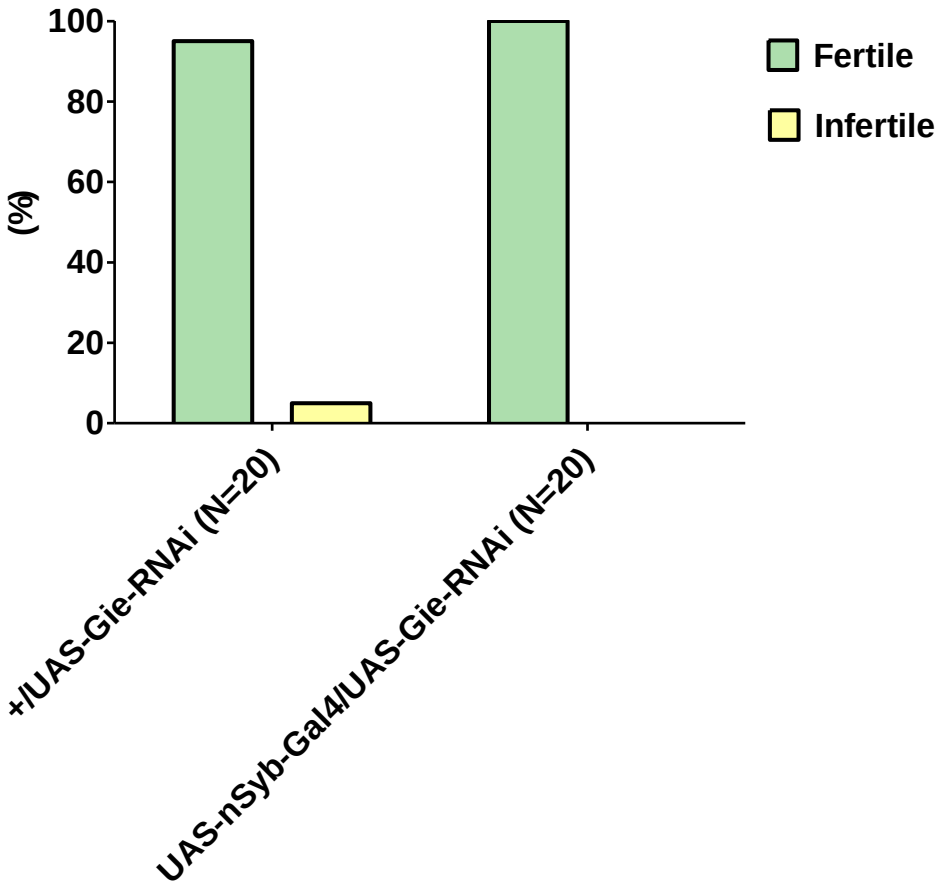


Figure 3.7.1 – Neuronal expression of Gie RNAi does not phenocopy the sterility phenotype of *dnaa30* mutant males. Results of single-male fertility assay performed with males

expressing or not Gie RNAi in the nervous system through induction with nSyb-GAL4 driver. As control, the fertility of mutant males with UAS-Gie RNAi but with no driver was evaluated. Results are represented in terms of percentage of sterile and fertile males observed.

4. Discussion

In this thesis we characterized the catalytic subunit of the *Drosophila* NatC complex (dNaa30). A *dnaa30* deletion mutant was generated and phenotypically characterized. In resume, we observed that a deletion in dnaa30 in *Drosophila* may lead to courtship behavior defects associated to sterility, reduction in longevity, mild locomotor behavior defects and mitotic phenotypes.

N-terminal acetylation is one of the most common protein modifications in eukaryotic cells, catalyzed by N-terminal acetyltransferases (NATs). The functional implications of N-terminal acetylation during the development of multicellular organisms are poorly known.

Previous works with functional characterization of NatC suggest that it functions to regulate the growth and development in wide range of organisms. However, as far as we are aware, the *Drosophila* NatC complex had never been identified or extensively characterized till now.

In this thesis we propose and describe dNaa30 as the catalytic subunit of *Drosophila* NatC complex. Our candidate subunit was co-immunoprecipitated with the *Drosophila* hNaa35 ortholog and functionally complements yeast Naa30 in a Δnaa30 mutant strain. This results strongly claim that dNaa30 (CG11412) is the *Drosophila* ortholog of yeast and human Naa30.

We observed that flies with a deletion in dnaa30 have dnaa30-specific fertility defects, which are not caused by problems in the germ-line development.

A possible role of NatA in the completion of spermatogenesis was already suggested for mouse [36, 37]. In both human and mouse, NatA catalytic substrates (*Naa10*) are located on the X chromosome and both contain paralogs (*Naa11*) on autosomal chromosomes. In mouse, evaluation of paralogs expression revealed that *mNaa11* is restricted to testis while *mNaa10* was found to be ubiquitously expressed [36]. It was proposed that *mNaa11* is induced to compensate for the loss of X-linked *mNaa10* during male meiotic sex chromosome inactivation. Interestingly, in *Drosophila,* the X-linked dnaa30 gene also has a testis-specific isoform (CG32319). The existence of this duplication event may in part explain why we found no spermatogenesis defects in *dnaa30* mutant males. In the mutant scenario, CG32319 may compensate the possible role of dnaa30 in spermatogenesis.

The progeny of *dnaa30* mutant males revealed to arrest early. Since no defects in spermatogenesis were detected, this observation could be the result of: (1) defects in the

transfer of sperm and seminal fluids from males to females during copulation, (2) fertilization or (3) behavioral defects.

We observed that the sterility phenotype observed in *dnaa30* mutant males is, at least in part, mediated by a neuronal role of dnaa30, which supports hypothesis (1) and (3). Although we cannot totally discard it, the possibility of defects in the transfer of sperm and seminal fluids seems less plausible since our preliminary results show that *dnaa30* mutant males have courtship behavior defects, which most likely explains the observed sterility. The contribution of a role of dnaa30 in other tissues cannot be excluded since the levels of fertility rescue observed with a pan-neuronal driver were lower than the ones observed with ubiquitous expression of dNaa30-Myc.

Importantly, we observed that the fertility, longevity and locomotor phenotypes observed in *dnaa30* mutant males are uncoupled events. So the reduction in courtship behavior/fertility is a specific phenotype and not a consequence of the reduction in locomotion and in longevity.

The NatC complex acetylates proteins with the N-terminal sequences Met-Leu, Met-Phe, Met-Ile, and Met-Trp. Some NatC substrates were already identified. mTOR protein was suggested to be a direct downstream target for NatC acetylation in *zebrafish*. Rapamycin phenocopied and overexpression of mTOR rescued the body length and vessel defects and the lethality observed in zNaa35 morphants [24]. Furthermore it was observed that hNatC can acetylate an oligopeptide which N-terminal is identical of the predicted mTOR, in *in vitro* acetyltransferase assays [8]. NatC-dependent N-Ac was shown to be a positive regulator of membrane attachment of the small GTPases Arl3p and Grh1p to the Golgi membrane [14, 15] and of Arl8a/b to the lysosomal membrane [8].

Having in account the known NatC substrates, our main hypothesis to explain the phenotypes observed in our *dnaa30* deletion mutant flies is that the dNaa30-mediated N-terminal acetylation of Gie (*Drosophila* Arl8) could be a common mechanism which regulates the activity of this small GTPase in diverse microtubule-dependent cellular processes (Fig. 4.1). We hypothesize that in *dnaa30* mutant flies, the activity of Gie is decreased due to the lower levels of N-terminal acetylation of this small GTPase. In this case, according to the results in *C. elegans* [20, 21], we would observe defects in presynaptic cargoes transport and, consequently, in presynapses assemble which could explain the courtship behavior/fertility phenotypes observed in our mutant, that are in part dependent of a dnaa30 neuronal role. Furthermore, the decreased activity of Gie would result in chromosome bridges and lagging

chromosomes, as was already described in S2 cells [19], which is in accordance with the mild mitotic phenotypes that we observed in maternal mutants for dnaa30.

A role of Arl8 in longevity was also documented in *zebrafish*. Knocking down Arl8 with RNAi decreased median lifespan by 35% in an insulin-receptor mutant [38]. So the reduction in longevity observed in *dnaa30* mutant males may also be explained by our hypothesis of decreased activity of Gie as consequence of lower levels of N-terminal acetylation.

Man Yan Wong and coworkers have suggested that the distribution of dense core vesicles among synaptic boutons at the *Drosophila* neuromuscular junction is regulated by coordinating cargo transport and capture [39]. Knowing that Arl8 controls the capture and dissociation of presynaptic proteins, we propose that Gie may have the same role at the *Drosophila* neuromuscular junctions and that the decreased activity of Gie, in a dnaa30 deletion scenario, could lead to locomotor defects, as we observed in our mutant males.

Although the results obtained in the single-male fertility assays, with males expressing Gie RNAi in the nervous system, are not encouraging, to determine the validity of our hypothesis, in the future we will evaluate if the ubiquitous expression of Gie can rescue zygotic dnaa30-associated phenotypes. Secondly, it would be important to determine if Gie is N-terminally acetylated in *Drosophila* and if the levels of acetylation are decreased in *dnaa30* mutant flies.

A role of TOR in the nervous system has been demonstrated in different works, suggesting that TOR activity can influence synaptic growth, function, and plasticity in postmitotic neurons and during disease [40]. It has been observed that the mTOR inhibitor rapamycin, through induction of macroautophagy, affects presynaptic structure and neurotransmission [41]. Jay Penney and coworkers demonstrated that TOR is required for the retrograde regulation of synaptic homeostasis at the *Drosophila* neuromuscular junction [42]. Thus TOR, through regulation of presynaptic and postsynaptic neurotransmission, could also be a substrate mediating the dnaa30-associated phenotypes in the nervous system: courtship behavior/concomitant fertility and locomotor defects. The regulation of TOR signaling by dNaa30 could occur directly, through acetylation of TOR, or indirectly, through acetylation of Gie. Arl8 may regulate mTORC1 signaling through its effect in lysosomal positioning [16, 43].

To support this hypothesis, it would be important to evaluate in the future if Gie is delocalized in *dnaa30* mutant flies and if this delocalization affects the positioning of lysosomes.

Deregulation of mTORC1 signaling in mutant flies could be evaluated by the levels of phospho-T398-S6K (readout of mTORC1 activity).

Hsp27 is a protein chaperone and an antioxidant. It plays a role in the inhibition of apoptosis and actin cytoskeletal remodeling. As far as we are aware, until know, Hsp27 had never been described as a substrate of NatC. When we co-immunoprecipitated dNaa30-Myc from *Drosophila* embryonic protein extracts, Hsp27 was detected in both replicas. It was also co-immunoprecipitated with dNaa30-Protein A (data not shown). Hsp27 was one of the 13 longevity candidate genes of *Drosophila* isolated by multiple stresses [44]. Overexpression of Hsp27 increased flies lifespan by 27% - 31% and the flies also displayed increased stress resistance. This way, we hypothesize that Hsp27 could be an effector of dnaa30 effect in longevity.

Drosophila neuropeptides have been implicated in the regulation of different physiological and behavioral processes: development, growth, feeding, metabolism, reproduction, longevity and in the modulation of olfaction, locomotor control, learning, and memory [45]. There are many examples of neuropeptides, in different types of organisms, which biological activity is regulated by N-terminally acetylation (α-melanocyte-stimulating hormone (MSH), β-endorphin, adrenocorticotropic hormone (ACTH)) [46, 47]. A neuropeptide circuit that coordinates sperm transfer and duration of copulation (regulated by the neuropeptide corazonin) was recently described in *Drosophila* [48]. Three peptides have been implicated in *Drosophila* courtship behavior: SIFamide, neuropeptide F (NPF) and sex peptide [49]. So we can imagine a scenario where dnaa30 could have effect in *Drosophila* fertility through regulation of N-terminal acetylation of neuropeptides involved in these two processes.

Collectively, our work of functional characterization of naa30 in *Drosophila* revealed that NatC may have a neuronal role which affects courtship behavior and fertility and also locomotion of flies. Furthermore, we observed that naa30 regulates the longevity of flies, which is in accordance with recent results obtained in *C. elegans*. As far as we are aware, this is the first time that an association between N-terminal acetylation and the behavior of a multicellular organism is made.

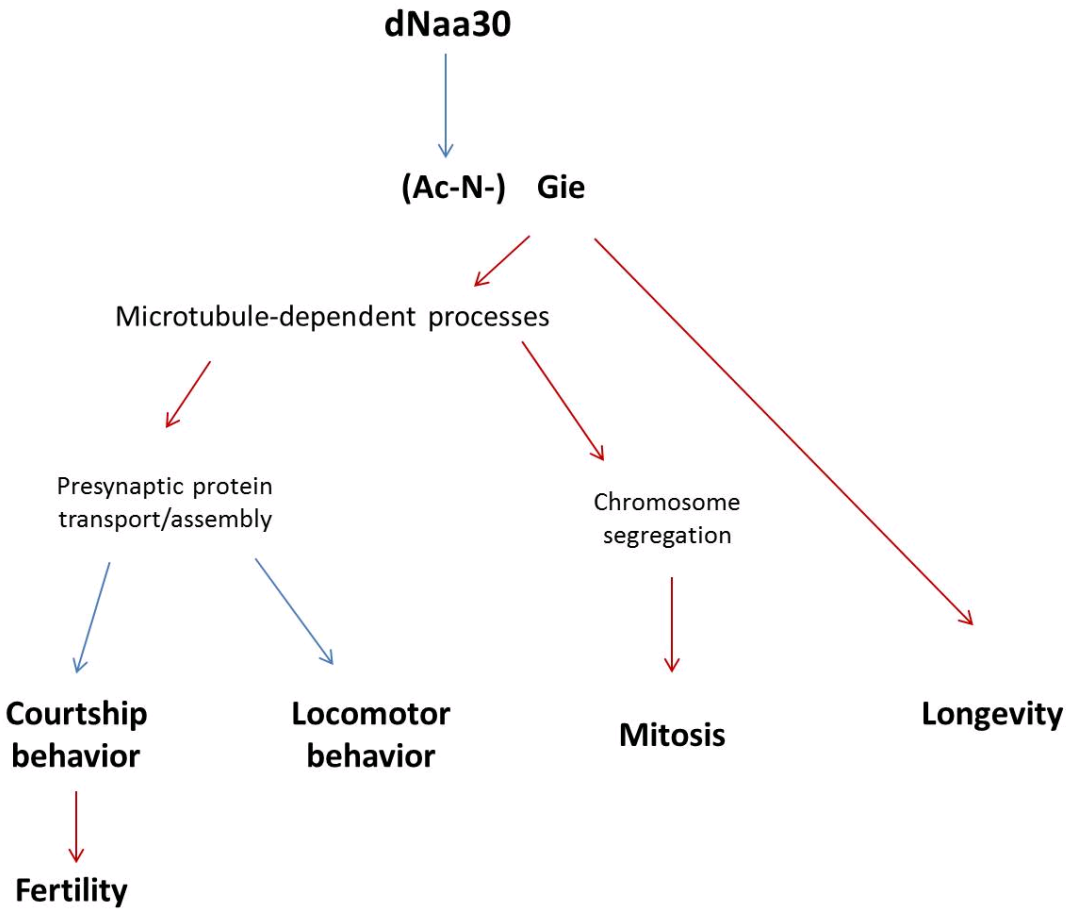


Figure 4.1 – Schematic representation of the proposing model: dNaa30-mediated N-terminal acetylation of Gie (*Drosophila* Arl8) could be a common mechanism which regulates the activity of this small GTPase in diverse microtubule-dependent cellular processes which could explain the phenotypes observed in *dnaa30* mutants: courtship behavior defects, infertility, mild locomotor and mitotic defects and longevity reduction. Previous publications suggest that Arl8 is involved in diverse microtubule-dependent cellular processes: it was observed that reduction of Gie1 activity causes abnormalities in chromosome morphology and segregation in *Drosophila* S2 cells and that, in *C. elegans* motoneuron DA9, a loss-of function mutant of arl8 leads to a premature accumulation of presynaptic cargoes. A role of Arl8 in longevity was also documented in *zebrafish*. Knocking down Arl8 with RNAi decreased median lifespan by 35% in an insulin-receptor mutant.

References

1. Arnesen, T., et al., *Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans.* Proc Natl Acad Sci U S A, 2009. 106(20): p. 8157-62.
2. Goetze, S., et al., *Identification and functional characterization of N-terminally acetylated proteins in Drosophila melanogaster.* PLoS Biol, 2009. 7(11): p. e1000236.
3. Van Damme, P., et al., *NatF contributes to an evolutionary shift in protein N-terminal acetylation and is important for normal chromosome segregation.* PLoS Genet, 2011. 7(7): p. e1002169.
4. Polevoda, B., T. Arnesen, and F. Sherman, *A synopsis of eukaryotic Nalpha-terminal acetyltransferases: nomenclature, subunits and substrates.* BMC Proc, 2009. 3 Suppl 6: p. S2.
5. Starheim, K.K., et al., *Composition and biological significance of the human Nalpha-terminal acetyltransferases.* BMC Proc, 2009. 3 Suppl 6: p. S3.
6. Arnesen, T., et al., *Induction of apoptosis in human cells by RNAi-mediated knockdown of hARD1 and NATH, components of the protein N-alpha-acetyltransferase complex.* Oncogene, 2006. 25(31): p. 4350-60.
7. Starheim, K.K., et al., *Identification of the human N(alpha)-acetyltransferase complex B (hNatB): a complex important for cell-cycle progression.* Biochem J, 2008. 415(2): p. 325-31.
8. Starheim, K.K., et al., *Knockdown of human N alpha-terminal acetyltransferase complex C leads to p53-dependent apoptosis and aberrant human Arl8b localization.* Mol Cell Biol, 2009. 29(13): p. 3569-81.
9. Arnesen, T., et al., *The protein acetyltransferase ARD1: a novel cancer drug target?* Curr Cancer Drug Targets, 2008. 8(7): p. 545-53.
10. Narita, K., *Isolation of acetylpeptide from enzymic digests of TMV-protein.* Biochim Biophys Acta, 1958. 28(1): p. 184-91.
11. Arnesen, T., *Towards a functional understanding of protein N-terminal acetylation.* PLoS Biol, 2011. 9(5): p. e1001074.
12. Starheim, K.K., K. Gevaert, and T. Arnesen, *Protein N-terminal acetyltransferases: when the start matters.* Trends Biochem Sci, 2012. 37(4): p. 152-61.
13. Forte, G.M., M.R. Pool, and C.J. Stirling, *N-terminal acetylation inhibits protein targeting to the endoplasmic reticulum.* PLoS Biol, 2011. 9(5): p. e1001073.
14. Behnia, R., et al., *Targeting of the Arf-like GTPase Arl3p to the Golgi requires N-terminal acetylation and the membrane protein Sys1p.* Nat Cell Biol, 2004. 6(5): p. 405-13.
15. Setty, S.R., et al., *Golgi targeting of ARF-like GTPase Arl3p requires its Nalpha-acetylation and the integral membrane protein Sys1p.* Nat Cell Biol, 2004. 6(5): p. 414-9.
16. Hofmann, I. and S. Munro, *An N-terminally acetylated Arf-like GTPase is localised to lysosomes and affects their motility.* J Cell Sci, 2006. 119(Pt 8): p. 1494-503.
17. Murthi, A. and A.K. Hopper, *Genome-wide screen for inner nuclear membrane protein targeting in Saccharomyces cerevisiae: roles for N-acetylation and an integral membrane protein.* Genetics, 2005. 170(4): p. 1553-60.
18. Aksnes, H., C. Osberg, and T. Arnesen, *N-terminal acetylation by NatC is not a general determinant for substrate subcellular localization in Saccharomyces cerevisiae.* PLoS One, 2013. 8(4): p. e61012.
19. Okai, T., et al., *Novel small GTPase subfamily capable of associating with tubulin is required for chromosome segregation.* J Cell Sci, 2004. 117(Pt 20): p. 4705-15.
20. Klassen, M.P., et al., *An Arf-like small G protein, ARL-8, promotes the axonal transport of presynaptic cargoes by suppressing vesicle aggregation.* Neuron, 2010. 66(5): p. 710-23.
21. Wu, Y.E., et al., *The balance between capture and dissociation of presynaptic proteins controls the spatial distribution of synapses.* Neuron, 2013. 78(6): p. 994-1011.

22. Lee, Y.J. and R.B. Wickner, *MAK10, a glucose-repressible gene necessary for replication of a dsRNA virus of Saccharomyces cerevisiae, has T cell receptor alpha-subunit motifs.* Genetics, 1992. 132(1): p. 87-96.
23. Pesaresi, P., et al., *Cytoplasmic N-terminal protein acetylation is required for efficient photosynthesis in Arabidopsis.* Plant Cell, 2003. 15(8): p. 1817-32.
24. Wenzlau, J.M., et al., *Embryonic growth-associated protein is one subunit of a novel N-terminal acetyltransferase complex essential for embryonic vascular development.* Circ Res, 2006. 98(6): p. 846-55.
25. Warnhoff, K., et al., *The DAF-16 FOXO Transcription Factor Regulates natc-1 to Modulate Stress Resistance in Caenorhabditis elegans, Linking Insulin/IGF-1 Signaling to Protein N-Terminal Acetylation.* PLoS Genet, 2014. 10(10): p. e1004703.
26. Nilsson, E.E., et al., *Fruitless is in the regulatory pathway by which ectopic mini-white and transformer induce bisexual courtship in Drosophila.* J Neurogenet, 2000. 13(4): p. 213-32.
27. An, X., et al., *The effects of ectopic white and transformer expression on Drosophila courtship behavior.* J Neurogenet, 2000. 14(4): p. 227-43,271.
28. Krstic, D., W. Boll, and M. Noll, *Influence of the White locus on the courtship behavior of Drosophila males.* PLoS One, 2013. 8(10): p. e77904.
29. Castrillon, D.H., et al., *Toward a molecular genetic analysis of spermatogenesis in Drosophila melanogaster: characterization of male-sterile mutants generated by single P element mutagenesis.* Genetics, 1993. 135(2): p. 489-505.
30. Wakimoto, B.T., D.L. Lindsley, and C. Herrera, *Toward a comprehensive genetic analysis of male fertility in Drosophila melanogaster.* Genetics, 2004. 167(1): p. 207-16.
31. White-Cooper, H., *Spermatogenesis: analysis of meiosis and morphogenesis.* Methods Mol Biol, 2004. 247: p. 45-75.
32. Mazumdar, A. and M. Mazumdar, *How one becomes many: blastoderm cellularization in Drosophila melanogaster.* Bioessays, 2002. 24(11): p. 1012-22.
33. Yamamoto, D. and M. Koganezawa, *Genes and circuits of courtship behaviour in Drosophila males.* Nat Rev Neurosci, 2013. 14(10): p. 681-92.
34. Nichols, C.D., J. Becnel, and U.B. Pandey, *Methods to assay Drosophila behavior.* J Vis Exp, 2012(61).
35. Ali, Y.O., et al., *Assaying locomotor, learning, and memory deficits in Drosophila models of neurodegeneration.* J Vis Exp, 2011(49).
36. Pang, A.L., et al., *Cloning, characterization, and expression analysis of the novel acetyltransferase retrogene Ard1b in the mouse.* Biol Reprod, 2009. 81(2): p. 302-9.
37. Pang, A. and O. Rennert, *Protein acetylation and spermatogenesis.* Reprod Syst Sex Disord, 2013. Suppl 1: p. 5.
38. Samuelson, A.V., C.E. Carr, and G. Ruvkun, *Gene activities that mediate increased life span of C. elegans insulin-like signaling mutants.* Genes Dev, 2007. 21(22): p. 2976-94.
39. Wong, M.Y., et al., *Neuropeptide delivery to synapses by long-range vesicle circulation and sporadic capture.* Cell, 2012. 148(5): p. 1029-38.
40. Swiech, L., et al., *Role of mTOR in physiology and pathology of the nervous system.* Biochim Biophys Acta, 2008. 1784(1): p. 116-32.
41. Hernandez, D., et al., *Regulation of presynaptic neurotransmission by macroautophagy.* Neuron, 2012. 74(2): p. 277-84.
42. Penney, J., et al., *TOR is required for the retrograde regulation of synaptic homeostasis at the Drosophila neuromuscular junction.* Neuron, 2012. 74(1): p. 166-78.
43. Korolchuk, V.I., et al., *Lysosomal positioning coordinates cellular nutrient responses.* Nat Cell Biol, 2011. 13(4): p. 453-60.
44. Wang, H.D., P. Kazemi-Esfarjani, and S. Benzer, *Multiple-stress analysis for isolation of Drosophila longevity genes.* Proc Natl Acad Sci U S A, 2004. 101(34): p. 12610-5.
45. Nassel, D.R. and A.M. Winther, *Drosophila neuropeptides in regulation of physiology and behavior.* Prog Neurobiol, 2010. 92(1): p. 42-104.

46. **Barnea, A. and G. Cho,** ***Acetylation of adrenocorticotropin and beta-endorphin by hypothalamic and pituitary acetyltransferases.*** **Neuroendocrinology, 1983. 37(6): p. 434-9.**
47. **Arends, R.J., et al.,** ***alpha-MSH acetylation in the pituitary gland of the sea bream (Sparus aurata L.) in response to different backgrounds, confinement and air exposure.*** **J Endocrinol, 2000. 166(2): p. 427-35.**
48. **Tayler, T.D., et al.,** ***A neuropeptide circuit that coordinates sperm transfer and copulation duration in Drosophila.*** **Proc Natl Acad Sci U S A, 2012. 109(50): p. 20697-702.**
49. **Terhzaz, S., et al.,** ***The neuropeptide SIFamide modulates sexual behavior in Drosophila.*** **Biochem Biophys Res Commun, 2007. 352(2): p. 305-10.**