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Cutoff and *Aubergine* mutations result in upregulation of retrotransposons and activation of a checkpoint in the *Drosophila* germline

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Abstract

Gametogenesis is a highly regulated process in all organisms. In the Drosophila ovary, signaling events between germline and somatic follicle cells establish the axes of the egg and the future embryo [1,2]. A meiotic checkpoint which monitors double stranded DNA breaks (DSB) and involves the Drosophila ATR and Chk2 homologs, coordinates the meiotic cell cycle with these signaling events. Activity of the checkpoint affects the translation of the transforming growth factor-alpha like Gurken signaling molecule which normally induces dorsal cell fates in the follicle cells ([3], [4], [5]). We found that mutations in the Drosophila gene *cutoff (cuff)* affect germline cyst development and result in ventralized eggs due to reduced Grk protein expression. Surprisingly, we found that *cuff* mutations lead to a marked increase in the transcript levels of two retro-transposable elements, *Het-A* and *Tart.* Tagged Cuff protein shows a peri-nuclear localization in the nurse cells, similar to components of the RNAi machinery. We found that a small interfering RNA against the *roo* element is still produced in *cuff* mutant ovaries. These results indicate that Cuff is involved in the rasiRNA pathway, most likely acting downstream of siRNA biogenesis. The eggshell and egg laying defects of *cuff* mutants are suppressed by a mutation in *chk2*. We also found that mutations in *aubergine (aub)*, another Drosophila gene implicated in the rasiRNA pathway, are also significantly suppressed by the *chk2* mutation. Our results indicate that mutants in rasiRNA pathways lead to elevated transposition incidents in the germline, which activates a checkpoint that causes a loss of germ cells and a reduction of Gurken protein in the remaining egg chambers.

Results and Discussion

Cutoff is a female sterile mutation affecting eggshell polarity, karyosome formation and female fecundity

Cutoff (cuff) mutations were isolated in a large scale female sterile screen of Drosophila [6, 7], and one additional allele was identified in a P-element insertion screen [8]. Females trans-heterozygous for *cuff* alleles lay eggs with various degrees of ventralization (Table 1 and data not shown). Dorso-ventral polarity of egg and embryo depends on the levels of the Gurken (Grk) ligand, which is produced and secreted by the germline and activates the EGF receptor (Egfr) in the overlying follicle cells ([9], [10]). To determine whether Grk-Egfr signaling was affected, we analyzed the *grk* expression pattern in a strong *cuff* mutant background. In wild

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type egg chambers, at stage 9 of oogenesis, *grk* RNA becomes restricted to the future dorsal anterior side of the oocyte, forming a cap around the oocyte nucleus (Fig. 1A). Grk protein is translated from the tightly localized RNA, and is also spatially restricted to the membrane overlying the oocyte nucleus (Fig. 1B) [11,12]. *Cuff* mutants do not significantly disrupt *grk* RNA localization (Fig. 1C). However, in many mid-stage egg chambers, the Grk protein level is greatly reduced, where between 10% to 40% of the egg chambers contain no detectable Gurken protein at all (Fig. 1D), consistent with defects in *grk* translation. In wild type egg chambers, by stage 3 of oogenesis, the oocyte nucleus forms a compact structure termed the karyosome (Fig. 1B inset). In *cuff* mutants, in 10–20% of the egg chambers, karyosome formation is affected, and instead, the DNA assumes various shapes and is often found in separate clumps (Fig. 1D inset).

Another prominent defect in *cuff* mutant females is a severely reduced fecundity (as reflected in the name of the gene). While *cuff* heterozygous females lay an average of around 20 eggs per day, newly eclosed *cuff* females of a strong allelic combination lay around 5 eggs per day. This phenotype becomes more severe as the females age. To address the cause of the reduced egg production, we analyzed the germaria, the anterior-most part of the ovarioles of the mutant females. In each ovariole, germ line stem cells divide asymmetrically, giving rise to another stem cell and a cystoblast. The cystoblast undergoes four rounds of mitosis with incomplete cytokinesis, forming an inter-connected 16 cell cyst which will differentiate into one oocyte and 15 associated nurse cells (for review of oogenesis see [13]). In the germarium, the 16 cells are connected by the fusome, a membraneous structure that connects the 16 cells of the cyst, which has been shown to be important for germ line cyst development [14,15]. To assay the division of the germ line stem cells and the cystoblasts, we analyzed fusome branching in *cuff* mutants. In wild type germaria (either *cn bw* females, or *cuff* heterozygous females), using an antibody against alpha Spectrin, we always observed highly branched fusomes in region 1 and region 2 of the germaria (Fig. 2A, N=146). In newly eclosed *cuff* mutant females, we observed similar patterns. However, as the females aged, we noticed a sharp increase in the percentage of mutant germaria without cysts that contain highly branched fusomes (Fig. 2B). In *cuff* mutant females one week after eclosure, 54% of the germaria did not have highly branched fusomes (N=35); the number increases to 83% in mutant females two weeks after eclosure (N=43). Instead, in older *cuff* mutant females, the mutant cysts appear to arrest in early stages in the germaria containing spectrosome-like structures. We also stained *cuff* heterozygous and trans-heterozygous females with an antibody against C(3)G. C(3)G is a component of the synaptonemal complex [16] which starts assembly in region 2A of the germarium. In *cuff* mutant females one week after eclosure, 44% of the germaria did not contain any C(3)G positive cysts (Fig. 2D, N=50); the number increases to 72% in females two weeks after eclosure (N=50). We stained *cuff* heterozygous and trans-heterozygous females with an antibody against Fasciclin (Fas) III, which marks immature follicle cells before stage 4 as well as the polar cells after stage 4 of oogenesis. In wild type ovaries, we regularly observed developing germ line cyst in region 2b and/or region 3 of the germaria (Fig. 2E). However, in *cuff* mutant females one week after eclosure, 72% of the germaria did not contain cysts of normal morphology in region 2b or region 3 (Fig. 2F, N=44); the number increases to 84% in females two weeks after eclosure (N=44). In addition, in *cuff* mutant females, we often observed a germarium attached to a mature egg chamber or an empty ovariole with only the germarium at the tip (Fig. 2F inset).

In older *cuff* mutant females, there is a gradual loss of germ cells even in the germaria. In mutant females three weeks after eclosure, around a third of the germaria did not contain any germ cells (N=74). Sometimes, we also observed germaria filled with cystoblast-like germ cells. Using the germline clone technique [17], we were able to show that Cuff is required in the germline for all of the functions described above, including Grk protein expression, reduced fecundity and karyosome phenotypes (data not shown).

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Characterization of cutoff

We determined that *cuff* corresponds to *CG13190*. Sequencing of *cuff* mutants revealed several DNA changes in the mutant alleles (Fig. 3A). In particular, $cuff^{WM25}$ is a strong allele based on the phenotypic analysis. In $cuff^{WM25}$ mutants, the start codon of CG13190 is mutated, making it very likely to be a null allele (see Fig. 3A for details). We generated transgenes carrying either the *cuff* genomic region or the predicted *cuff* coding sequence under the control of a UAS enhancer. Both the genomic transgene as well as the *cuff* coding sequence driven by nanos-Gal4-VP16 fully rescued the eggshell defects of *cuff* mutants. The genomic transgene also rescues the female sterility.

Genomic database searches identified the yeast gene Rai1 as a homolog of *cuff*. This gene has been shown to interact with a nuclear 5′-3′ exoribonuclease (Rat1) which is involved in rRNA processing and transcriptional termination [18–20]. A cytoplasmic homolog of Rat1, Xrn1, has also been described in yeast and vertebrates and has been implicated in mRNA regulation that is localized to cytoplasmic processing bodies [21,22]. We generated an HA-tagged *Drosophila* Rat1 (CG10354) construct and overexpressed it with a fully functional FLAG tagged Cuff in the ovary. Using immuno-precipitation (IP), we failed to detect any interaction between the exo-ribonuclease and Cuff (data not shown). It is therefore possible that Drosophila Rat1 is not the correct partner for Cuff. This is also supported by our observation that over-expressed Rat1, as expected, localizes to the nucleus, whereas over-expressed Cuff localizes to the cytoplasm. We were not able to detect endogenous Cuff protein with an anti-Cuff antibody, presumably due to low levels of protein expression. However, over-expressed HA-tagged Cuff partially co-localizes with perinuclear puncta in the nurse cells in younger egg chambers. A similar localization pattern has been described for the helicase Vasa, and we found that Cuff partially co-localizes with Vasa in the cytoplasm (Fig. 3B, C). The peri-nuclear localization pattern, also designated as nuage in the germ cells and related to mammalian P-bodies [23], has been previously described for components of the RNAi machinery and for genes involved in RNA degradation [24,25].

Given the eggshell ventralization and the karyosome defect, *cuff* has similar mutant phenotypes as a group of mutants known as the spindle class genes [26]. Several members of this group encode DNA repair genes [27,28] for instance *spindle(spn)* B *(XRCC3)*, and *okra (DmRad54)*. In these mutants, the DSBs that are created during recombination persist, activating Chk2 through the *Drosophila ATR* homolog *mei-41*. The activity of these kinases negatively regulates the translation of Grk, possibly through a post-translational modification of Vasa, which in turn, leads to ventralization of the eggs laid by mutant females [4,29]. Inactivation of the checkpoint, for instance through mutations in *chk2* or *mei-41*, suppresses the egg shell defects of the spindle class DNA repair mutants. In addition, in double mutants of the DNA repair genes and the genes required for initiating the DSBs, such as *c(3)*g, *mei-W68* or *mei-P22*, DSBs are not generated, therefore the checkpoint is not activated, and the egg shell morphology is normal, even in the presence of the repair mutants. To check whether Cuff is involved in the repair of DSBs initiated in prophase of meiosis I, we generated *mei41; cuff* and *cuff; c(3)*g double mutants. While both mutations suppress the eggshell defect of *spnB* or *okra* to wildtype morphology [3]neither suppresses the eggshell defect of *cuff*, indicating that Cuff does not function in the meiotic repair pathway. To our surprise, however, a mutation in *chk2* partially suppresses the eggshell defect of *cuff* (Table 1) as well as the defects in cyst development (Fig. 2G, H). *chk2 cuff* double mutants lay mostly wild type looking eggs (Table 1), they have cysts with highly branched fusomes in the germaria (N=52) (Fig. 2G, H), and the females lay more eggs compared to *cuff* single mutants although the rescue is not 100% (data not shown). In certain allelic combinations, we were able to observe a dominant effect in the *chk2* suppression of *cuff* eggshell defect. To check whether the eggshell defect of other RNAi machinery components can be suppressed by *chk2*, we generated double mutants of

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chk2 and *aub*. The *chk2* mutation partially suppresses the eggshell defect (Table 1) as well as the laying defect of *aub* mutants (data not shown). As in *chk2 cuff* double mutants, we also observed a partial dominant suppression in the *chk2 aub* combination. Compared with *aub* mutants, *chk2 aub*/+ *aub* flies lay more eggs, a higher percentage of which are of wild type-like morphology. In contrast, no significant suppression of the *aub* egg shell phenotype was observed in combination with two alleles of *mei-41*. However, it should be noted that *mei41; aub, mei41; cuff* and *cuff; c(3)*g females continued to lay very few eggs, such that a small percentage of suppression would have gone unnoticed.

Previously, work from our lab suggested that the DNA repair checkpoint, upon activation, regulates Grk translation through a post-translational modification of Vas, resulting in slower Vas electrophoretic mobility [29]. To address whether in *cuff* mutants, the checkpoint acts in the same manner, we assayed Vas mobility in *cuff* mutant combinations. In *cuff* mutants, Vas migrates slightly slower compared with wild type control consistent with the modification seen in the DNA repair mutants. The mobility is not changed in *mei41; cuff* double mutant background, which is consistent with the fact that *mei41* mutants do not significantly suppress the egg shell phenotype of *cuff*. However, Vasa mobility is restored to wild type in the *chk2 cuff* double mutant (Fig. 3D). This suggests that although in *cuff* mutant, the checkpoint is activated through a different sensing mechanism, upon activation, the checkpoint involves Chk2 and acts through similar pathways to affect Gurken translation in the egg chambers that escape the early arrest.

Cutoff mutations lead to an increased level of retro-transposable elements in the female germ line

Several of the Spindle Class genes, such as *spnE* and *aub*, have been shown to be essential components of the RNAi machinery [30]. Since over-expressed Cuff has a peri-nuclear localization, we wanted to check whether Cuff might also be required in RNAi pathways. Recently, a specific branch of the RNAi pathways, the repeat associated small interfering RNA (rasiRNA), has been implicated in the control of retro-transposable elements in the *Drosophila* germline [31]. Using qRT-PCR we studied the level of *Het-A* and *Tart*, two of the retro-transposable elements responsible for maintaining the telomere in *Drosophila*. Previously, it has been shown that in *spnE* and *aub* mutants, *HetA* and *Tart* transcripts are de-repressed, resulting in a marked elevation in the transcripts level. Compared with heterozygous controls, in *spnE* homozygous mutant females, *Het-A* and *Tart* transcripts are up-regulated by approximately ten-fold, while in *aub* mutants only *Het-A* is significantly upregulated. In *cuff* mutant females, the elevation for both transcripts is even more pronounced. Compared with the heterozygous control, *HetA* levels are elevated by more than 800-fold in *cuff* mutants, and *Tart* transcript levels increase by more than 20-fold (Fig. 3E). Transposable elements are normally silenced in the Drosophila germline by the rasiRNA pathway. This silencing process appears to be strongly impaired in the *cuff* mutants. We further tested whether the upregulation of the transposable elements in *cuff* mutants could be due to a reduction in the level of rasiRNAs. However, we found that the levels of the 25 nt long *roo* interfering RNA are not reduced in *cuff* mutant ovaries, in contrast to ovaries mutant for *aub* (Fig 3F). This indicates that Cuff is not involved in the biogenesis of the rasiRNAs and points to a function for Cuff in the actual silencing process. Since high transcript levels of the retro-transposable elements in the germline are correlated with elevated transposition incidents, which in turn lead to decreased chromosomal integrity [31,32], it is possible that such chromosomal defects activate the checkpoint involving *chk2*. Such a model has recently been proposed by Klattenhoff et al. [33]. In addition, since transposable elements are involved in chromatin structure regulation [34], it is also possible that a chromatin checkpoint exists that involves Chk2 activity. Once Chk2 is activated, either by the mutants in DNA repair pathways, or by RNAi components such as Cuff and Aub, Chk2 activity leads to post-translational Vas modification, and a negative

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regulation of Grk translation. However, unlike DNA repair mutants, *cuff* and *aub* mutations are not suppressed to wildtype morphology and fecundity by mutations in *mei41*, suggesting that they activate the checkpoint through a different, or additional, sensing mechanism. Furthermore, most of the mutants in DNA repair pathways do not cause defects in cyst development or germline stem cell maintenance. These additional defects seen in *cuff* mutants could be due to the timing of checkpoint activation. DNA repair mutants activate the meiotic checkpoint during prophase of meiosis which initiates after the formation of the 16 cell cyst, while *cuff* and *aub* mutants appear to act earlier in oogenesis, given that they have effects already during the mitotic cycles preceding the onset of meiosis. The transposon activated checkpoint leads to not only translational arrest of Grk, but also mitotic cell cycle arrest. Many of the arrested germline cells and cysts eventually undergo apoptosis, leading to gradual loss of germline stem cells and developing cyst in *cuff* mutants. However, germ cells that escape the early arrest encounter the second checkpoint effect that leads to a reduction in Gurken translation.

It was recently discovered that there are a large number of different small RNAs generated in the germline of both mammals and flies [35–38]. Many of them are associated with Piwi family proteins, and most have no known functions. Since the germline represents a special cell type that will pass its DNA on to future progeny, it is possible that selfish elements have developed a high propensity to remobilize in the germ line. It is further very plausible that in most organisms, the germline has evolved sophisticated mechanisms to defend itself against such transposable elements. Many of the small RNAs found in the germline may be involved in the defense against transposable elements, as well as in the regulation of transcription and translation. When the machinery to generate these small silencing RNAs or the effector complexes that are responsible for transcript degradation are disrupted, chromosomal integrity might be at risk. Here we have found that in Drosophila a checkpoint involving the conserved Chk2 kinase monitors the RNAi mediated events in the germline, and ensures the genomic integrity of the progeny. Chk2 therefore acts as a surveillance factor for both transposon generated problems as well as DNA repair problems in the germline. Whether Chk2 has a similar role in the mammalian germline will be interesting to investigate in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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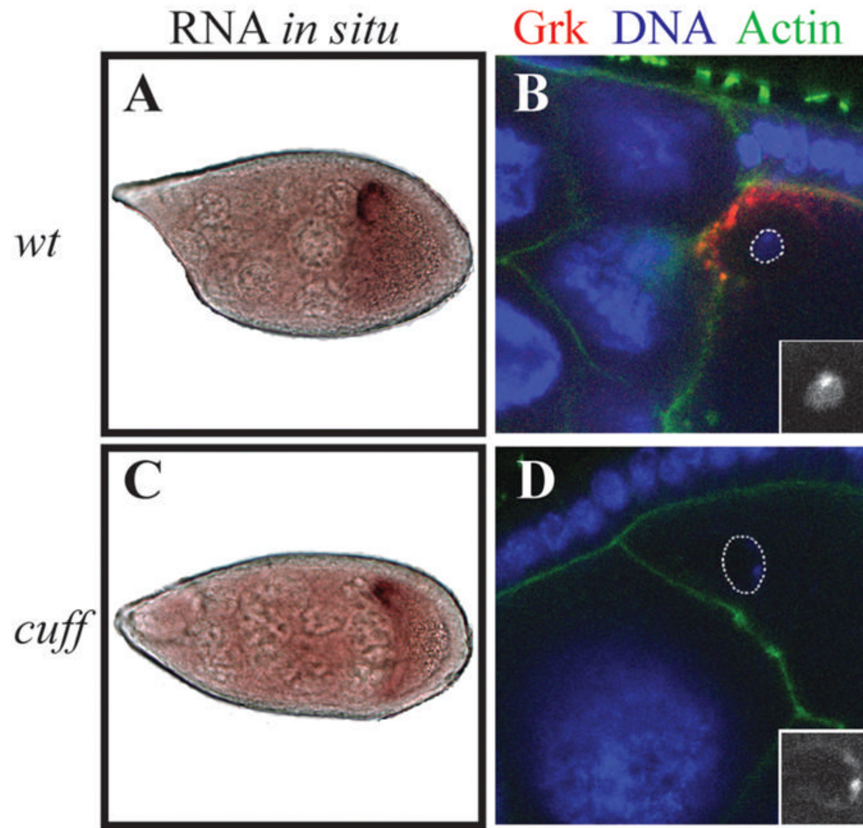

Fig 1. Grk expression in *cuff* mutant egg chambers

grk RNA *in situ* hybridization (A, C) and Grk antibody staining (B, D).

(A, B) *Cuff* heterozygous females were used as wild type control: In Stage 9 wild type egg chambers, *grk* transcripts form a tight cap around the oocyte nucleus at the dorsal cortex (A). Grk protein is translated from the localized mRNA, and is also restricted to the dorsal anterior region of the oocyte (B). At this stage of oogenesis, the chromatin of the oocyte nucleus forms a compact, round structure termed the karyosome (B inset).

(C, D) *cuff* $^{WM25/KG05951}$. In Stage 9 *cuff* mutant egg chambers, *grk* transcript localization appears mostly normal (C), however, in 10–40% of the egg chambers, Grk protein is undetectable (D). Some 10–20% of the oocyte nuclei at this stage also assume a defective morphology. Often, the DNA seems to localize to the periphery of the nucleus (D inset). In B and D, the oocyte nucleus is marked by dotted lines.

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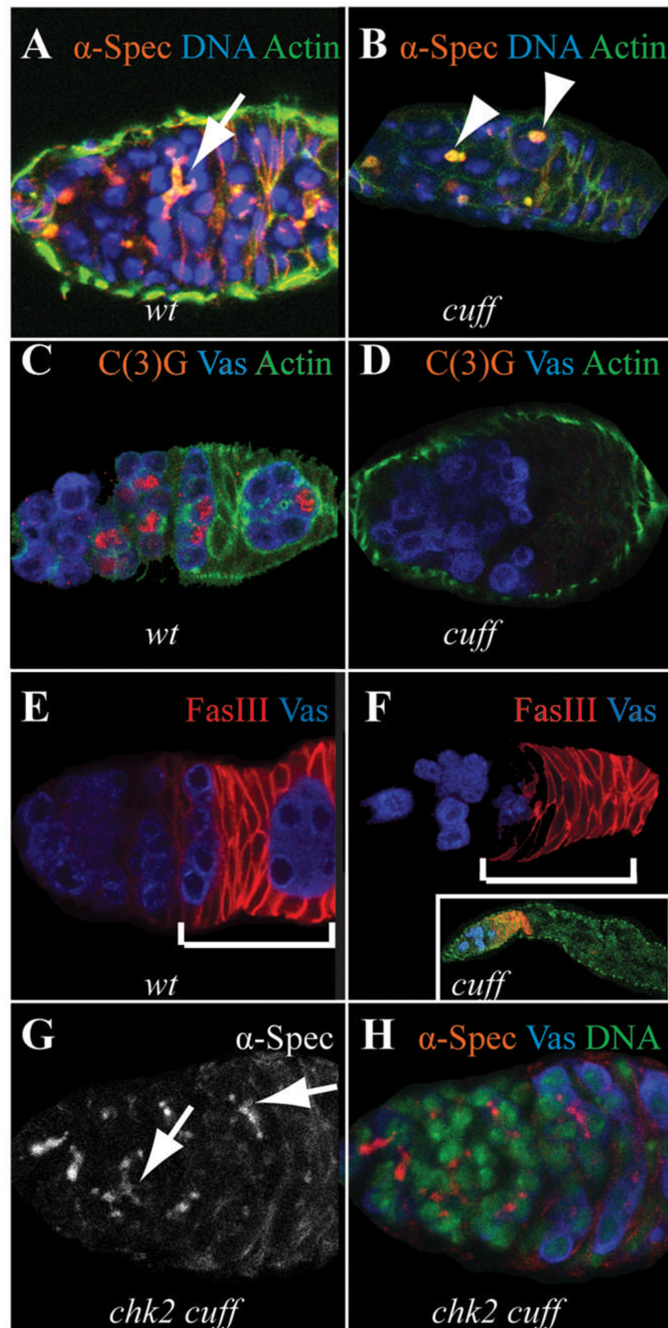

Fig 2. Germline cyst development in *cuff* mutants

(A, B) Using an antibody against α-Spectrin (red), we always observed highly branched fusomes (arrow) in wild type germaria in the anterior regions (A). In contrast, in *cuff* mutant germaria, many of the cysts fail to divide normally and form branched fusomes, instead often retaining a round spectrosome-like fusome (arrowheads) (B).

(C, D) Using an antibody against C(3)G (red), we monitored meiotic progression in wild type and *cuff* mutant backgrounds. In wild type germaria, we consistently observed multiple cysts initiating meiosis (C); while in *cuff* mutant background, although germ cells are present (as marked by Vas staining in blue), the cysts lack C(3)G staining, indicating an early arrest (D).

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(E, F) In wild type germaria, developing germline cysts migrate to the posterior of the germaria (E), and subsequently bud off, forming an egg chamber. In *cuff* mutants, this process is disrupted. In corresponding regions marked by the bracket (region 2b and 3 as marked by FasIII staining in red), there are few if any normal looking cysts in *cuff* mutant germaria (F).

(G, H) The defects in cyst development of *cuff* mutants is partially suppressed by a mutation in *chk2*. In *chk2 cuff* double mutants, we observed many highly branched fusomes (arrows in G), and females also lay many more eggs than the corresponding *cuff* mutant.

All flies were growing under optimal nutritional conditions. *Cuff* heterozygous females were used as wild type control, while *cuff* $^{WM25/KG0595}$ and *chk2 cuff* ^{WM25}females were used for phenotypic analysis.

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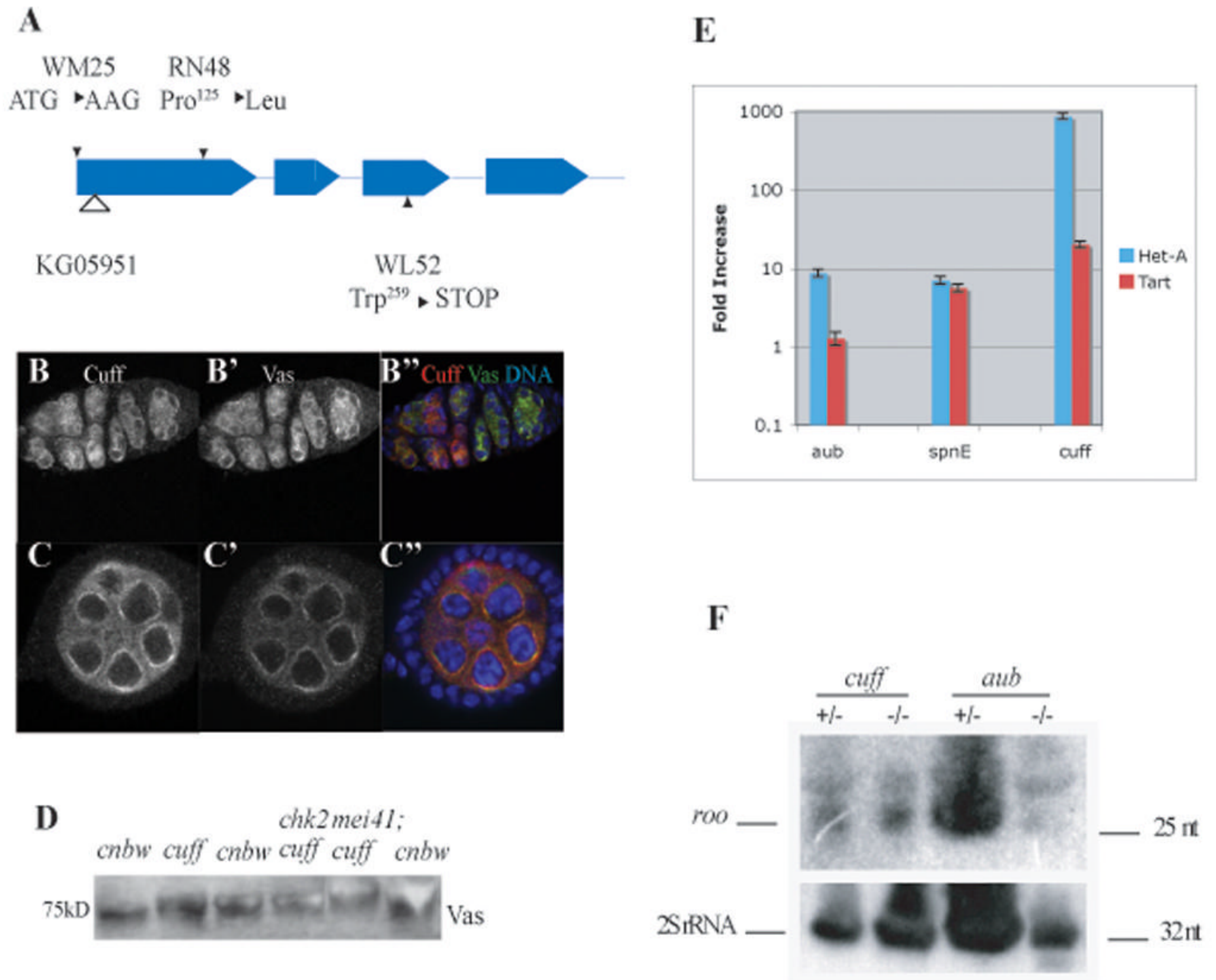

Fig 3. Characterization of *cuff*

A. Sequencing of *cuff* mutants. $cuff^{WM25}$, $cuff^{RN48}$, and $cuff^{WL52}$ were induced by EMS [6]. $cuff^{KG05951}$ was derived by the BDGP Gene Disruption Project.

B–C. In order to analyze Cuff localization, we expressed HA-tagged Cuff protein under the control of *nanos*-Gal4 VP16. When expressed in this manner, Cuff shows a prominent peri-nuclear pattern, co-localizing with Vas protein in the germarium (B) and early stage egg chambers (C).

D. In *cuff* mutants, Vas protein electorophoretic migration is slower than in wild type. A very similar phenotype was observed in *spnB* mutants (Ghabrial and Schupbach, 1999). The Vas migration defect is suppressed by a *chk2* mutation, but not by a mutation in the *Drosophila ATR* homolog *mei41*.

E. Mutations in *cuff* lead to a strong up-regulation of *Het-A* and *Tart* retrotransposable elements in the Drosophila germline. In *cuff* mutants, *Het-A* displays a 800-fold increase in transcript levels, while *Tart* is up-regulated approximately 20 fold. A deregulation of these elements can also be detected in the germline of *spnE* and *aub* mutants (see also [31]). In *spnE* ovaries both *Het-A* and *Tart* are up-regulated approximately 10 fold, while in *aub* only *Het-A* levels are significantly increased.

F. rasiRNAs production is not affected in *cuff* mutant ovaries. Similar levels of the *roo* rasiRNA can be detected in ovaries heterozygous (lane 1) and homozygous (lane 2) mutant for *cuff*. In contrast, *roo* rasiRNAs are expressed in ovaries heterozygous mutant for *aub* (lane 3), but are absent from the homozygous mutant.

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