Hecw controls oogenesis and neuronal homeostasis by promoting the liquid state of ribonucleoprotein particles

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Specialised ribonucleoprotein (RNP) granules are a hallmark of polarized cells, like neurons and germ cells. Among their main functions is the spatial and temporal modulation of the activity of specific mRNA transcripts that allow specification of primary embryonic axes. While RNPs composition and role are well established, their regulation is poorly defined. Here, we demonstrate that Hecw, a newly identified Drosophila ubiquitin ligase, is a key modulator of RNPs in oogenesis and neurons. Hecw depletion leads to the formation of enlarged granules that transition from a liquid to a gel-like state. Loss of Hecw activity results in defective oogenesis, premature aging and climbing defects associated with neuronal loss. At the molecular level, reduced ubiquitination of the Fmrp impairs its translational repressor activity, resulting in altered Orb expression in nurse cells and Profilin in neurons.
NP granules play a fundamental role in both germ and somatic cells as they are responsible for the mRNA transport and local translation required for neuronal and oocyte maturation. Oogenesis has been intensively studied in Drosophila where many genes essential for germ line development belong to these membrane-less granules. Mutations in critical RNP components have been linked to neurological diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), highlighting the crucial contributions of RNA localisation and translational control to long-term neuronal integrity. Furthermore, liquid-to-solid-state transitions are emerging as the key events in the formation of intracellular pathological protein aggregates. Whereas the ubiquitin-proteasome system has been demonstrated to play a major role in pathological aggregates, our understanding of the role/s played by the ubiquitin system in the RNP's metabolism and dynamics is limited.

In the ubiquitination cascade, E3 ligases act as catalysts and molecular matchmakers of the reaction. They carry out the final step of covalently binding ubiquitin (Ub) to substrates, usually to lysine (K) residues, and destrabeculate E3 ligases is known to contribute to a wide range of physiological and pathologic processes, including immune regulation, viral infection, tumorigenesis and neurological disorders.

In this study, we investigate the molecular, cellular and organismal functions of a previously uncharacterised member of the NEDD4 family in Drosophila to reveal an unexpected and pivotal role for non-degradative ubiquitination in maintaining the liquid state of germ- and neuronal RNP granules.

Results
CG42797/Hecw is the Drosophila ortholog of HECW1 and HECW2. By searching the Drosophila genome, we identified a single ortholog of human HECW1 and 2, encoded by the uncharacterised gene CG42797, whose protein product shares co-linearity and 40% overall similarity with the corresponding human proteins. Extensive amino acid sequence identity is present in two WW domains required for substrate interaction and in the catalytic HECT domain. Similar to the C. elegans Ce01588, the Drosophila protein lacks the C2 lipid binding domain, which is characteristic of the NEDD4 family, suggesting that CG42797 may be unable to bind membranes.

To confirm that CG42797 is a catalytically active HECT ligase, we performed an in vitro self-ubiquitination assay. We found that CG42797 is able to ubiquitinate itself and to generate free polyubiquitin chains, whereas a mutation in the evolutionary conserved catalytic cysteine abrogates both activities. Analysis of various Drosophila organs by qPCR and immunoblotting, revealed a preferential expression of Hecw in the fly gonads and central nervous system. In fly ovaries, Hecw displays a broad distribution in the cytoplasm of both somatic and germline tissues. Similarly, the adult brain showed cytoplasmatic staining exclusively in elav-positive neuronal cells. Interestingly, as previously reported for several components of the Ub proteasome system and autophagy pathway controlling protein homeostasis, the level of Hecw expression in fly heads decreases with aging.

Loss of Hecw causes age-dependent neuronal degeneration. To study the consequences of loss of Hecw, we generated catalytic inactive (CI) mutants and knock out (KO) flies using the CRISPR/Cas9 system. As all the tested mutants and KO lines exhibit identical defects, we hereby describe HecwCI and HecwKO as representative examples.

Both HecwCI and HecwKO homozygous (mutant from now on) flies are viable and do not show macroscopic morphological defects, indicating that Hecw is a non-essential gene. Prompted by the reduced expression of Hecw in adult flies, we investigated the behaviour of the mutant flies during aging. To minimise the influence of genetic background, environment, nutrition and mating conditions, we performed a lifespan assay with mixed-sex groups in standard cornmeal food. HecwCI and HecwKO mutant animals displayed reduced longevity with respect to isogenic control lines with a 22% and 24% decrease in median survival, respectively. Mutant lifespan reduction was even higher at 29°C (28% median survival reduction).

Drosophila display an aging-related decline in climbing whose anticipation is considered a hallmark of neuronal dysfunction. Remarkably, in climbing assays, both Hecw mutant flies displayed motor function impairment, which was already visible in 20-day-old flies and became extremely severe as the flies aged. Again, the phenotype was exacerbated at 29°C. Next, we analysed frontal brain paraffin sections and observed that mutants presented extended tissue vacuolisation, which resulted from neuronal death in the CNS. The size and number of vacuoles are significantly higher in mutants compared to isogenic control flies and tissue vacuolisation progressively increases with fly age.

Importantly, defective neuronal function and morphology are rescued by the reintroduction of a wild-type copy of Hecw in homozygous HecwKO animals. Moreover, overexpression of Hecw with the pan-neuronal driver elav-GAL4 causes reduction in longevity, similarly to that observed in Hecw mutants. Importantly, the expression level of Hecw in fly gonads prompted us to assess gamete formation in mutant flies. In females, egg-laying varies with age. After a peak at day 4 post eclosion, there is a physiological decline in egg production, which is generally reduced by 50% at 40 days. When compared to matched control flies, 20-day-old Hecw mutant females lay a
significantly reduced number of eggs (Fig. 3a). To determine whether the reduced egg-laying exhibited by Hecw mutants is accompanied by structural defects, we immunostained ovaries of 3- and 30 day-old well-fed mated wild-type and mutant flies to detect the CPEB protein Orb ( oo18 RNA binding), which accumulates in the cytoplasm of the developing oocyte. Already at day 3, 20% of egg chambers derived from mutant flies showed an altered number of germ cells (Fig. 3b). Defective egg chambers present either reduced or increased number of nurse cells and ring canals, and compound egg chambers (Fig. 3b and Supplementary Fig. 4a). In addition, we detected the presence of late-stage apoptotic egg chambers that are usually absent in nutrient-rich conditions (Fig. 3b), indicating that defective egg chambers are likely culled. Consistently, the hatching rate of mutants is not
significantly affected (Supplementary Fig. 4b). The penetrance of these defects increases with age, reaching about 40% in 30-day-old flies. Classification and quantification of the aberrant phenotypes observed are summarised in Supplementary Table 1.

To attribute these functional alterations either to the somatic or germline cells (GC), we performed tissue-specific knock down in follicle cells (FC), using the *traffic-jam-Gal4* driver (tj>), and in GC, using the *nanos-Gal4* driver (nos>). Depletion of Hecw in GC (Fig. 3c) but not in FC (Supplementary Fig. 4c) recapitulates *Hecw^CI* or *Hecw^KO* mutant phenotypes, demonstrating that aberrant oogenesis defects are mostly caused by the absence of Hecw function in the germline.

The oogenesis defects of chronically Hecw-depleted flies are fully rescued by the reintroduction of a wild-type copy of *Hecw*...
expressed at physiological levels in the whole organism (Supplementary Table 1 and Fig. 3d). As shown in neurons, unscheduled expression of Hecw is also detrimental in gonads, in which ectopic expression of Hecw leads to oogenesis aberrations similar to those observed in HecwCI animals (Fig. 3e and Supplementary Fig. 4d). These results indicate that tight regulation of Hecw expression in the germline is necessary to allow oocyte development.

Loss of Hecw changes the state of RNPs in germ cells. In developing egg chambers, Orb protein expression is usually confined to the oocyte thanks to the repressive action of Cup and Fmrp on selected mRNAs, as part of RNPs assembled in nurse cells. Interestingly, we observed the presence of suprumerary Orb-positive cells in about 30% of HecwCI mutants (Fig. 3b), as well as discrete Orb-positive puncta in the cytoplasm of nurse cells in both HecwCI and HecwKO mutant flies (Fig. 4a). To characterise the origin of Orb-positive puncta, we generated HecwKO mutant flies expressing the RNP marker Me31B::GFP [ref. 26] in homozygosis. In wild-type flies, association of Orb with RNPs selectively occurs in the oocyte27,28. By contrast, in Hecw mutant animals altered Orb puncta fully colocalise with Me31B in the cytoplasm of nurse cells of previtellogenic and early vitellogenic egg chambers (Fig. 4b). Importantly, HecwKO and HecwCI egg chambers contain significantly larger Me31B::GFP RNPs when compared to control (Fig. 4b, c and Supplementary Fig. 5a).

The dynamic composition of germline RNPs is linked to the biophysical status of membrane-less organelles, which allows free diffusion in and out of the particles29,30. To investigate the nature of RNPs dynamics in the presence or absence of Hecw during Drosophila oogenesis, we performed live imaging of Me31B::GFP, HecwCI::Me31B::GFP or HecwKO::Me31B::GFP egg chambers (Supplementary Movie 1 and 2). Wild-type RNP particles appear mostly spherical and occasionally undergo fusion (Supplementary Movie 3 and Supplementary Fig. 5b), two features of organelles with liquid-like properties31. This behaviour is altered in HecwKO RNPs, which grow more irregularly shaped aggregates during the coarsening process (Supplementary Movie 2 and Supplementary Fig. 5c).

To determine the nature of the interactions within RNP particles, egg chambers were treated with 2,5% 1,6 hexanediol, an aliphatic alcohol that disrupts weak hydrophobic bonds, typical promoters of liquid droplets32. While the size and number of wild-type granules is drastically reduced upon alcohol treatment, the larger HecwCI and HecwKO RNPs barely dissolve, mirroring their solid-like nature (Fig. 4d, e).

This result prompted us to evaluate the movement of Me31B, which is a combination of free diffusion and active, microtubule-dependent transport33. Live observation revealed no statistical differences in granule speed between control and HecwKO flies. (Supplementary Movie 1, 2 and Supplementary Fig. 5d). Consistently, immunofluorescence analysis of the microtubule cytoskeleton structure in HecwKO egg chambers showed no major alteration when compared to control (Supplementary Fig. 5e). RNP mobility in each egg chamber was quantified with Differential Dynamic Microscopy (DDM) analysis34 that provides the average mean square displacement $\text{MSD}(\Delta t)$ of the granules, from which we extract an effective diffusion coefficient (Fig. 5a–c). Results indicate that RNP transport is not impaired in the absence of Hecw (Fig. 5d).

To measure the exchange rate of Me31B::GFP molecules inside the RNP particles, we performed fluorescence loss in photobleaching (FLIP) measurements (Supplementary Movie 4), as the fast movement of the granules precluded direct FRAP analysis. Initial analysis performed considering the neighbouring regions of the bleached area showed that the mobile fraction of Me31B::GFP is reduced in HecwKO egg chambers (Supplementary Fig. 6a). As the difference cannot be unambiguously attributed to a different rate of exchange in the granules, we refined our analysis considering smaller regions of interest (ROIs) that selectively include either RNPs or cytoplasm (Fig. 6a). No difference in bleaching efficiency is observed on the directly irradiated area (Fig. 6b) while examination of single RNP showed a significant difference in fluorescence decay time between wild-type and HecwKO flies ($\tau_{\text{WT}} = (1.3 \pm 0.1) \cdot 10^2$ vs $\tau_{\text{KO}} = (4.2 \pm 0.1) \cdot 10^2$. $P < 10^{-6}$ (Fig. 6c). Since we matched control and HecwKO egg chambers for size or spatial distribution of RNPs (Supplementary Fig. 6b–d), this behaviour could only be attributed to a reduction in the exchange rate of the fluorescent protein between the RNP interior and the cytoplasm. This alteration is limited to the granules as the movement of cytoplasmic Me31B::GFP is comparable between wild-type and HecwKO flies (Fig. 6d). Thus, in the absence of Hecw, RNP particles may undergo a transition to a less fluid, gel-like state. Overall, these results indicate that Me31B::GFP-positive RNPs possess liquid droplet properties that are regulated by Hecw-mediated ubiquitination.

Hecw interacts and colocalises with RNPs components. The altered physical properties of Me31B::GFP RNPs prompted us to investigate whether Hecw may interact with RNP proteins. Consistently, in ovarian extract, Hecw co-immunoprecipitates with Me31B::GFP together with other known RNP components, such as Orb and Fmrp [ref. 2] (Supplementary Fig. 7a). The interaction is maintained upon RNase treatment, suggesting that
Hecw interacts directly with RNPs via protein-protein interactions (Fig. 7a).

To identify Hecw binding partners, we performed a pull-down assay using a GST construct spanning the two WW domains as a bait, followed by mass spectrometry (Supplementary Table 2). The majority of Hecw interactors are mRNA binding proteins involved in multiple steps of RNA processing (Supplementary Table 2), among them Fmrp. Strikingly, two other interactors, Hrp48 and Glorund, are translational repressors previously implicated in translational control during oogenesis.\textsuperscript{35} These findings provide compelling evidence for a critical role of Hecw in RNPs regulation.

**Hecw-dependent ubiquitination modulates Orb via Fmrp.** How could Hecw-dependent ubiquitination regulate RNPs? We focused on Fmrp, a known RNA binding repressor of the Orb autoregulatory loop in nurse cell\textsuperscript{25}. *Fmr1\textsuperscript{Δ113}* loss of function

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**Fig. 3 Aberrant expression of Hecw impairs proper oogenesis.**

(a) Fertility assay of 20-day-old females of the indicated genotypes. Results of four biological replicates are expressed as mean ± SD, **P < 0.01 by unpaired two-tailed t-test (n = 6 females/genotype/replicate).

(b) IF analysis of wild-type (ctrl, upper right panel), Hecw\textsuperscript{CI} (CI, left panels) and Hecw\textsuperscript{KO} (KO, right panels) egg chambers. Mutants show aberrant number of nurse cells (top panels), compound egg chambers (middle panels), oocyte misspecification and apoptotic egg chambers (bottom panels). Blue, DAPI; red, phalloidin; white, Orb; asterisks indicate oocytes in compound egg chambers; white arrow indicates an Orb-positive nurse cell. Scale bar: 25 µm. c–e IB and IF analysis of the indicated genotypes. Hecw depletion in the germline causes oogenesis defects, as in (b), upper panel. Example of compound egg chamber in a 3-day-old Hecw-depleted fly (nos>RNAi Hecw) (c). The oogenesis defects are rescued in the Hecw\textsuperscript{KO;DC504} genetic background (d). Ectopic expression of Hecw in the germline (nos>RFP-Hecw) induces oogenesis defects (e). Blue, DAPI; red, phalloidin. Asterisks indicate oocytes of the compound egg chamber. Scale bar: 25 µm. See also Supplementary Table 1 for the quantification.
mutant flies display oogenesis defects that resemble the ones of Hecw mutants (Fig. 7b). Corroborating the idea that the two proteins act on the same axis, genetic interaction revealed no worsening of the phenotypes (Supplementary Fig. 7b). In addition, we found that endogenous Hecw and Fmrp from fly ovaries coimmunoprecipitate (Fig. 7c) and that Fmrp is a substrate of Hecw, as measured by in vitro ubiquitination assay (Fig. 7d). We confirmed this result in vivo by isolating endogenous ubiquitinated targets using tandem-repeated Ub-binding entities (TUBEs), a sensitive method that demonstrates the covalent

Fig. 4 RNP morphology and composition is altered in HecwKO. a IF analysis of egg chambers from 3-day-old flies of the indicated genotypes with anti-Orb antibody (n = 3). White arrows indicate ectopic Orb puncta in nurse cells of HecwKO flies. b IF analysis of the indicated genotypes (n = 3). Green, Me31B::GFP which marks the RNPs; red, Orb which marks the oocyte (in white in the first panel); blue, DAPI; white, phalloidin. Scale bar: 25 μm. c Quantification of RNPs size of egg chambers (stages from 6–9) from 3-day-old flies of the indicated genotypes. Results are expressed as mean ± SD. n = 477 (ctrl Me31B::GFP), n = 559 (HecwKO;Me31B::GFP), n = 444 (HecwCI;Me31B::GFP). 5 egg chambers/genotype from two biological replicates. ***P < 0.0001 by two-tailed Mann–Whitney test. d IF analysis of egg chambers of 3-day-old flies as indicated (n = 3), not treated (nt) or treated with 1,6 hexanediol (1,6 hex). Scale bar: 50 μm. e Quantification of RNPs size in egg chambers (stage 7–9) of the indicated genotypes and conditions. Treatment significantly reduces RNP size in control but not in HecwKO and HecwCI flies (KO;Me31B::GFP and CI;Me31B::GFP). Particles analysed: n = 505 ctrl (nt), n = 49 ctrl (1,6 hex), n = 642 HecwKO (nt), n = 536 HecwKO (1,6 hex), n = 314 HecwCI (nt), n = 553 HecwCI (1,6 hex) (3 egg chambers/genotype). Size mean ± SD is reported. ****P < 0.0001 by two-tailed Mann–Whitney test.
attachment of polyubiquitin chains to proteins modified at low stoichiometry. While Fmrp is clearly detectable in the pull-down from wild-type ovaries, the lane corresponding to the HecwKO ovaries displays a remarkably reduced amount of protein (Fig. 7e), indicative of reduced Fmrp ubiquitination in the absence of the E3 ligase.

To evaluate the impact of Hecw-dependent ubiquitination on Fmrp, we examined its protein levels in wild-type and Hecw mutant ovaries. As predicted by Hecw specificity towards K63-linked polyubiquitination (Fig. 7d), Fmrp stability is not affected by the lack of Hecw activity (Fig. 7f). In contrast, the downstream target Orb accumulates in Hecw mutant ovaries (Fig. 7f), in good agreement with ectopic presence of Orb puncta in nurse cells (Fig. 4a, b). Regulation by Hecw appears to occur at the protein level, since both Fmr1 and Orb mRNA expression does not change significantly when compared with wild-type controls (Supplementary Fig. 7c). Consistent with the antagonistic effect exerted by Hecw on Orb through Fmrp, overexpression of Hecw in the germline strongly downmodulates Orb protein levels (Fig. 7g) without affecting its mRNA (Supplementary Fig. 7d) and treatment of ovaries with the proteasome inhibitor MG132 does not rescue Orb expression (Supplementary Fig. 7e).

Me31B-positive RNPs in ovaries control spatial and temporal translation of key mRNAs during development. To test whether the aberrant RNP structure and composition of Hecw mutant tissue alters translation control, we analysed the localisation of the two major Orb target proteins: Gurken (Grk) and Oskar (Osk). While the mRNA levels are not altered (Supplementary Fig. 7c), both proteins show aberrant localisation in Hecw mutant oocytes. Grk is found ectopically in the ventral portion of the oocyte (Fig. 7h), while Osk localises away from the posterior margin (Fig. 7i). Remarkably, in stage 8/9, ectopic Osk colocalises with Me31B::GFP positive particles in Hecw mutant egg chambers, suggesting premature translation (Supplementary Fig. 7j), as is the case of Orb. To prove that these phenotypes are causally linked to aberrant Orb expression, we reduced Orb levels by crossing both KO and CI Hecw flies with Orb<sup>343/+ </sup>flies (Supplementary Fig. 8a). The results showed that we could largely rescue the defective egg chambers present in Hecw mutant flies, leaving altered RNPs unaffected (Supplementary Table 1 and Supplementary Fig. 8b, c). Overall, these results indicate that Hecw is a positive regulator of Fmrp repressor activity on Orb and its targets.

**Hecw modulates Fmrp activity in neurons.** Neuronal synapses, like oocytes, depend heavily on translating stored mRNA. Fmrp exerts a critical role in the presynaptic compartment where it regulates the transport and translation of specific mRNAs, which have an impact on synaptic strength and connectivity. One of the relevant Fmrp neuronal mRNA targets is Profilin, whose mutations cause familiar ALS. We thus wondered if, also in neurons, the Hecw mutant may modulate Fmrp activity and, as a consequence, the expression of its repressed target. Remarkably, while Fmrp stability is not affected by an altered expression of Hecw, Profilin shows a twofold increase in the HecwKO line and a similar decrease upon Hecw overexpression in neurons, without significantly affecting its mRNA (Fig. 8a). We then monitored RNPs status, revealing enlarged Me31B-positive RNPs in the neurons of young adult Drosophila HecwKO brains (Fig. 8b). In addition, treatment with 2.5% 1,6 hexanediol significantly reduced the size and number of wild-type granules, but had minimal effect on the larger granules present in the Hecw mutant brains (Fig. 8c, d). These findings confirm the critical role of Hecw in maintaining the liquid state of RNP granules and suggest Profilin as one of the pathologically relevant proteins downstream of Hecw activity in neurons.

To prove that Orb and Profilin dysregulation in Hecw mutants depends on the translational repression of Fmrp in the granules, we decided to downmodulate Fmrp by RNAi using nanos- and clav-Gal4 driver, respectively. Strikingly, immunoblot analysis showed that the levels of both Orb and Profilin in ovaries and neurons, respectively, are restored upon Hecw overexpression when Fmrp levels are strongly reduced (Fig. 8e). Thus, our genetic analysis reveals the existence of a Hecw-Fmrp-Orb/Profilin axis that impacts on RNP translational repression activity.

**Fig. 5 Lack of Hecw does not alter RNPs mobility.** a Representative intermediate scattering functions f(q, Δt) obtained from Differential Dynamic Microscopy (DDM) analysis of an image sequence of a KO;Me31B::GFP egg chamber plotted as a function of the time delay Δt for different values of the wave vector q in the range 0.4 – 0.8 μm<sup>-1</sup>. b From each of the curves shown in (a), a q-dependent estimate of the mean square displacement MSD<sub>q</sub>(Δt) = −Δq<sup>2</sup>/2 ln(f(q, Δt)) can be obtained (coloured symbols). Black circles correspond to the best estimate for the average mean square displacement MSD<sub>q</sub>(Δt) of the RNPs, obtained by extrapolating MSD<sub>q</sub>(Δt) to q = 0 at fixed Δt. The extrapolation procedure is illustrated in the inset for the representative case Δt = 10s. c Average mean square displacement MSD<sub>q</sub>(Δt) (symbols) of the RNPs in the considered egg chamber, displaying a characteristic linear dependence on the delay time Δt, which is indicative of a random, diffusive-like motion. An effective diffusion coefficient D<sub>e</sub> can be obtained by fitting a linear model MSD<sub>q</sub>(Δt) = 4D<sub>e</sub>Δt to the data (continuous black line). d DDM analysis of N = 18 egg chambers shows no significant difference in the RNP mobility between wild-type (Me31B, D<sub>e</sub>WT = 0.023 ± 0.006 μm<sup>2</sup>/s N = 9) and HecwKO egg chambers (KO;Me31B, D<sub>e</sub>KO = 0.021 ± 0.005 μm<sup>2</sup>/s, N = 9). See also Supplementary Fig. 5h, i.
Fig. 6 Lack of Hecw alters the RNP biophysical properties. a FLIP analysis was performed by repeatedly bleaching a randomly selected ROI (indicated by a yellow square) and by monitoring and quantifying the fluorescence level as a function of time in the same region, in single RNPs outside the bleached area (green circle), and in neighbouring portions of the cytoplasm (blue circle). One representative image for each genotype is shown. In (b), (c) and (d), the relative intensity of Me31B::GFP fluorescence in the three regions: bleached area, RNPs and cytoplasm, respectively, is plotted as a function of time for both control and HecwKO. Data are reported as mean ± SD. ****P < 0.0001 by two-tailed Mann-Whitney test (n = 18 samples/genotype). Both in the bleached area and in the cytoplasm, the loss curves show no statistically significant difference (P = 0.07 by t-student) between control and HecwKO, whereas a highly significant difference is observed for RNPs.
Ub controls the RNP liquid state and Fmrp repressor activity. To determine whether ubiquitination may directly affect Fmrp activity in RNP granules, we reconstituted a model biomolecular condensate by combining a purified recombinant fragment of Drosophila Fmrp, a lysate of reticulocytes and a luciferase mRNA as a reporter for protein synthesis. Previous studies have shown that the isolated disordered region containing the RGG motif of human FMRP is sufficient for both phase separation and translational repression in vitro. Based on sequence alignment, we generated the corresponding Drosophila Fmrp construct as well as a chimeric version with Ub fused in frame at the C-terminus as a proxy for ubiquitinated Fmrp (Fig. 9a). We then compared the two constructs using differential interference contrast (DIC) and luminescence assay to examine formation of phase-separated protein-rich condensates (Fig. 9b) and to measure translation inhibition (Fig. 9c). Confirming previous reports, a switch-like translation inhibition response that parallels phase separation is observed increasing the concentration of the RGG construct (Fig. 9b, c). Intriguingly, the RGG-Ub phase separates at a approximate twofold lower concentration shifting the inhibitory curve to the right (Fig. 9b, c). Thus, ubiquitination appears to increase Fmrp phase-separation propensity together with its
inhibitory activity in vitro, and, by extension, it may be a critical determinant that facilitates RNPs assembly in ovariess and neurons.

**Discussion**

We report here the identification and molecular characterisation of Hecw, the fourth member of the NEDD4 family in Drosophila. Flies lacking Hecw activity show neurodegenerative traits, shorter lifespan and reduced fertility. We provide compelling evidence that Hecw-mediated ubiquitination is needed for the diffusive exchange in and out of RNP compartments during oogenesis and in neurons. Mechanistically, Fmrp ubiquitination by Hecw is required for the translational repression of Orb and Profilin (Fig. 9d), two known Fmrp targets. Indeed, evaluation of RNP granules liquid state:

Ubiquitins RNP controls liquid state. In the Drosophila ovariess, sponge bodies are marked by the de-capping activator/RNA helicase Mc31B [ref. 28]. These are first synthesised in the nurse cells and then transported and localised posteriorly in developing oocytes, where their mRNAs are translated, ultimately dictating the establishment of germ cells and embryonic axes. While their composition has been recently investigated, the mechanism behind the dynamic nature and biophysical properties of sponge bodies are largely unknown. Recent literature underlined a liquid-like behaviour for RNP granules in Caenorhabditis elegans, as well as for germ granules in fly embryos. We now show that also Drosophila sponge bodies behave like liquid droplets.

Our in vivo data provide evidence for the pivotal role of Hecw and Ub in maintaining the liquid-like nature of RNPs, and show that, in the absence of Hecw, they transition into a less dynamic gel-like state. The lower exchange rate between RNPs and the cytoplasm is specific of Hecw mutant granules and possibly reflects an altered internal rearrangement and activity of the proteins present inside these membrane-less particles. Indeed, the Orb protein that is undetectable in the nurse cells granules is clearly present in the larger Hecw-depleted RNPs, suggesting a lack of local translational repression. Genetic validation demonstrates that in an Orb heterozygous background, we could largely rescue the defective phenotype of egg chambers observed in HecwKO and HecwCI homozygous flies.

Our in vitro reconstitution studies further sustain the idea that Hecw-mediated ubiquitination favours phase-separated transitions, and promotes translation repression of granules generated by recombinant Fmrp. Indeed, the protein concentration required for LLPS is shifted to lower values when Ub is coupled to the RGG domain of Fmrp. These data suggest that Ub acts as a stabiliser of phase separation, in good accordance with its peculiar biophysical and thermodynamic properties.

An important question that remains to be addressed is the impact of linkage-specific polyubiquitin chains on RNP granules. Longer chains are predicted to increase multivalency further promoting LLPS, in particular in the case of K63-linked chains whose moieties are arranged in a beads-on-string scaffold. Other chains may have alternative effects depending on their different structural conformations and on the nature of the RNPs components, as already demonstrated for UBQLN2, hHR23B, and p62, whose condensates could either assemble (hHR23B, and p62) or disassemble (UBQLN2) via noncovalent interactions with polyubiquitin chains.

Although our study is limited to the analysis of Mc31B in oocytes and neurons, it is conceivable that other RNPs may behave similarly and that Hecw-mediated ubiquitination may modulate liquidification of other phase-separating complexes, thus promoting specific regulated events.

**Hecw acts in the Fmrp-Orb and Fmrp-Profilin axis.** We identify Fmrp as interactor, substrate and effector of Hecw activity in RNPs. Importantly, a Hecw mutant phenocopies Fmrp loss-of-function flies, both in terms of phenotypic defects and molecular targets. Indeed, Orb, a known target of Fmrp repressor activity, shows an inverse correlation with Hecw expression, an effect that cannot be ascribed to a direct activity of Hecw on Orb. Same evidence was obtained in neurons where Profilin appears to be a relevant Fmrp target. Thus, similar to Fmrp, changes in Hecw expression levels are deleterious and induce pathological effects. Based on this evidence, we proposed and genetically validated a linear cascade of events in which Hecw ubiquitimates Fmrp to maintain Orb and Profilin mRNAs in a repressed state. Such K63-linked, non-proteasomal function of Hecw in Fmrp ubiquitination is in good accordance with the previously reported activity of this enzyme family, and is clearly different from the proteasome-mediated degradation of Fmrp identified in neurons.

Fmrp represses translation both during ovary and neuronal development, however, the underlying mechanism remains unclear. We envisage that Ub, by adding an additional surface of interaction to Fmrp, could enable precise spatiotemporal recruitment of repressive components and ribosomes. Indeed, while Drosophila Fmrp directly binds the 80S ribosome near the tRNA and translation factor binding site, post-translational modifications (PTMs) have been suggested to modulate the affinity of Fmrp for the ribosome or target mRNAs, thereby “turning off” protein synthesis locally. A Ub-dependent Fmrp association to ribosomes might occur in nurse cells and be
**Fig. 8 Lack of Hecw alters RNP in neurons.**

**a** Upper panel: IB of wild-type (ctrl), HecwKO (KO) and RFP-Hecw-overexpressing (elav>RFPHecw) fly heads with the indicated antibodies. Profilin levels increase in HecwKO and decrease upon Hecw overexpression. Values reported below each blot are normalised for tubulin and represent the fold change relative to the control, presented as mean of three experiments. Bottom panel: mRNA level of profilin was measured by qPCR in the adult heads of the indicated genotypes. The reported expression levels relative to control are expressed as mean ± SD calculated over three experiments. Ns, not significant by unpaired two-tailed t-test. **b** IF analysis of adult brains from 1-day-old flies with the indicated antibodies (n = 2). Neurons are marked with the anti-elav antibody (purple); red, Hecw; blue, DAPI. Scale bar, 5 μm. **c** IF analysis of adult brains from 1-day-old flies of the indicated genotypes, not treated (nt) or treated with 1,6 hexanediol (1,6 hex). (n = 3). Scale bar, 5 μm. **d** Quantification of RNP size in fly neurons of the indicated genotypes and conditions. RNP size is significantly increased in HecwKO flies (KO;Me31B::GFP **P < 0.01** and HecwCI (CI;Me31B::GFP **P < 0.01**). 1,6 hexanediol treatment significantly reduces RNP size in control flies. Size mean ± SD is reported. ****P < 0.0001 by two-tailed Mann–Whitney test (ten heads/genotype from two biological replicates). **e** Profilin and Orb levels are restored upon concomitant Hecw overexpression and Fmr1 silencing. Left panel: Profilin expression in fly brains of the indicated genotypes. Values reported below each blot are normalised for tubulin and represent the fold change relative to the control, presented as mean of three experiments. Right panel: Orb expression in fly ovaries of the indicated genotypes. Values reported below each blot are normalised for tubulin and represent the fold change relative to the control, presented as mean of two experiments.
released by deubiquitination in the oocyte when translation is due to progress. Similar cycles of ubiquitination/deubiquitination have been identified and characterised in other cellular processes. Thus, the existence of Ub receptors, effector components capable of recognising Ub, is predicted. Supporting this notion, one of the Hecw interactors is the P-body component Lingerer (Supplementary Table 2), which is known to associate with Fmrp and other RNA-binding proteins to modulate their repressive functions and is endowed with a Ub associated (UBA) domain (Fig. 9d).

While our data are fully compatible with this model, it is also conceivable that Ub may impinge on the physical properties of Fmrp inside RNP granules, as we demonstrated in vitro (Fig. 9a–c). Recent studies showed that phosphorylation, methylation, and sumoylation could control Fmrp phase-separation propensity, and regulation of its intrinsically disordered regions. In the less mobile Hecw mutant RNPs, Fmrp may be disordered or otherwise uncappable of interacting with repressive complexes and ribosomes. Further studies are needed to test the behaviour of ubiquitinated vs non-ubiquitinated Fmrp and define functional links with different PTMs.

**Hecw and disease.** Hecw mutant flies show premature aging, neuronal loss, and neuromotor defects, all reminiscent of neurodegenerative processes in humans. Remarkably, evidence links the human ortholog HECW1 to neurodegeneration. Transgenic mice overexpressing HECW1 display neuronal loss in the spinal cord, muscular atrophy and microglia activation, and the HECW1 protein has been shown to ubiquitinate mutant superoxide dismutase 1 (SOD1), typical of familial ALS patients. Similar to Hecw, the expression of HECW1 seems to decline with aging, suggesting the existence of a positive and protective role for this E3 ligase in the maintenance of neuronal homeostasis. Our molecular dissection in *Drosophila* supports a mechanism by which Hecw regulates the spatiotemporal translation of Fmrp...
targets. Among them, of particular interest is Profilin, a known ALS causative gene. Interestingly, profilin mutant mouse models replicates the key features of ALS including adult-onset, progressive motor neuron loss accompanied with progressive motor weakness ending in paralysis and death. While the exact role of Profilin mutants at the onset of the disease remains to be established, the identification of a specific Ub ligase acting upstream Profilin expression reveals a new avenue to therapeutically investigate this devastating neurological disease.

Beside ALS, it is worth mentioning that FMR1 alterations are at the basis of the Fragile X syndrome, characterised by intellectual disability, autism as well as premature ovarian failure. Based on the basis of the Fragile X syndrome, characterised by intellectual disability, autism as well as premature ovarian failure. Based on findings, we hypothesise that the severity of these disorders may depend on Hecw status both in neurons and in ovaries. Future studies on Hecw/HECW1 in flies and mammalian models are predicted to illuminate new pathogenetic aspects of these diseases.

Methods

**Drosophila strains and cell lines.** Flies were maintained on standard flyfood containing cornmeal, molasses and yeast. All experiments were performed at 25°C, unless differently specified. The following fly strains were used in this study: y w; Bloomington Drosophila Stock Center [BDSC] #11685), nano-natal-GAL4-VP16 (kindly provided by A. Ephrussi), nano-GAL4-VP16 (BDSC #32180), elav-GAL4;UAS-Dicer2 (BDSC #25750), traffic jam-GAL4 (Kyoto stock center, [DGRC] #6929), Orb53 (BDSC #58477), UAS-Hecw::CG42797 RNAi (Vienna Drosophila Stock Centre [VDRSC] #8933). The rescue line HecwKO;UAS-Dicer5 was generated by crossing Hecw mutant flies with the Dp(l3)DC504 (BDSC #32313) flies, which contain a the CG42797 locus region inserted on the third chromosome. Transgenic flies were generated by injecting the UASTattB-RFP-CG42797 construct into embryos carrying the attp-P; zkh65Fb #c31 docking site (BestGene,inc.). Transgenic offspring was screened by eye-colour (white marker) and sequenced. Strain details are reported in Supplementary Table 3. Drosophila S2 cells obtained from Invitrogen, were cultured in Schneider’s medium (Gibco) supplemented with 1% Glutamine (Euroclone) and 10% of Fetal Bovine Serum (FBS) (Euroclone) and maintained at standard culture conditions (28°C).

Ethics approval was not required for experiments on invertebrates.

**Hecw** and HecwKO and generation by CRISPR/Cas9 editing. Guide sgRNAs for CRISPR/Cas9 mutagenesis were designed using the MIT CRISPR design tool (http://crispr.mit.edu/). Target sequences were cloned in the pBFvU6.2 vector (NIG-Healthcare). Constructs

To generate a pGEX6P1-Hecw (1-130aa) construct for use in antibody production, the N-terminal region of the protein was amplified from pUASTattB-RBP-CG42797 with the primers CG42797 EcoRI_F and CG42797 XhoI_Nterm_R and cloned by enzymatic digestion into a pGEX6P1(GE Healthcare).

To generate pET43-His-MBP-Fmrp construct, full-length Fmrp was amplified from cDNA of y w/ovaries using the primers Fmrp BamHI_F and Fmrp EcoR1_R and cloned by enzymatic digestion into a pET43-His-MBP vector (kind gift of Sebastiano Pasqualato, European Institute of Oncology, Milan).

To generate pGEX6P1-Fmrp97–984 construct, the region corresponding to the RGG domain of Fmrp was amplified using full-length Fmrp template with primers 387_slices_F and Fmrp EcoR1_R and cloned by enzymatic digestion into pGEX6P1(GE Healthcare).

To generate pGEX6P1-Fmrp97–984, Ub (RGG-Ub) fusion protein, Fmrp fragment was amplified from full-length construct using the oligos 387_slices_F and dFmrp NcoI_R and Ub sequence was amplified from pGEXET3-Ub WT using the oligos NcoI_Ub_F and Ubsmall_R.

The two obtained fragments were cloned by enzymatic digestion into pGEX6P1 vector (GE Healthcare).

Full sequence of the primers used in this study are reported in Supplementary Table 4. All constructs were sequence-verified. The remaining constructs were previously described.

**RNA extraction and qPCR.** RNA was extracted from Drosophila tissues with Maxwell RSC simplyRNA Tissue kit (Promega) and quantitative PCR (qPCR) analysis was performed with the following Taqman Gene Expression Assay (Thermo Fisher Scientific): Dm01837441_g1 (CG42797/Hecw), Dm019181193_g1 (jumped), Dm02136373_m1 (Fmr1), Dm002124583_g1 (oskar), Dm01250375_g1 (Su(Hw)).

**Lifespan assay.** 1 to 3-day-old flies were kept at 25°C or 29°C at a density of 25 flies/vial (4 vials/group), with mixed-sex groups in standard cornmeal food to minimise the influence of genetic background, environment, nutrition and mating conditions. Flies were flipped and scored every two/three days for survivorship assay. The assay was repeated twice, and data were analysed with PRISM (GraphPad software). Survival fractions were calculated with product limit Kaplan–Meier method and log-rank test was used to evaluate the significance of differences between survivorship curves.

**Climbing assay.** Fifteen flies/group were placed in a 9 cm plastic cylinder. After a 30 s rest period, flies were tapped to the bottom of the cylinder. Negative geotaxis was quantified by counting the number of flies that can cross a 7 cm threshold during a 15 s test period. The climbing index was calculated as the total number of succeeding flies over the total. The test was performed on 6-9 independent groups of animals for each genotype, as described in the legends. The climbing ability was measured the first day of life and monitored every 5 days. Significant differences between Hecw mutants and control started to emerge after the 14th day of the lifespan.

**Fertility assay.** Fertility was assessed by counting the eggs laid in 24 h by 20-day-old flies. 6 females and 3 males per genotype were kept in a cage on a 3 cm molasses plate with fresh yeast for a 24 h egg collection after 24 h of adjustment to the cage. The test was performed on three groups per genotype. The hatching rate was calculated as the number of eggs hatched into larvae in 3 days over the total number of eggs.

**Immunostaining and treatments.** Fly ovariode section and staining was performed as previously described. Briefly, ovaries were fixed in 4% paraformaldehyde (PFA) in PBS (HIMEDIA) for 20 min at room temperature, permeabilised in 1% Triton X-100 in PBS, followed by 1 h block in PBST (PBS-Triton X 0.1%) containing 5% (w/v) bovine serum albumin (BSA). Primary antibodies were incubated overnight at 4°C, secondary antibodies were incubated for 2 h and DAPI (Sigma) was incubated for 15 min at room temperature. Adult brains (Figs. 1e and 8b-c) were dissected in Drosophila S2 medium with 10% of FBS (Euroclone) and processed as described before. Ovary microtubule detection (Supplementary Fig. 3e) was adapted from. Briefly, ovaries were incubated in BRB80-T buffer (80 mmol PIPES pH 6.8, 1 mmol MgCl2, 1 mmol EGTA, 1% Triton X100) for 1 h at 25°C without agitation. Then, ovaries were fixed in MeOH at −20°C for 15 min and rehydrated for 15 h at 4°C in PBST and blocked for 1 h in PBST containing 2% (w/v) BSA before incubation with primary and secondary antibodies overnight in PBST-2% BSA. In case of drug treatments, fly ovaries were dissected in Drosophila S2 medium with 10% of FBS (Euroclone) and incubated with 2.5% 1,6-hexanediamine on an orbital shaker at room temperature for 5 min (Fig. 4d and Supplementary Fig. 8c) prior to fixation.

All tissues were mounted in 20% glycerol, 50 mM Tris, pH 8.4 to avoid mechanical sample deformation. Images were acquired using 40x and 60x objectives on a Leica TCS Sp2 upright confocal microscope. The outlines of the oocytes and the anteroposterior (AP) axis were manually specified.
For 52 immunostaining (Supplementary Fig. 1e), cells were plated on coverslips coated with poly-ornithine. S2 cells were rinsed twice with PBS and fixed in 4% PFA for 5 min, permeabilised with 0.1% Triton X-100 in PBS at 4 °C for 30 min. After 30 min, the coverslips were washed once in PBS, incubated in 1%BSA for 2 h at room temperature and then washed again in PBS. Coverslips were mounted on slides using Mowiol Mounting Medium (70% glycerol in PBS, 1.5% DABCO (1,4-Diazabicyclo[2.2.2]octane from Sigma)).

TUNEL staining was performed as previously described. Briefly, fixed brains from 30-day-old flies were permeabilized in 0.5% Tween 20 containing 0.5% Triton X-100 PBS at 4 °C for 45 min. The slides were then incubated with TUNEL reagent (In Situ Cell Death Detection Kit, TMR red, Sigma) for 14–16 h at 37 °C in dark humid chamber, washed in PBS and incubated with Hoechst 33342 (Life Technologies, 2 μg/ml in PBS) 10 min at 25 °C. Finally, brains were rinsed in water and mounted on glass slides with ProLong Gold fluorescence anti-fading reagent (Invitrogen). Images were acquired with a Nikon Eclipse C1si confocal microscope. To quantify TUNEL+, cells, 200–500 cells from individuals from n = 5 individuals/genotype were scored using the FIJI Software (https://imagej.net/Fiji version 2.1.0/I.53c).

Immunohistochemical analysis. Adult heads of 1-day-, 30-day- and 60-day-old flies were dissected in PBS and fixed in 4% PFA (HIMEDIA) overnight, at 4 °C. Samples were embedded in 12% low-melting agarose: while the agarose solidified, heads were properly oriented. Heads-containing agarose blocks were dehydrated in serial dilutions of ethanol (from 70 to 100%) prior to paraffin embedding (70% ethanol for 30 min to avoid phenotypic changes due to stress).

Fluorescent loss in photobleaching (FLIP). FLIP experiments were performed on the UltraView VOS spinning-disk confocal system (Perkin Elmer) equipped with an EclipseTi inverted microscope (Nikon), provided with an integrated FLIP PhotoKinesis unit (Perkin Elmer) and a Hamamatsu CCD camera (C9100-50) and driven by Velocity software (Improvision; Perkin Elmer). Photo bleaching was performed on a square region of the egg chamber of a fixed nurse cell at the maximum laser power to bleach the GFP signal every 5 s, for a total of 100 bleaching events. Initially, five images were acquired to determine the levels of pre-bleach fluorescence. Images were acquired through a 60x oil-immersion objective (Nikon Plano Apo VC, NA 1.4) every 2.5 s for 8 min.

In order to analyse the fluctuations over time in FLIP experiments, two homemade Fiji plugins were developed; one to analyse fluorescence either in selected and manual tracked RNPs or in the cytoplasm and the other one to evaluate the fluorescence in the bleached area and in its neighbourhood regions. During each acquisition, a background intensity Ibg(t) and a reference intensity Iref(t) were measured. The amplitude of the background noise was obtained as the average intensity within a small ROI in a corner of the image outside the egg chamber. Ibg(t) monitors potential systematic changes in the fluorescence emission not directly related with the FLIP experiment and was calculated as the average intensity within a small ROI inside the sample, far from the bleached region and in a different nurse cell. In all of the experiments, we found that both Ibg(t) and Iref(t) were fairly constant over time, with no increasing or decreasing net trends. Iref(t) showed very small fluctuations (with RMS amplitude below 5% of the mean value), while in some cases, the profile of Ibg(t) was perturbed by the presence of granules moving in and out the ROI. Thus, we adopted a constant value Ibg for the background intensity (obtained as a time average of Ibg(t)) and we did not apply further corrections for systematic intensity drifts.

In each experiment, we selected N (6 < N < 8) ROIs of average area 2.5 μm² outside of the bleached area (half of them in the cytoplasm, half of them in correspondence to RNPs). The area of a ROI associated with an RNP was kept constant over time, while its position was adjusted in order to follow the moving granule. For the n-th ROI, the average intensity within the ROI before bleaching.

The fluorescence recovery after photobleaching (FRAP). For FRAP recordings, 3-day old Me31B::GFP and HecwKO/Me31B::GFP females were prepared as described above. Time series were recorded using a Confocal Spinning Disk microscope (Olympus) equipped with IX83 inverted microscope provided with an IXON 897 Ultra camera (Andor) and a I3X FRAP module equipped with a 405 nm laser using 60x objective. The system is driven by the Olympus CellSens Dimension 1.18 software (Build 16666). The recording frame rate of the GFP signal, excited by 488 nm laser, was 66 ms. Photobleaching was performed via the 405 nm laser for two cycles of 500 ms on a square region of the cytoplasm (3.04 x 3.04 μm) to bleach GFP signal.

Before bleaching, 15 frames were acquired, obtaining a pre-bleaching reference image IB(t). After bleaching, time-lapse observation was continued for 1500 frames. We consider the azimuthally-averaged intensity profile of the bleached region (r, t) = (r, t) = μ(r, t) = |μ(r, t)|, where r is the image intensity at time t after bleaching, r0 is the center of the bleached region and the symbol ( ) represents an average over pixels in the image at a distance r from the center r0 of the bleached region. The process of fluorescence recovery was monitored by following the progressive widening of the bleached spot over time. For each fixed t, μ(r, t) was fitted by a Gaussian profile A(σ²/t + 7)BT, where the parameter σ(r) provides an estimate of the width of the bleached spot. In all cases, we found that the increase of over time of σ(t) was well captured by the simple equation σ(t) = σf + 4Dr, describing the diffusive-like broadening of a Gaussian concentration profile. From a linear fit of σ(t) over a time interval of 30 seconds, we evaluated the effective diffusion coefficient D was obtained, as shown in the inset of Supplementary Fig. 5d. Compared to standard data treatment, where the FRAP process is monitored by measuring the average intensity within the bleached region, the described procedure, which is based on the time-resolved estimation of a parameter obtained from the fit of a spatial feature, is more robust with respect to the local intensity fluctuations introduced in the images due to the randomly-moving, highly-contrasted RNPs, whose appearance in the vicinity of the bleached ROI can significantly alter the shape of the recovery curve. The distributions of the obtained values of D are shown in Supplementary Fig. 5e, showing no statistically significant difference between the wild-type and HecwKO nurse cells (D = 0.24 ± 0.03 μm²/s).
scenario of biological samples. Rather than reconstructing trajectories followed by the individual particles during their motion in direct space, DDM extracts quantitative mobility information from the analysis of the temporal correlations of the spatial Fourier transforms of the direct space images. We used DDM to estimate the average mean square displacement $MSD(\Delta t)$ of the RNP. For each image sequence, DDM analysis was performed over a square ROI of average size 45 µm within the egg chamber. For each time delay $\Delta t$, multiple of the delay $\Delta t$, we performed the inverse Fourier transform of the average intensity function $D(q, \Delta t) = \langle 1/(q^2 + \Delta t^2) - 1/(q^2) \rangle$, where $b(q, \Delta t)$ is the 2D Fourier transform of the image at time $t$, $q = |\mathbf{q}|$, and the symbol $\langle ... \rangle$ indicates a combined temporal and azimuthal (i.e., performed over the orientation of $q$) average. The obtained image structure function can be written as

$$D(q, \Delta t) = A(q) \left[ 1 - f(q, \Delta t) \right] + B,$$

where $A(q)$ is an amplitude term determined by the optical properties of the sample and by the microscope collection optics, $B$ is a term accounting for the delta-correlated noise in the detection chain, and $f(q, \Delta t)$ is the intermediate scattering function (ISF) [ref. 80], whose decay with $\Delta t$ mirrors the motion of the moving entities in the image.

As a first step of our DDM analysis, we estimated the noise term $B$ from an exponential fit of the tail of $D(q, \Delta t)$ for $q \gg 0.12$ µm$^{-1}$, a procedure that exploits the fact that $A(q)$ vanishes for large enough $q$ due to the finite numerical aperture of the microscope objective. Once $B$ was known, we proceeded with the determination of the amplitude $A(q)$ as the large $\Delta t$ limit of $D(q, \Delta t)$, a procedure that exploits the fact that $f(q, \Delta t)$ decays almost completely to zero for large $\Delta t$. In our experiments, for which this typically happens for $q > 0.4$ µm$^{-1}$, we estimated $A(q)$ from a stretched-exponential fit of $D(q, \Delta t)$ for $\Delta t > 100$ s (Supplementary Fig. 5h).

$M(\Delta t)$ and $B$ were known, Eq. (1) was inverted to obtain the ISF $f(q, \Delta t)$, as shown in Fig. 5a. For identical particles with Gaussianly distributed displacements, the particle mean squared displacement can be obtained directly from $f(q, \Delta t)$ via a simple algebraical inversion. In less ideal cases, the following relation still holds:

$$MSD(\Delta t) = \lim_{\mu \rightarrow 0} \int_s^\infty q^2 f(q, \Delta t) dq,$$

where $MSD(\Delta t)$ is a mean square displacement averaged over the entire population of particles present in the image. In this case, the limit for $q \rightarrow 0$ was calculated via a linear extrapolation over the wave vector range 0.4–0.8 µm$^{-1}$, as shown in the inset of Fig. 5b.

The results of this procedure, enabling the automatic determination of the average mean square displacement are shown in Supplementary Fig. 5f. For all the egg chambers considered in this study. In all cases, the $MSD(\Delta t)$ display a linear dependence on $\Delta t$, indicating a diffusive-like behaviour with negligible persistence, at least for the investigated time scales. The diffusion coefficient $D_0$ was estimated from the linear fit $MSD(\Delta t) = 4D_0\Delta t$. After averaging the obtained values overall examined cells of the same type, we found $D_0 = 0.023 \pm 0.006$ µm$^2$/s and $D_{\Delta t=0} = 0.021 \pm 0.005$ µm$^2$/s, for wild-type and $hgt^{600}$, respectively. These two values are fully compatible within the experimental uncertainty, indicating no significant difference in the RNP dynamics between wild-type and $hgt^{600}$ egg chambers.

**Statistics and reproducibility.** Statistical analyses were performed with Prism (GraphPad software version 9.1.2). Unless differently specified, all the statistical significance calculations were determined by using either unpaired Student’s t-test or the non-parametric Mann–Whitney test, after assessing the normal distribution of the sample with Normal (Gaussian) distribution test. Sample sizes were chosen not blind to the group allocation during the experiments and data analyses.

**Antibodies.** Anti-Hecw was produced in rabbit by immunisation with the N-terminal fragment of Hecw (aa 1-130) protein fused to GST protein (Eurogentec S.A.). The resulting polyclonal antibody was affinity purified and validated (Supplementary Fig. 1e) on S2 cells depleted of Hecw according to the protocol described in [ref. 81]. Briefly, for double-stranded RNA production, the following T7- and T3-tagged primers were used to amplify the Hecw region of interest: HecwT7: 5′-ttaacgtactaatagggagGAATAATTTGACATTTTG-3′, HecwT3: 5′-catagctactaatagggagGGATAATTTGACATTTTG-3′. PCR products were transcribed in vitro with T7 and T3 polymerases (Promega) according to the manufacturer’s instructions. To generate double-stranded RNAs (dsRNAs), T3 and T7 transcripts were annealed at 68°C for 15 min and at 37°C for 30 min. For dsRNA treatments, we starved S2 cells for 30 min in serum-free medium. We then added medium with 20% serum and dsRNA at the concentration of 15 µg/106 cells and incubated it for 96 h on coverslips for IF analysis.
puromycin (Sigma) or buffer (25 mM NaPO4 pH 7.4, 50 mM NaCl, 2 mM DTT). Reactions were assembled at different concentration of RGG or RGG-UB (20-10-5-2.5-1.2-0.6-0.3 mM) or RBA (10 µM). All in vitro translation reactions were prepared on ice, followed by incubation at room temperature for 3 h before luminiscence measurement. Luciferase translation was measured with luciferase assay system (Promega) by mixing 75 µl of luciferase substrate with 3 µl of unpurified translation mixture in a black 96-well plate (Corning). End-point luminiscence measurements were carried out in triplicate using Victor3 multilabel plate reader (Perkin Elmer).

**DIC imaging of phase separation samples.** 10 µl of translation mixtures of the in vitro reactions described above were transferred on imaging chambers prepared as described in ref. [83] and visualised for the appearance of liquid droplets with DIC microscopy. Images were acquired with DeltaVision Elite microscope (GE Healthcare) equipped with a Cool SNAP HQ2 camera using UPlanSapo 100x: 1.4NA oil-immersion objective and driven by Softwox 6.5.2 version. Phase separation in different conditions was defined by the observation of round droplets. All images represent a single focal plane focused on the surface of the glass slide. Images were processed with FIJI Software (https://imagej.net/Fiji version 2.1.0/ 1.53c).

**Western blots and immunoprecipitations.** *Drosophila* tissues, collected as described in ref. [72], were homogenised with a pestle and incubated for 20 min on ice in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, and 0.1% SDS) supplemented with a protease inhibitor cocktail (CALBIOCHEM), clarified by centrifugation and analysed by immunoblotting.

For co-immunoprecipitation experiments (Fig. 7a and Supplementary Fig. 7a), *Drosophila* ovaries were lysed in JS buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 µM MgCl2, 5 mM EGTA) supplemented with protease inhibitors (Calbiochem). After extensive washes with JS buffer, beads were re-suspended in Laemmli-buffer and proteins analysed by SDS-PAGE and immunoblotting.

In case of drug treatments, fly ovaries were dissected in *Drosophila* S2 medium with 10% of PBS (Euroclone) and incubated with 50 µM MG132 on an orbital shaker for 2 h (Supplementary Fig. 7e) prior lysis.

For immunoprecipitation of ubiquitinated proteins, 40 µl of Agarose-TUBE2 (UM402, LifeSensors) previously equilibrated in JS buffer, were incubated overnight with 1 mg of ovary lysate on an orbital shaker at 4 °C. After overnight incubation, the lysate was spin down and the supernatant was re-incubated with additional 30 µl of TUBE2 for 1.5 h at 4 °C. Beads were then washed, re-suspended in Laemmli-buffer, loaded on SDS-PAGE and analysed by immunoblotting.

**LC-MS/MS analysis.** For identification of Hecw interactors, a GST fusion construct encompassing the two WW domains of Hecw (636-834aa) was used. Briefly, 2 µg of GST proteins were incubated with 1 mg of S2 lysate for 2 h at 4 °C in YY buffer (50 mM Na-HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-100). After four washes of the GST proteins with YY buffer (50 mM Na-HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 µM MgCl2, 3 mM EGTA) supplemented with protease inhibitors (Calbiochem). After extensive washes with JS buffer, beads were re-suspended in Laemmli-buffer and proteins analysed by SDS-PAGE and immunoblotting.

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A.O., Em.M., and D.P. aided in all the imaging acquisition and FLIP experiments; F.G.
and R.C. analysed and quantitatively elaborated the live images; F.N. performed tunnel
experiment; G.C. prepared genetic crosses for revision; ELM. and T.V. contributed to
planning and interpretation of data; S.P. coordinated the team, designed and supervised
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**Competing interests**
The authors declare no competing interests.

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