**Drosophila** exoribonuclease *nibbler* is a tumor suppressor, acts within the RNA\(^i\) machinery and is not enriched in the nuage during early oogenesis

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**Abstract**

**Background:** micro RNAs (miRNAs) are important regulators of many biological pathways. A plethora of steps are required to form, from a precursor, the mature miRNA that eventually acts on its target RNA to repress its expression or to inhibit translation. Recently, *Drosophila nibbler* (*nbr*) has been shown to be an important player in the maturation process of miRNA and piRNA. Nbr is an exoribonuclease which helps to shape the 3′ end of miRNAs by trimming the 3′ overhang to a final length.

**Results:** In contrast to previous reports on the localization of Nbr, we report that 1) Nbr is expressed only during a short time of oogenesis and appears ubiquitously localized within oocytes, and that 2) Nbr was is not enriched in the nuage where it was shown to be involved in piwi-mediated mechanisms. To date, there is little information available on the function of *nbr* for cellular and developmental processes. Due to the fact that *nbr* mutants are viable with minor deleterious effects, we used the GAL4/UAS over-expression system to define novel functions of *nbr*. We disclose hitherto unknown functions of *nbr* 1) as a tumor suppressor and 2) as a suppressor of RNA\(^i\). Finally, we confirm that *nbr* is a suppressor of transposon activity.

**Conclusions:** Our data suggest that *nbr* exerts much more widespread functions than previously reported from trimming 3′ ends of miRNAs only.

**Keywords:** *Drosophila*, Nibbler, Tumor formation, Nuage

**Background**

In eukaryotes, three main RNA\(^i\) pathways have received considerable attention in the past: microRNAs (miRNAs), small interfering (siRNAs) and Piwi-interacting RNAs (piRNAs) [18]. All three pathways reveal difference in their biogenesis, type of Argonaute family proteins, mode of target regulation and substrates [17]. The RNA\(^i\) machinery and mechanisms associated with it are evolutionarily conserved in most eukaryotic organisms, including insects [42].

During the last decade, microRNAs (miRNAs) were found to be important regulators of development, pathology and physiology of plant, as well as humans (reviewed by [22, 48]. Despite their small size of about 22 nucleotide (nt), these RNA molecules exert complex functions by binding preferentially to the 3′ untranslated region (UTR) of target RNAs to block their function. miRNAs are initially synthesized by RNA polymerase II to yield a precursor miRNA of about 70 nt length which are 5′ capped and which are also 3′ polyadenylated which subsequently folds into a structure with a partially-paired stem, a single-stranded loop and a 2 nucleotide 3′ overhang. These 3 features are characteristic for the primary miRNA.

As a next step, these primary miRNA are exported from the nucleus to the cytoplasm. There, Dicer, a RNase III processes the pre-miRNA to a 22 nt mature miRNA/miRNA\(^*\) duplex [20, 27]. Subsequently, miRNA...
duplexes are assembled in a complex with Argonaute (Ago) to form the precursor RNA-induced silencing complex (RISC) [11]. This complex formation appears to be uncoupled from the synthesis of the miRNA. It is also within the RISC complex where one of the strands is chosen as the active silencing complex. Finally, the active miRNA within RISC binds preferentially to the 3′ UTR of their target mRNAs which leads either to repression of the transcription or by inhibiting its translation [14].

When measuring the length of the end of the 3′ overhangs, it was noted that there was an unusually high heterogeneity within the per-miRNA molecules which was ascribed to a sloppy mode of action of RNase III. This necessitated to postulate the presence of yet another enzyme that would account for the precise outcome of the 2 nt overhang. In 2011, two groups presented Nibbler (Nbr) protein in Drosophila as a candidate enzyme belonging to the class of exoribonucleases which likely represented the missing link [23, 32]. In vitro assays showed that Nbr trims many miRNAs to a 22 nt product, when bound to Ago [23], exemplified on a preferred target, miR-34 [23, 32] which itself exists in several isoforms. In absence of nbr, all smaller isoforms of miR-34 are lost [32], indicating that Nbr has a specific function on trimming miR-34, but it would not exclude that nbr would have a broader set of targets. Interestingly, Nbr was predicted to contain no RNA-binding activity, therefore, it was suggested that Ago would exert this job, and only the binding of Nbr to Ago in an complex would allow to act on miRNAs. nbr flies were first reported to be semi-lethal and sterile [23, 32]. Later nbr flies were found to be viable, but showing accelerated age-related effects [15, 24, 49].

Recently, research on the Piwi protein, a protein functionally and structurally close to Ago, and the associated piwi pathway furthered our understanding on the mechanisms of the biogenesis of small interference RNAs [24]. The piwi pathway and the associated piRNAs have mainly been studied in Drosophila. piRNAs are 23–29 nt small RNAs expressed predominantly in the oocyte [6, 34]. Concomitantly, piRNAs were discovered as master regulators to repress transposable elements (TEs) in Drosophila as well as in mice, rats, nematodes, and zebra fish [4, 19, 21, 31, 37, 39, 47]. It appears that there are thousands of distinct piRNA sequences present in the genomes of Drosophila [4]. To date, no structural or sequence similarity between the sequences of different piRNAs was found, except for a stronger bias for uracil in the first nucleotide [4]. In Drosophila, piRNAs recognize their targets, which predominantly are mRNAs of TEs through perfect or nearly perfect antisense matching. Hence, interfering with the piwi system may change the activity of transposon which may have deleterious effects on the organisms. piRNAs undergo several steps of maturation including formation of the primary piRNAs which are loaded onto Piwi [28]. As a further maturation process, the “Ping-Pong” cycle, reported to occur in the nuage of Drosophila germ cells [4], amplifies secondary piRNAs and thereby silences targets [12, 43]. Due to the amplification of the piRNAs, it is thought that the process consumes transcripts of TEs, thereby leading to a silencing of TEs. Conversely, interfering with the Ping-Pong cycle has likely the opposite effect, i.e. TE transcripts are present at unusually high levels. This in turn increases the probability of TEs to insert into developmentally-important genes or tumor-suppressor genes which may have deleterious effects such as generating cancer in tissues which otherwise would not happen if the regulation of TE activity was in balance.

Given the importance of nbr, little information is available as to the overall function of nbr for development or cellular mechanisms in a broader context. We therefore thought to shed some light onto possible mechanisms. Instead of using classical mutants where information on the function is very limited, we used inducible nbr RNAi and dominant-negative versions of Nbr, and employed the GAL4/UAS over-expression system [3]. Using these approaches, we disclose hitherto novel functions of nbr in (i) regulating TE activity, and in (ii) suppressing tumors. Moreover, we show that Nrb is expressed very early during oogenesis and that nbr is involved in regulating small interfering RNA (siRNA) activity. Taken together, our data suggests that nbr reveals a broader involvement in regulative cellular processes than just trimming specific miRNAs.

Methods
Drosophila stocks
All transgenic stocks were obtained using conventional transformation techniques [36] and were maintained as balanced stocks. MS1096-GAL4, apterous-GAL4, tubulin-GAL4, paired-GAL4, en-GAL4 and UAS-nbr were obtained from the Bloomington stock center. UAS-Dg flies were obtained from Martina Schneider [40].

The Drosophila Gene Collection clone GM01690 containing the complete ORF of nbr was used to generate the vectors pUAST-EGFP-nbr-D expressing the wild-type Nbr protein fused to EGFP, and pUAST-EGFP-nbr-N expressing the inactive Nbr protein fused to EGFP. Mutation of the catalytic domain was done by conventional inverse PCR using primers (5′-tcatatacttgatctgaatgt-3′) and (5′-acctttcctgatctgcctg-3′) to produce the amino acid substitution D435N (colored D in Fig. 1b). Both coding sequences were amplified with primers 5′-GGGCGCCCGCGAATGGCCAGCGAAG-3′ and 5′-CCTCTAGAGGCGCACGTTCTCAATC-3′. After restriction with Not I/Xba I, the amplified products were ligated in frame with EGFP, generating the vectors pUAST-
Fig. 1 Nbr harbors an exonuclease domain and is conserved from worms to humans. a Schematic presentation of some 3'-5' exonuclease-containing proteins, drawn to scale. From top: human Werner syndrome protein (accession number L76937.1), human FLJ371119 mRNA (accession number AK094438.1, corrected for some sequencing errors, based on genomic DNA), Drosophila melanogaster nibbler (nbr), (CG9247, accession number NM_136250.2), Caenorhabditis elegans ribonuclease-like protein ZK1098.3 (accession number NM_066703.1), Caenorhabditis elegans Mut7 protein (accession number NM_066704.1) and E. coli RNase D (accession number X07055). Identified domains are indicated on the right part and comprise the 3'-5' exonuclease domain, the DEAD box, the Helicase C domain, the HDRC domain and a low homology region common to CG9247, human FLJ20433 and C. elegans ZK1098.3. Note that human FLJ20443 and C. elegans ZK1098.3 are likely nbr orthologues as they also contain a low-homology region (shaded oblique) common to all Nbr proteins.

b Amino acid sequence alignment of the catalytic part of the 3'-5' exonuclease domain of the 6 proteins in Fig. 1a. Subdomain I-III nomenclature taken from [53]. Identical amino acids appear black, conservative changes appear in grey. Critically conserved amino acids appear in bold under the alignment. The critical amino acid D of subdomain I that was exchanged to N in Fig. 2 is highlighted in red/yellow.

c Exon-intron structure of nbr. Gray boxes represent exons. The line above exon 1 represents the probe used for in situ hybridization, as well as for the product of the RT-PCR for silencing quantification. The double line indicates the dsDNA fragment used for silencing in S2 cells. The hairpin line above indicates the dsRNA generated in the RNAi transgenic lines. The asterisk represents the position of the aspartic acid in domain I mutated in UAS-EGFP-Nbr-N construct.

d Silencing effect of transgenic RNAi embryos. Agarose gel electrophoresis of the RT-PCR products amplified for 29 cycles or 32 cycles. Genotypes: UAS-nbr/; UAS-nbr; tub-GAL4/TM3 (lanes 1 and 3); and UAS-nbr/UAS-nbr+/+; tub-GAL4/TM3 (lanes 2 and 4). Amplification of nbr generates a 430 bp band (see Fig. 1c), compared to the 388 bp band of the internal control from ribosomal protein 49 (rp49).

e Deduced amino acid sequence of the boundary of EGFP-Nbr used in the over-expression analysis of Fig. 2. Left side C terminus of EGFP (capital letters), middle linker region (small letters), right side N-terminus of Nbr (capital letters).
**EGFP-nbr-D** and **pUAST-EGFP-nbr-N**. Sequencing of the constructs was performed before the establishment of transgenic lines.

**RT-PCR**
Total RNA was isolated from ovaries or adult males and females using TRIzol (Invitrogen) and treated with DNase I (Ambion) to remove DNA. First strand cDNA was prepared using the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer’s instructions with 500 ng total RNA and 50 ng random hexamer primers in a 10 μl reaction.

The primers for amplification of *copia* were: (5′-ATT CAACCTAAAATAACG-3′) and (5′-ATTACGGTT TAGCCTTGT-3′), producing a product of 438 bp. The primers for amplification of the control, the ribosomal protein-encoding gene *rp49* were: (5′-GACCATCCGCC CAGCATAAGGC-3′) and (5′-GAGAAGCGAGGGG ACCGTGGG-3′) producing a product of 388 bp. In order to compensate for the distinct abundance of transcripts, primers for *copia* were used at 200 nM and for *rp49* at 40 nM.

**In situ hybridization**
Riboprobes were generated using a DIG-labeling kit (Roche). Two templates were amplified from cDNA and cloned into pBS (KS). The 5′ template including the signal peptide sequence of *nbr* was amplified with primers (5′-TGGTACCTCCGCAATGAGTGATTTAC-3′) and (5′-TATGGATCCTGAGTTCTCTAGT-3′) generating a 467 base pair probe. The 3′ template from the cytoplasmic part of the gene was amplified with the primers, (5′-CAAAGTCTGTCGATACGAGG-3′) and (5′-GACCATCGCCTGGTTGTCATATG-3′) generating a 343 base pair probe. The procedure for in situ hybridization was carried out according to [46]. A sense probe was used as a negative control.

**Generation of antibodies and immunohistochemistry**
A NH2-terminal peptide CNFDATLDKAEEFFKLRE KWNM comprising aa 46-69 of Nbr was used to immunize a rabbit. Crude serum from the 2nd bleed was used in all experiments. For *Drosophila* whole-mount staining, a Nbr monoclonal antibody [24] was used at 1:100 and detected using 2nd antibodies coupled to Alexa 555. For all immunofluorescence pictures, a Zeiss LSM 710 was used. For super-resolution recording, an Airy-Scan (Zeiss™) assembly was used in combination with a 63× lens. For Western analysis, embryos from the cross of the **paired (prd)**-**GAL4 > UAS-nbr** strain (Bloomington stock #16587) were used for 4-8 h extracts which, together with a 4-8 h wild-type extract of similar protein concentration were separated on a 10% PAGE and probed with the Nbr antiserum at a 1:2000 dilution.

**RNA interference in cells**
S2 cells were propagated in 1× Schneider’s *Drosophila* medium (GIBCO), supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin at 27 °C. dsRNAs were produced from amplified DNA templates using a MEGASCRIPT T7 transcription kit (Ambion) following the protocol of [10]. DNA templates were amplified with primers containing a 5′ T7 RNA polymerase binding site (5′-TAATACGACTCTATAGGGAGAC CAC-3) followed by sequences specific for the targeted genes. The following primers were used: *nbr* (5′-TA ATACGACTCCTATAGGGAGACCAGCAGGCTATA ctcg-3′) and (5′-TAATACGACTCCTATAGGGAGA CCAAGTCTGTCGATACGAGG-3′); *GFP* (5′-TAATACGACTC ACTATAGGGAGACCAGCAGGCTATAcgcg-3′) and (5′-TAATACGACTCCTATAGGGAGACCAGCAGGCTATA ctcg-3′) and mock-lacZ (5′-TAATACGACTCCTATAGGGAGACCAGCAGGCTATA ctcg-3′) and (5′-TAATACGACTCCTATAGGGAGACCAGCAGGCTATA ctcg-3′), amplifying the 838 bp sequence of the vector pBluescript SK downstream of the T7 RNA polymerase binding site that includes the lacZ alpha gene. dsRNA products were DNase-treated, ethanol-precipitated and resuspended in DEPC water. The dsRNAs were analyzed by agarose gel electrophoresis to ensure that single bands of the expected size were present. S2 cells were transfected using FuGENE 6 (Roche) in 3 cm dishes at 50%–70% confluence, following the manufacturer’s recommendations. The standard transfection reaction contained 2 μl of plasmid expressing GFP (pAC-EGFP), 2 μg of dsRNA targeting GFP and 2 μg of either *nbr* dsRNA or mock dsRNA.

**Transgenic RNA interference construct**
A 408 bp fragment at the 5′ of the *nbr* gene (Fig. 1c) was amplified from cDNA with primers 5′-TGGTACCTCCGCAATGAGTGATTTAC-3′ and 5′-TATGGATCCTGAGTTCTCTAGT-3′) generating a 467 base pair probe. The 3′ template from the cytoplasmic part of the gene was amplified with the primers, (5′-CAAAGTCTGTCGATACGAGG-3′) and (5′-GACCATCGCCTGGTTGTCATATG-3′) generating a 343 base pair probe. The procedure for in situ hybridization was carried out according to [46]. A sense probe was used as a negative control.

**Results**
*Castillejo-Lopez et al. Hereditas (2018) 155:12**
transcript, originally termed CG9247 by the *Drosophila* sequencing consortium (BDGP; [9]), which contained a typical 3′-5′ exonuclease domain found in many proteins from worms to humans (Fig. 1a), subsequently termed *nibbler* (*nbr*) [23, 32]. Analysis of the *nbr* open reading frame (ORF) revealed a protein of 625 amino acids with two domains shared by other proteins in the animal kingdom. At the amino terminus, there is no indication of a signal peptide indicative of a secreted or a transmembrane protein, suggesting that it is an intracellular protein. The first third of the protein (amino acids 1-181) does not contain any homology to any known protein, while the second third (amino acids 182-409) contains low homology to human protein FLJ20433 and *C. elegans* nuclease ZK1098.3. The last third of the protein contains the 3′-5′ exonuclease domain which was the initial searching bait. This domain is a widespread domain found in diverse proteins such as human Werner syndrome protein [51] or *E. coli* RNaseD [52]. Due to the fact that human FLJ20433 and *C. elegans* ZK1098.3 contain a similar domain arrangement as CG9247 and because lengths of all three proteins are similar, we presume that these three proteins represent the respective *nbr* orthologues.

Analysis of the *nbr* 3′-5′ exonuclease domain revealed that the sequence homology is fairly good (Fig. 1b), in particular the absolutely preserved amino acids that are the typical characteristics of an exonuclease domain. This domain is part of a large DEDD subfamily of exoribonucleases [53], owing to the fact that they contain invariant acidic amino acids at certain position such as the aspartic acid D and the glutamic acid E within domain I (Fig. 1b) and two aspartic acids D in domain II and III, respectively (Fig. 1b). This DEDD subfamily includes the proof-reading domains of many DNA polymerases as well as other DNA exonucleases and shares a common catalytic mechanism characterized by the involvement of two metal ions [44]. Of the different members of the DEDD subfamily, the Nbr protein resembles most the RNaseD proteins which further subdivide the DEDD subfamily into the DEDDy sub-subfamily [53], due to the presence of an invariant tyrosine Y within the catalytic domain III (Fig. 1b).

*nbr* exhibits nuclease activity

To explore the function and activity of the exonuclease domain, we inactivated the domain through a change of the invariant amino acid D to N within the catalytic domain I (highlighted in Fig. 1b, c), and by fusing the enhanced green-fluorescent protein (EGFP) to this altered Nbr protein as an EGFP-Nbr-N fusion protein (Fig. 1e). We then used the *Drosophila* wing imaginal disc as a model tissue to test the nuclease activity of Nbr and its catalytic-dead variant, Nbr-N, by using the GAL4/UAS over-expression system [3]. We over-expressed Nbr-EGFP in the proximal part of the wing disc using the *apterous-(ap) GAL4* line which drives transgenes in the proximal compartment (P) of the wing. In *ap-GAL4 > UAS-Nbr-EGFP* wing discs, we detected strong nuclease activity in the proximal compartment of the wing disc (Fig. 2d). Upon propidium-iodine (PI) staining, we observed substantial nucleic acid (NA) degradation in the proximal compartment such that only the green color from the EGFP-Nbr fusion protein remained visible. Moreover, this NA degradation led to extensive apoptosis, demonstrated by a collapsed proximal part of the wing disc. Conversely, the distal part was not affected by the ectopic expression of Nbr and thus appeared normal. These data suggest that Nbr is an potent nuclease. Conversely, a mutant form of Nbr, Nbr-N, is unable to exert any nuclease activity upon over-expression with *ap-GAL4* (Fig. 2e). Hence, even though Nbr is overexpressed in the proximal compartment, it shows intact cells, evidenced by positive staining of PI, intact NA and the change of the merged color of PI and EGFP into yellow. Moreover, this defective nuclease activity does not compromise cell survival, therefore the shape of the proximal part of the wing disc appears normal.

Localization of the *nbr* mRNA and protein

We employed in situ hybridization to detect the spatial transcript pattern of *nbr* in *Drosophila* whole mount embryos. *nbr* is expressed ubiquitously in the early developing embryo (Fig. 2a), suggesting a maternal deposition, also confirmed by FlyBase [1]. At stage 5, i. e. at cellular blastoderm stage, *nbr* expression drops considerably, and transcripts are only detected ubiquitously at low level during the remaining embryonic stages (FlyBase, [1]; data not shown). To ease analysis of the localization and function of *nbr*, we constructed *EGFP-Nbr* flies under control of the GAL/UAS system that allows ectopic expression of any protein [3]. To test the functionality of the **EGFP-nbr-N** transgene, we monitored the expression of the ectopic transcript driven by the *paired (prd)-GAL4* driver using in situ hybridization. As is evident from Fig. 2b, the **EGFP-nbr-N** transgene is faithfully expressed in 7 stripes, compared to the low-level ubiquitous expression of the endogenous *nbr* transcripts.

To investigate the subcellular localization of the Nbr protein, we first analyzed the EGFP-Nbr wild-type fusion protein in salivary gland cells from third instar larvae using a *tubulin (tub)-GAL4* driver line. As evident in Fig. 2c, fluorescence was detected at low levels in the cytoplasm and at a perinuclear localization, similar to the localization of Nbr in the nuage, as described by [24].
To evaluate the nature and appearance of the protein, we used an antiserum which was directed against a peptide residing at the NH2-terminal part of the Nbr protein. On Western analysis, a 60 kD band was readily detected in wild-type 4-8 h extracts (Fig. 3k) in agreement with other reports [15]. The intensity of the 60 kD band was substantially increased when Nbr was overexpressed using prd-GAL4 driving an UAS-nbr construct during an identical time interval and upon equal protein amount loaded (Fig. 3k).

To monitor the subcellular expression, we stained Drosophila tissues using a monoclonal antibody against Nbr [24] along with a nuclear stain, DAPI. We first focused on oogenesis, since nbr was reported to play a critical role during this stage [15, 24]. We detected the protein during the earliest stages in the germarium and in stage 2 oocytes only (Fig. 3a), while all subsequent stages were devoid of Nbr. Using super-resolution microscopy aimed to detect Nbr localization subcellularly and in the nuage [24], the Nbr protein was detected uniformly in the cytoplasm of stage 2 oocytes (Fig. 3b). This localization data are inconsistent with the data of [24] who reported staining in the nuage surrounding the early nurse cells, but otherwise devoid of any other cellular localization. Moreover, our subcellular Nbr localization data do not entirely fit the localization pattern of EGFP-Nbr (Fig. 2c), however, the data was recorded in two different tissues, the salivary gland vs. the oocyte. Moreover, both the data by [24] and the data of Fig. 2c were based on the use of GFP-Nbr constructs rather than true antibody detection. Hence, we propose that the Nbr-EGFP fusion proteins tend to accumulate
in the nuage and thus lead to a misinterpretation of the location where Nbr acts. On the other hand, our antibody data fit another report that revealed Nbr ubiquitous expression in the cytoplasm of oocytes [15] using a Nbr-HA fusion protein and anti HA staining, hence the localization does not include an EGFP-tag, but rather a small and binding-neutral HA-tag. In summary, there are marked differences between the EGFP-Nbr localization and the true antibody staining which question the nuage staining by [24].

Later during oogenesis, i.e. during stage, Nrb was detected at low levels at the cortex of the oocyte (Fig. 3e) which persisted during later stages of oogenesis (data not shown). In freshly-laid embryos, the cortical pattern was particularly pronounced (Fig. 3h), due to the strong maternal loading of nbr mRNA (Flybase; [1]. The cortical pattern persisted during early nuclear stages, including nc 11 embryos (Fig. 3i) when the majority of the nuclei have reached the periphery of the blastoderm. At cellular blastoderm, this cortical staining persisted (Fig. 3j), and Nrb was detected on the basal as well as on the apical side of the nuclei, however, at low levels, leaving the nuclei free of Nbr staining.
Down-regulation of the nhr nuclease activity can lead to tumor formation.

To determine the function of nhr for development, we used a GAL4/UAS-inducible knock-down strategy [29]. To this end, a hairpin cDNA construct (Fig. 1c) was cloned into the pUAST vector [3] and transgenic lines were established. The knockdown activity of this construct was measured in a reverse transcriptase-PCR approach and was found to lead to a 60% reduction of nhr mRNA (Fig. 1d). Interestingly, one of these lines, UAS-nhr19 showed incidences of melanotic tumors (Fig. 4a) or solid tumors (Fig. 4b) (arrows), when crossed to an ubiquitously-expressed tub-GAL4 driver line. Due to the widespread occurrence, we then sought to limit the occurrence of cancer to well-defined areas. To this end, an engrailed (en)-GAL4 driver line which is active in posterior wing compartments (P, Fig. 4d) was crossed to UAS-nhr19, and the wings of the progeny were examined. We found small tumors exclusively in the posterior compartments of the wings (Fig. 4d, arrows), consistent with en expression in the posterior compartment of the wing. Thus, it appeared that down-regulation of nhr increased the incidence of cancer. We reasoned that the activity of Nbr might be needed for control of cell divisions or for control of activity of transposons which randomly affect the activity of cell cycle regulators. Alternatively, the increase of incidences of melanotic tumors in nhr-depleted tissues could be a consequence of the impairment of normal aging processes as previously reported by [15].

Knockdown of nhr reduces the effect of RNAi

Next, we pondered if nhr might be involved in mechanisms of the RNAi machinery. For this reason, we set up two parallel assay systems, a cell-culture based system and a transgenic fly approach to test if nhr is involved in RNAi. Drosophila Schneider cells S2 were transfected with a reporter plasmid driving EGFP by an actin promoter. In parallel, two RNAi, one against the RNA of EGFP and another one against the RNA of a mock gene, LacZ, were applied simultaneously.

The expression of the GFP transgene is complete suppressed when the cells are co-transfected with RNAi against EGFP, or RNAi against EGFP and RNAi against the mock gene lacZ (Fig. 5a). The efficiency of RNAi is reduced when the mock RNAi is substituted with the RNAi against nhr (Fig. 5b), demonstrating that suppression of nhr suppresses the RNAi machinery.

When then assayed nhr function using an in vivo system and took advantage of the fact that knock-down of the Drosophila dystroglycan (Dg) gene affects the formation of the posterior cross vein (PCV) in wings of the progeny of MS1096-GAL4 > UAS-Dgi flies (Fig. 5c, arrows) [13]. In this genetic combination, the variability of loss of PCV tissue ranged from total absence to slight reduction, as indicated in Table 1. In ~30% of the flies, a complete reduction of the PCV was observed (Fig. 5c), while in ~16% of the cases, the PCV was present halfway (Fig. 5d). In about 53% of the cases, more than half of the PCV was present (Fig. 5e). However, when nhr was knocked-down using UAS-nhr19 flies, the loss of...
PCV was greatly ameliorated and only ~15% showed complete PCV loss (Table 1). More than 72% of the flies showed more than half of the PCV present, compared to 53% in UAS-Dgi (Table 1). An unrelated gene, GC3505, a serine protease not involved in wing development (C. Castillejo-Lopez, unpublished), did not significantly alter the loss of PCV upon knock-down in the same genetic background, as compared to the UAS-Dgi reference flies (Table 1). This data demonstrated that lowering nbr activity caused a reduction of the effect of RNAi. It also suggested that the exonuclease activity of nbr was tightly linked to the process of RNAi.

**nbr affects the levels of transposon RNA intermediates**

The close similarity of Nbr to *C. elegans* Mut-7 (Fig. 1), a gene involved in TE silencing, and the elevated levels in occurrence of tumors in Fig. 4 prompted us to investigate if theses tumors were caused by elevated levels of transposon activity. We reasoned that the increased TE hopping frequency into genes regulating cell cycles would

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**Figure 5** nbr knockdown reduces the silencing effect of RNA interference. a and b Reduction of the silencing effect of nbr in S2 cells. a cells transfected with the reporter plasmid pAc-EGFP and RNAi against EGFP and mock RNAi. Expression of EGFP is completely abolished. b When mock RNAi is replaced with nbrRNAi, expression of the EGFP reporter gene is increased in a number of cells. c-f phenotypic classes of males flies expressing dsRNA against *Drosophila* dystroglycan (Dg, CG18250) related to the occurrence of the posterior cross vein (PCV) in wings, divided into 4 classes of decreasing severity: (c) <5%, (d) 5-50%, (e) 50-100% and (f) 100% = wild-type. In wings of Dg-knockdown flies, PCVs extending more than 50% are observed in ~53% of the wings (see Table 1). In wings of doubly Dg, nbr knockdown flies, this number is increased to ~73%, demonstrating that the RNAi activity of Dg is suppressed by lowered activity of nbr.

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**Table 1** Occurrence of posterior cross vein (PCV) in Dg knockdown flies, assayed together with 3 different genetic backgrounds

| Genotype | PCV < 5% | PCV < 50% | PCV > 50% | Total wings |
|----------|----------|-----------|-----------|-------------|
| MS1096-GAL4 > Dg/Y, ftl/Y | 30.1% | 16.5% | 53.4% | 402 |
| MS1096-GAL4 > Dg/Y, 3505-2a/Y | 37.5% | 7.6% | 54.9% | 662 |
| MS1096-GAL4 > Dg/Y, nbr/Y | 14.9% | 12.4% | 72.7% | 442 |

Allelic classes are subdivided into 3 classes: those flies that show <5% PCV occurrence, those <50% PCV occurrence and those with >50% occurrence. As read out system, a MS1096-GAL4-driven Dg knockdown construct (13) was used. This construct creates loss of PCV to various extent (Fig. 4c). Lane 1, an unrelated ftl gene [8]. Lane 2, CG3505-2a (unpublished data). Both unrelated genetic backgrounds were used as a negative controls. Lane 1 and 2 show that 54% of all flies have 50% or more PCV tissue present. Lane 3, in doubly ds-nbr; ds-Dg flies, this value increased to 73%, indicating that lowering nbr activity suppresses the knock-down activity on Dg, suggesting that nbr interferes with the RNAi machinery.
ultimately lead to an increase of incidences of tumors. It is well known that the *Drosophila* germ line is constantly exposed to high activity of transposons [33, 38, 50], hence, mechanisms must exist that limit the hopping of transposons. We therefore investigated the activity of TEs within the female germ line and compared the intermediate RNA level of the *copia* transposons in wild-type and *nbr* knockdown ovaries. As is evident in Fig. 6, the level of *copia* RNA intermediates is 33% higher in *nbr* ovaries compared to wild-type ovaries after 31 PCR cycles, after calibration with a reference gene, *rp 49*. However, after 34 nuclear cycles, the PCR reaction is close to saturation and only a 1% increase was observed, after calibration with *rp 49*. These semi-quantitative data suggests that *nbr* serves as a suppressor of *copia*. Moreover, a comparison between tissues revealed that the relative rate of transposition is considerably higher in ovaries, compared to that of whole adult females (Fig. 6, Aw).

**Discussion**

Using in vivo studies and by exploiting the GAL4/UAS system in *Drosophila*, we have analyzed the function of the Nibbler protein for development and for cellular mechanisms. We have undisclosed novel functions of this protein which suggest more wide-spread functions than hitherto anticipated.

Data from Fig. 2 indicate that Nibbler possesses a general nuclease activity and is probably more widely involved in cellular activities than only involved in trimming small RNA ends [23, 32]. This result is not surprising, as the protein possesses an exonuclease domain, however, this report shows for the first time that Nbr shows a broader involvement in trimming mRNAs. Discussed as a possibility by [15] that Nbr affects the length of not only miRNAs, but also that of *piRNAs*, it was speculated that Nbr could potentially trim the 3’ ends of a much broader species of RNA substrates, including other long and short non-coding RNAs and mRNAs. However, presumed to be instructive for the *piRNA* pathway, *nbr* has received little attention in the context of general function of RNA trimming.

Nbr appears to control the expression levels of TEs, as exemplified by *copia* in Fig. 6. As noted by [49], *copia* expression is also increased in *nbr* mutants which is in line with our observation (Fig. 6) that *nbr* controls the activity of TEs. Whether the regulation is direct or indirect via the *piwi* pathway which is involved in regulating the levels of TEs in germ cells, in currently unknown, but we favor an involvement of the *piwi* pathway.

Our data on Fig. 4 indicated that knock-down of *nbr* provokes the formation of tumors. Our current hypothesis is that ablation of *nbr* increases the rate of transposition. In these cases, the *piRNA* pathway is probably not involved, as the pathway is restricted to germ cells and extremely little *piwi* expression was observed during larval stages (Flybase; [1}). Instead, we envision that *nbr* is involved in the *miRNA* pathway, by controlling any of the multi-isoform miRNAs that are expressed during larval and pupal stage [23, 32]. These are then thought to control genes regulating cell-cycles or cell-cycle check points.

Since a while, it is known that miRNAs are involved in tumorigenesis, where the focus is mainly in humans [25, 30, 45]. To date, in *Drosophila*, only a handful of miRNA genes are known to be involved in the formation of cancer. One of them is the *bantam* gene, identified
by a conventional gain of function screen which constitutes a miRNA gene that positively regulates cell proliferation and suppresses apoptosis – two features typical of oncogenes [5, 26]. However, with the advent of the availability of systematic studies by applying inducible Drosophila miRNA transgenes, scores of uncovered of surprisingly specific, dominant phenotypes were discovered [2, 16]. These surveys suggest that miRNA gain of function may generate diseases much more frequently than miRNA loss of function.

Our sensitive assay on Dg-RNAi-mediated depletion of the posterior cross vein (PCV) of Fig. 5c-e and Table 1 confirms a direct involvement of nbr in RNAi-mediated gene silencing. If nbr is reduced, the activity of the RNAi machinery is weakened and depletion of PCV structures is reduced substantially. Likewise, we could confirm the mechanistic action of nbr in cell culture assays which revealed that effect of RNAi was weakened when nbr activity was compromised (Fig. 5b). Hence, for the first time, we can demonstrate that nbr is involved in patterning processes involving whole tissues. Moreover, our data demonstrate that nbr is part of a general RNAi machinery and not just involved in trimming selected miRNAs [23, 32].

In the past, there has been considerable disagreement as to the localization of Nbr [15, 24]. The nuage-based localization of Nbr [24], based on its involvement in the piwi-pathway was born by the necessity to reveal colocalization of Nbr with Aub/Ago3 in the nuage, and to adapt its localization to fit the model. Arguably, for localization studies, it is not recommended to use a fusion protein involving EGFP as in [24], as it can lead to substantial localization artefacts due to oligomerization [35, 41]. Consistent with this observation was the fact that our EGFP-Nbr fusion protein, apart from general cytoplasmic localization, also showed perinuclear localization in 3rd instar salivary glands (Fig. 2c), similar to the EGFP-Nbr localization reported in the nuage [24]. In fact, there is not an immediate necessity to describe Nbr enrichment in the nuage as claimed by [24]. Instead, it would have sufficed to imply ubiquitous Nbr localization which also includes localization in the nuage, in order to fulfill the model. This argument was put forward by [15] who observed ubiquitous Nbr localization within oocytes as well, however, their expression profile differed slightly from ours and Nbr was reported to be ubiquitously expressed beyond oocyte stage 2. Ubiquitous Nbr expression rather than accumulation in the nuage also makes sense from another perspective: Given the wide-spread involvement [15, 49] (this report), the function of Nbr is needed in the whole cytoplasm and not just in the nuage.

Conclusions
We have shown that nbr is a tumor suppressor gene, and that the protein is involved in the RNAi machinery and controls the levels of transposons. Nbr is expressed only during a short time window during oogenesis and is not enriched in the nuage. Hence, we have described novel functions of nbr that go beyond from what was expected from previous knowledge on the mode of action of nbr. The ubiquitous localization of Nbr in oocytes necessitates a further careful analysis as to the mode of action of this protein. While it is not excluded that indeed it localizes to the nuage, it is not the sole subcellular location where Nbr resides which asks for further functions of this protein in other areas of the cytoplasm. Moreover, our data will encourage studies to show that Nbr is involved in many cellular processes.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
CCL designed, analyzed and interpreted the data and was a major contributor in writing the manuscript. XC was responsible for data of Fig. 3. KF was involved in experiments related to Fig. 5. SB was responsible for the data of Fig. 3 and writing of parts of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
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Consent for publication
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