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Drosophila Dynein light chain (DDLC1) binds to *gurken* mRNA and is required for its localization

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Abstract

During oogenesis in *Drosophila*, mRNAs encoding determinants required for the polarization of egg and embryo become localized in the oocyte in a spatially restricted manner. The TGF-alpha like signaling molecule Gurken has a central role in the polarization of both body axes and the corresponding mRNA displays a unique localization pattern, accumulating initially at the posterior and later at the anterior-dorsal of the oocyte. Correct localization of *gurken* RNA requires a number of *cis*-acting sequence elements, a complex of *trans*-acting proteins, of which only several have been identified, and the motor proteins Dynein and Kinesin, traveling along polarized microtubules. Here we report that the cytoplasmic Dynein-light-chain (DDLC1) which is the cargo-binding subunit of the Dynein motor protein, directly bound with high specificity and affinity to a 230-nucleotide region within the 3′UTR of *gurken*, making it the first *Drosophila* mRNA-cargo to directly bind to the DLC. Although DDLC1 lacks known RNA-binding motifs, comparison to double-stranded RNA-binding proteins suggested structural resemblance. Phenotypic analysis of *ddlc1* mutants supports a role for DDLC1 in *gurken* RNA localization and anchoring as well as in correct positioning of the oocyte nucleus.

Keywords: *gurken*; mRNA localization; *ddlc1*; *Drosophila*

1. Introduction

mRNA localization plays a major role in spatially targeting the expression of specific proteins to subcellular locations, a process essential for the polarization and proper function of many cell-types (reviewed in [1–3]). In *Drosophila* several classes of transcripts encoding proteins that are required for the establishment of axial polarity, germ cell formation and early embryonic patterning become spatially restricted in the egg during oogenesis (reviewed in [3,4].

The *Drosophila* ovary consists of a number of ovarioles, each containing a chronologically ordered series of egg chambers which develop into mature eggs during oogenesis. Each egg chamber is composed of a germline cyst of a single oocyte and fifteen nurse cells (NCs), which is surrounded by a somatic epithelial cell layer (follicle cells) (oogenesis is reviewed in [5]). Egg polarization depends on cell–cell communication between germline and soma [6] via the germline specific TGF-α signaling protein Gurken (GRK) and the EGF receptor (Egfr) on the follicle cells [7,8]. Restricted spatial expression of the GRK ligand in the oocyte, which is essential for correct patterning, is achieved by strictly localizing the mRNA within the oocyte [9]. In early oogenesis *grk* RNA is localized to the posterior of the oocyte where GRK instructs cells to adopt a posterior cell fate [7,8], while in mid-oogenesis *grk* becomes localized to the anterior-dorsal (AD) cortex, where the protein induces dorsal follicle cells fates [9,10]. This localization pattern is unique to *gurken*, whereas all other mRNAs localized in the oocyte are targeted either to the anterior (including *bicoid* and *fs(1)K10*) or to the posterior pole (including *oskar* and *nanos*) (Reviewed in [3,4])

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Localized mRNAs contain *cis*-acting localization signals that reside primarily within their 3′-untranslated regions (UTRs). The secondary structures of these elements (rather than their primary sequences) are recognition sites for specific trans-acting factors required for localization (Reviewed in [4,11]). *grk* mRNA localization was shown to depend on sequences that map to the 5′UTR, 3′UTR and to the protein coding region [12,13]. Several trans-acting factors are specifically required for the localization of *grk* to the AD in mid stages of oogenesis. These include the nuclear-cytoplasmic shuttling RNA binding proteins (RBPs) Squid (SQD) [14] and HRB27C/HRP48 which interact with each other and bind to the 3′UTR of *grk* [15,16], the SQD-binding nuclear protein FS(1)K10 [17], Ovarian tumor (OTU) protein [16] and the splicing factor Half pint (HFP) which regulates *grk* and *otu* splicing [18]. Mutations in the genes encoding any of these proteins interfere specifically with *grk* mRNA localization to the AD, leading to the accumulation of *grk* at the anterior margin (oocyte and NCs boundary) where it is inappropriately translated, inducing ectopic dorsal fates. Interestingly, all of these trans-acting proteins appear to function in translational repression of unlocalized *grk* RNA [15,16,18,19].

Studies in a number of systems revealed that mRNAs are trafficked in large RNA-protein (RNP) complexes which may include proteins required for regulating stability, transport and translation of the associated RNAs (Reviewed in [20–22]). Directional trafficking of these RNP complexes requires an intact polarized actin or microtubule (MT) cytoskeleton and motor proteins of the Myosin, Dynein and Kinesin families (Reviewed in [23]). In *Drosophila*, localization to the anterior of *bicoid* (*bcd)* and *grk* (briefly localized to the anterior) and the re-positioning of the oocyte nucleus (ON) from posterior to anterior (in mid-oogenesis) requires MTs, the Dynein motor (for transport) and Kinesin (for recycling Dynein) [24–26]. Live imaging of *grk* mRNA localization suggests that it localizes in two steps; first moving from posterior to anterior and then turning towards the ON positioned at the AD [27]. Given the distribution of MT minus ends in the oocyte, it was further proposed that MTs emanating from the asymmetrically positioned ON may present a distinct subpopulation that would differentially promote the second step of *grk* transport towards the AD [27].

Cytoplasmic Dynein is a multi-subunit, minus end-directed molecular motor that consists of two MT-binding Dynein heavy chains (DHCs) with ATPase and motor activities, several Dynein intermediate chains (DIC), a group of light intermediate chains and several light chains (DLCs) [28]. The smallest Dynein light chain (DLC) of 8 KD (real MW-10 KD) (termed LC8 in vertebrates and DDLC1 in *Drosophila*) is highly conserved from chlamydomonas to man [29]. It is present in two copies in each Dynein complex and attaches itself to the motor via binding to the intermediate chains [30]. LC8 binds to a versatile collection of proteins involved in diverse biological processes (see Table 1 and references in [28,30] which led to the proposal that LC8 may be involved in targeting the Dynein motor complex to various cargoes ([28,31] and references within). In *Drosophila*, DDLC1 binds to Swallow (SWA), an RBP involved in *bcd* mRNA localization [32], to Egalitarian (EGL) which is required for oocyte specification [33] and to Spindle-F (SPN-F) which colocalizes with MT minus-ends and may function in MT organization [34].

Table 1
ddlc1 alleles

Allele	Nature[a]	% expression[a]	Viability	Egg laying
$ddlc1^{ins1}$	p-insertion	30	Viable	No
$ddlc1^{exc39}$	p-excision	50	Viable	Few-ventralized
$ddlc1^{DIIA82}$	p-excision	20	Lethal	NA

Expression-Refers to *ddlc1* RNA levels relative to 100% expression in the wt. [35,45]. Expression, viability and egg-laying were tested for flies homozygous for the indicated alleles. NA—not applicable.
[a] These data were taken from previous publications [35,45].

Here we report that DDLC1 was picked up in a screen designed to identify proteins that directly bound to the 3′UTR of *grk* mRNA. UV cross-linking and RNA competition analysis indicated that a DDLC1-GST fusion protein specifically bound to *grk* with high specificity and affinity but did not bind to anteriorly localized *fs(1)K10* or posteriorly localized *osk* transcripts, suggesting that *grk* RNA may be a direct target of DDLC1. Examining RNA localization in hypomorphic *ddlc1* mutants revealed that *grk* localization to the AD and its anchoring were compromised, as was the anterior positioning of the ON, implicating DDLC1 in all these processes.

2. Materials and methods

2.1. Fly stocks

OreR flies were used as wt. *ddlc1* mutants ($ddlc1^{ins1}$, $ddlc1^{exc39}$, $ddlc1^{DIIA82}$) were obtained from Krishanu Ray [35].

2.2. Molecular methods

2.2.1. Probe synthesis

^{32}P-labeled RNA probes and unlabeled RNA for competition assays were synthesized as in the 'Riboprobe In Vitro Transcription Systems' protocol (Promega). Synthesis continued for 2 h at 37 °C, followed by 30 min of incubation with RQ1 RNase-free DNase (Promega). Reaction was stopped by adding EDTA (4 mM) and incubating at 65 °C for 10 min. Unincorporated nucleotides were removed on a 1-ml Sephadex G-50 (Amersham Biosciences) spin column. Typical yield was $0.5–2\times10^9$ cpm/μg ($\sim4.5\pm10^7$ cpm/nmol) of RNA. Integrity of the probe and unlabeled RNA competitors was confirmed by gel electrophoresis.

2.2.2. RNA ligand-binding screen

An ovarian cDNA expression library (originally from Peter Tolias) in λgt22A was plated, induced to express proteins, blotted to nylon filters and probed for RNA binding as previously described [36]. A total of 3×10^5 PFUs were probed with a non-denatured (native) ^{32}P-labeled sense-strand probe representing the full length *grk*-3′UTR (500 bases). Clones, positive after three rounds of re-screening, were amplified by PCR and sequenced.

2.2.3. DDLC1-GST fusion proteins

Full length *ddlc1* cDNA amplified with 5′ primer acccgggaaacagccaaaatgtctgatcg and 3′ primer tggttgttgctgtttctgct was cloned into pGEM-T (Promega), excised with *Sma*I–*Eco*RI, introduced into the *Sma*I and *Eco*RI sites of pGEX1 expression vector (Pharmacia Biotech) (frame was verified by sequencing) and transformed into *Escherichia coli* BL21 (Invitrogen). To induce expression of DDLC1-GST and SQD-GST (obtained from Trudi Schüpbach; [15]), 0.1 mM of

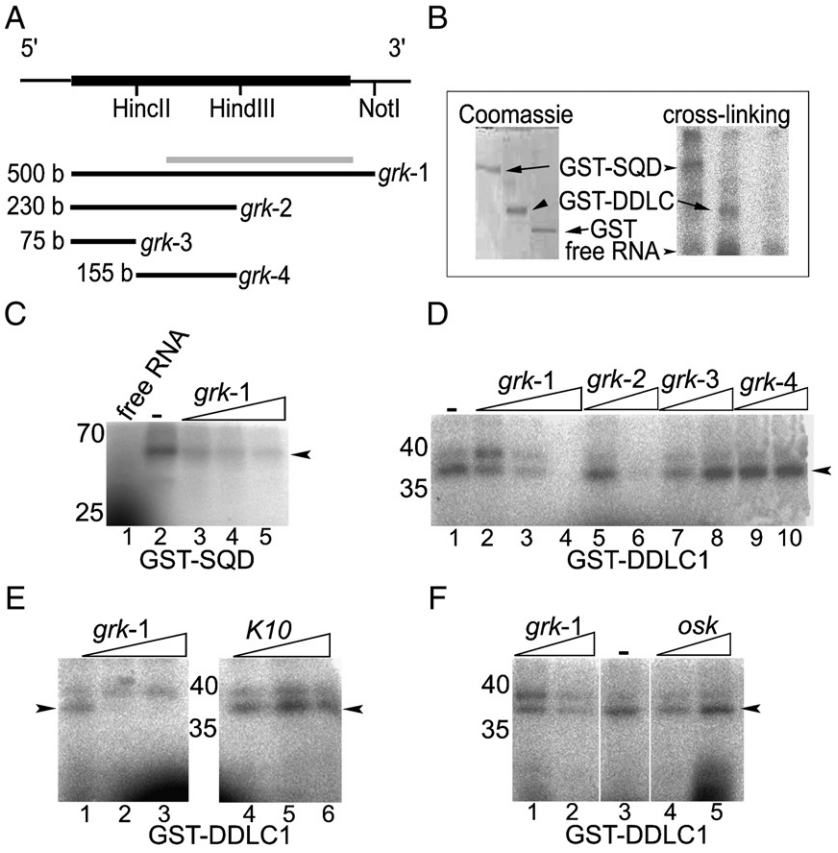

Fig. 1. DDLC1 specifically binds to a 230-nucleotide region within *grk* 3′UTR. (A) Regions in the 3′UTR of *grk*, that were used as probe (*grk1*) and as RNA competitors (*grk-2*, *grk-3*, *grk-4*) in the grk/DDLC1 binding experiments. Gray bar (above *grk1*) indicates region proposed to bind to SQD protein [15]. (B) SQD-GST and DDLC1-GST fusion proteins and GST protein, shown in the left panel, were UV cross-linked to radiolabeled *grk1* probe. Both SQD-GST and DDLC1-GST fusions bound to *grk-1* probe, while GDT alone did not bind (right panel). (C–F). RNA binding competition assays. (C) Binding of SQD-GST to radiolabeled *grk-1* in the absence (−) or increasing amounts of *grk-1* RNA competitor (lanes 3–5: x25, x50, x150 cold RNA molar excess). (D–F) Binding of DDLC1-GST to radiolabeled *grk-1* in the absence (−) or increasing amounts of cold *grk-1* RNA (D lanes 2-4 and E lanes 1–3: x25, x50, x150 RNA excess; F lanes 1, 2: x50, x150), *grk-2* (D lanes 5, 6: x25, x50), *grk-3* (D lanes 7, 8: x50, x150), *grk-4* (D lanes 9, 10: x50, x150), full length *fs(1)K10* 3′UTR (900 nucleotides) (E lanes 4–6: x25, x50, x150) and full length *osk* RNA (3Kb) (F lanes 4, 5: x50, x150). In D–F, the band marked by the arrowhead is the only band expected for dynein-GST fusion protein. The nature of the slower migrating band that appears sometimes above the dynein-GST is not completely clear.

IPTG was added to cells grown for 2 h at 37 °C under no selection conditions (no antibiotic). Following another 3–4 h incubation, cells harvested by centrifugation were lysed by freeze–thawing in MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4) after which 0.5% of Lysozyme, protease inhibitor cocktail and 0.9% of Triton-X were added. GST fusions were purified by incubating the supernatants with glutathione-coated agarose beads (Sigma). Evaluation of integrity and quantification of fusion proteins was done by PAGE and Coomassie staining, using known BSA standards.

2.2.4. UV cross-linking and RNA-binding competition assays

2×10^5 cpm. radiolabeled RNA probe and 25 ng (0.68 pmol) of GST-DDLC1 fusion protein were used in RNA binding and competition assays. UV cross-linking was performed as in [37]. For competition assays, unlabeled competitor RNA at increasing molar excess was pre-incubated with the protein samples for 10 min prior to adding the radiolabeled probe. Following UV cross-linking, unbound RNA was digested and protein–RNA complexes fractionated by SDS-PAGE and analyzed by PhosphorImager.

2.2.5. Gel shift assay

Determination of the equilibrium binding dissociation constants (K_d) for GST-DDLC1—*grk* 3′UTR was performed as described [37]. Briefly, serially diluted GST-DDLC1 protein samples were preincubated for 10 min at RT in 5 μl of 3 mM $MgCl_2$ supplemented with 5 units of RNasin (Promega). The samples supplemented with 8 μg of yeast tRNA (Sigma) were then added to the radiolabeled RNA probe (10,000 cpm) in a final volume of 15 μl. After 15 min of equilibration at room temperature, 2 μl of loading buffer (0.25 μg/μl xylene cyanol, 0.25 μg/μl bromphenol blue, and 6% (v/v) glycerol) were added and the samples were loaded onto a native 5% polyacrylamide gel in 1X TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8) and run at 25 mA for 2–3 h. Gels were exposed to a PhosphorImager and radioactivity associated with protein complexes was quantified using ImageJ program (available online from the NIH; http://rsb.info.nih.gov/ij/). The K_d value was determined from best fit of the data to the single-site binding curve defined by the equation; Fraction of RNA Bound=(maximum RNA bound×[protein])/(K_d+[protein]). The K_d was assumed to be equal to the protein concentration that gave 50% binding.

2.3. In situ hybridization, immunohistochemistry and imaging

In situ hybridization was as in [38]. Antisense digoxigenin-labeled RNA probes were prepared with a kit from Boehringer Manheim. Antibody staining of ovaries was performed as previously described, using a primary anti GRK antibody made in rat [19] (1:3000) and secondary Cy3-conjugated anti rat (1:1000; Jackson ImmunoResearch Laboratories). Micrographs were taken with a Spot RT-slider digital camera on a Leica DMR microscope. Confocal images were generated on a Zeiss LSM510 inverted confocal microscope. Pictures were minimally manipulated using Photoshop.

3. Results and discussion

3.1. A screen for grk-3′UTR-binding proteins.

Screening an ovarian cDNA library (in phages) for proteins that could bind to radiolabeled 3′UTR of *grk* mRNA, we have picked up a number of different candidate proteins. One of them corresponded to SQD protein which was previously shown to bind to the 3′UTR of *grk* [15], indicating that our screen could identify relevant *grk*-3′UTR interacting proteins. Another positive clone encoded *Drosophila* cytoplasmic DLC (DDLC1), the focus of this manuscript.

3.2. DDLC1 specifically binds to the 3′UTR of grk and may structurally resemble RNA binding domains found in dsRBPs

Binding of DDLC1 to *grk* 3′UTR was confirmed by UV cross-linking experiments demonstrating that a GST-DDLC1 fusion protein directly bound to a full length radiolabeled *grk* 3′ UTR probe (*grk-1*) (Fig. 1A, B, D–F). Similar binding was observed with the SQD-GST fusion protein and *grk*-3′UTR (Fig. 1B, C). In both cases, interaction was abolished upon preincubation of the proteins with unlabeled competitor RNA corresponding to full length *grk* 3′UTR (SQD; Fig. 1C, DDLC1; Fig. 1D–F). Further competition assays with RNA corresponding to smaller domains within the *grk* 3′UTR (Fig. 1A) revealed specific binding of DDLC1-GST to a 230-nucleotide region (*grk-2*) (Fig. 1A, D) which only partially overlaps with the mapped SQD binding domain (Fig. 1A, based on [15]). When the 230 nucleotide region was split into two smaller probes (*grk 3* and *grk 4*), no binding to DDLC1-GST was detected (Fig. 1A, D). Binding of DDLC1 to *grk* appeared rather specific since neither posteriorly localized *osk* (whose localization machinery shares some of the transacting factors involved in *grk* localization such as SQD and HRB27C/HRP48: [39,40], nor anteriorly localized *fs(1)K10* interacted with DDLC1-GST, as indicated by their inability to compete for binding to the protein (Fig. 1E, F). Likewise, neither of the additional competitors tested, including *osk* antisense RNA and a nonsense transcript of 100 nucleotides generated for a region in the pBluScript vector, were able to compete with *grk* for binding to DDLC1-GST (not shown). In addition to the high binding specificity of DDLC1-GST to *grk* 3′UTR, the protein exhibited high affinity for the RNA, with an apparent K_d of ~5.5 nM as calculated from the gel shift assay (Fig. 2). Collectively, the results presented suggested that DDLC1 may function as an RBP with high specificity and affinity for a 230-nucleotide region with the *grk* 3′UTR. Notably, LC8 (the mammalian homolog of DDLC1) was previously shown to bind to the 3′UTR of the parathyroid hormone (PTH) mRNA and was suggested to function in the co-localization of this mRNA with MTs [41]. Along these lines, it is possible that direct binding of DDLC1 to the *grk* RNA may be sufficient to recruit the *grk* cargo to the Dynein motor complex without the need for an additional adaptor RBP. The fact that *grk* is only the second mRNA ever proposed to directly bind to DLC would suggest, however, that direct binding of RNA to DLC may not present a general mechanism for linking RNA cargos to the Dynein motor.

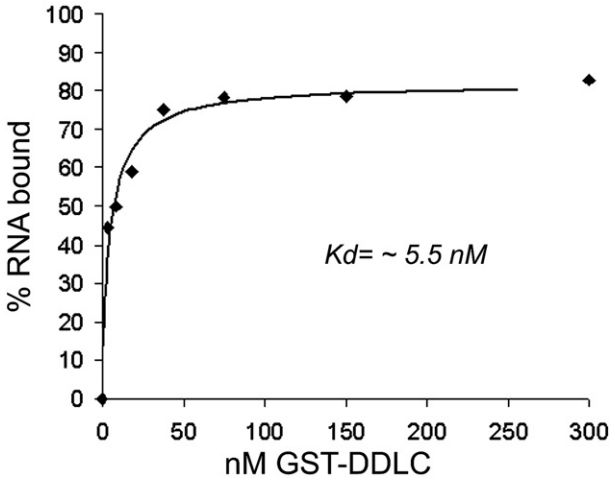

Fig. 2. Gel mobility shift Assay. Serially diluted GST-DDLC1 protein samples (final concentration 4–300 nM) were allowed to bind to the 230-nucleotide *grk* 3′UTR (*grk-2*) radiolabeled RNA probe (10,000 cpm). Following a 15-min equilibration period and gel electrophoresis, the relative amount of bound radioactivity was determined and plotted (diamonds) against the protein concentration. The dissociation constant (K_d) was calculated from best fit of the data to the single-site binding curve and was assumed to be equal to the protein concentration that gave 50% binding.

Since there are no known RNA binding motifs in DDLC1, its binding to RNA was quite unexpected. Recently, the NMR structure of DDLC1 was determined [42] and the protein was classified as an α-β 2-layer sandwich protein (SCOP: Structural Classification of Proteins; http://scop.mrc-lmb.cam.ac.uk/scop/). Many proteins of this class are RBPs including ribosomal proteins and double stranded RBPs (dsRBPs). Comparing the 3D structure of a full length pH-induced monomer of DDLC1 (1RHW in PDP: Protein Database Bank) (Fig. 3A) to those of dsRBPs, represented here by dsRNA binding domain III of *Drosophila* Staufen (STAU) (1STU in PDB) (Fig. 3B), suggested some structural resemblance. Both proteins have two α-helices segregated from a domain of several β-sheets. Moreover, a loop in DDLC1 which includes Lys-48 and Lys-49 (Fig. 3A) may be analogous to a loop with two lysine residues (Lys-50 and lys-51; Fig. 3B) of known significance for RNA binding by STAU [43,44]. Further experiments involving site directed mutagenesis of these residues will be needed to test this prediction.

3.3. Mutations in ddlc1 interfere with grk mRNA localization and anchoring and with positioning of the oocyte nucleus

To find out whether mutations in *ddlc1* specifically affected *grk* RNA localization, we examined the patterns of *bcd*, *osk* and *grk* transcripts in egg chambers from *ddlc1* mutants [35]. Three hypomorphic *ddlc1* alleles were used; $ddlc1^{ins1}$ (contains a P-element inserted 40 bp upstream of the 2.4-kb transcript; [45], $ddlc1^{exc39}$ and $ddlc1^{DIIA82}$ (both generated by imprecise excision of the P-element) [35]. Expression of *ddlc1* RNA was reported to be ~50%, 30% and 20% that of wild-type (wt) for $ddlc1^{exc39}$,

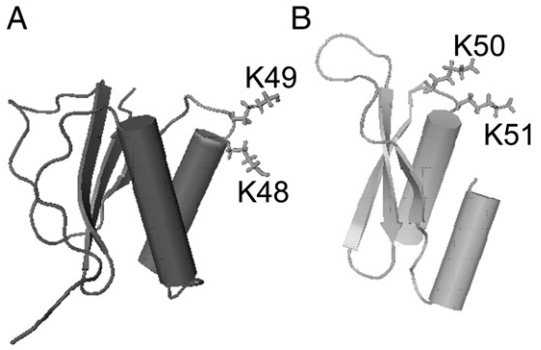

Fig. 3. Modeling of RNA binding by DDLC1. Some structural elements of DDLC1 resemble those found in dsRBPs such as STAU. Both DDLC1 (full length protein) (A) and the dsRNA binding domain III of STAU (B) have two α-helices (cylinders) segregated from a domain of several β-sheets. Analogous loops with two lysine residues of know significance for RNA binding by STAU are depicted.

ddlc1^{ins1} and *ddlc1*^{DIIA82}, respectively [35], (Table 1). RNA localization was examined in egg chambers from *ddlc1*^{exc39}/*ddlc1*^{ins1} trans-heterozygotes (weakest), *ddlc1*^{ins1} homozygotes (intermediate) and *ddlc1*^{DIIA82}/ *ddlc1*^{ins1} trans-heterozygotes (most severe) allele combinations, all of which laid no eggs. In contrast to the strict AD localization of *grk* in wt, starting at stage 8 (Fig. 4A–C), in ~42% of *ddlc1*^{exc39}/*ddlc1*^{ins1}, 33% of *ddlc1*^{ins1}/*ddlc1*^{ins1} and 20% of *ddlc1*^{DIIA82}/ *ddlc1*^{ins1} egg chambers, *grk* RNA was mislocalized to the anterior margin (Table 2, Fig. 4E, F), suggesting that normal levels of DDLC1 may be essential for efficient execution of the second step of *grk* RNA localization, namely, transport to the AD of the oocyte. The mislocalized RNA was apparently not translated, as no GRK protein could be detected at the anterior (data not shown), indicating that translational regulation of the *grk* transcript was intact in the *ddlc1* mutants. This is contrary to the situation in *sqd* and *fs(1)K10* mutants, where *grk* mRNA mislocalized to the anterior is efficiently translated, inducing dorsalization. Intriguingly, the accumulation of *grk*, *bcd* and *osk* transcripts in the oocyte and

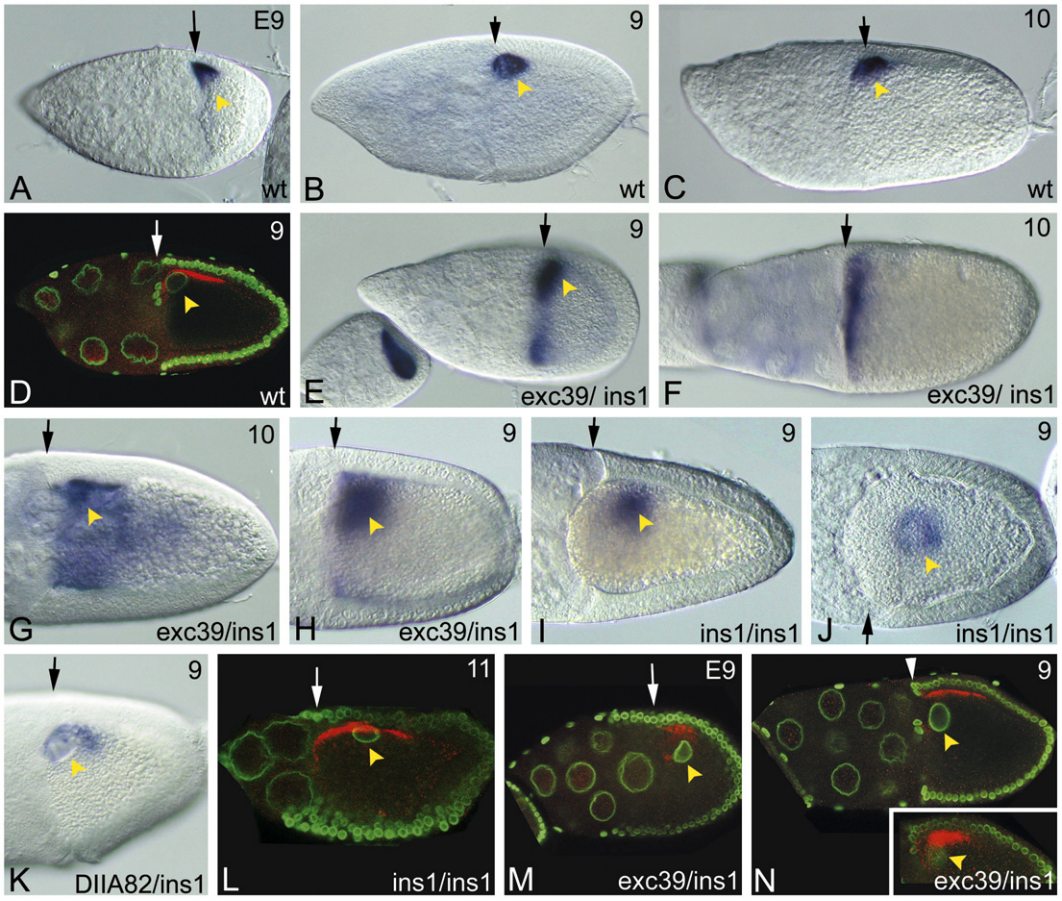

Fig. 4. Mutations in *ddlc1* interfere with *grk* RNA localization and anchoring and with positioning of the oocyte nucleus. *grk* RNA (A–C, E–K) and GRK protein (D, L–N) localization patterns in wt (A–D) and *ddlc1* mutants (E–N). In wt egg chambers *grk* RNA is tightly localized to the AD (A–C), whereas in a fraction of *ddlc1* mutant chambers *grk* resides at the anterior margin (E, F), or displays a diffused pattern around the anterior (G) or the ON (H, I, K). Additionally, contrary to wt (A–D), in mutant chambers the ON may reside at various positions along the AP axis (I, J, L). GRK protein (red), in wt, tightly associates with the AD cortex and with the ON. In the mutants association of GRK with the nucleus is lost while association with the cortex is still maintained (M, N). Inset in N shows an additional section of the same oocyte closer to the surface. Nuclear membrane is stained for lamin (green). Egg chambers were from *ddlc1*^{exc39}/*ddlc1*^{ins1} (E–H, M, N), *ddlc1*^{ins1}/*ddlc1*^{ins1} (I, J, L) and *ddlc1*^{DIIA82}/ *ddlc1*^{ins1} (K). Chambers are at early stage 9 (A and M), middle to late stage 9 (B, D, E, H–K, N), stage 10 (C, F, G) or stage 11 (L). Anterior is to the left and dorsal is up in A–E, G–I and K–N. The black and white arrows in each panel indicate anterior margin. Yellow arrowheads indicate the ON.

Table 2
RNA and ON localization phenotypes associated with hypomorphic mutation in *ddlc1*

Genotype	wt-S>8	Anterior	Diffused	Defective ON position	*n*
$ddlc1^{exc39}/ddlc1^{ins1}$	36.7	42.2	21.1	3.1	128
$ddlc1^{ins1}/ddlc1^{ins1}$	46.8	32.6	20.6	6.4	126
$ddlc1^{DIIA82}/ddlc1^{ins1}$	55.4	19.6	25.0	7.1	56

The percentages of the following phenotypes are presented; wt-S>8 — Stage 8 and older egg chambers with apparently wt *grk* RNA localization pattern, Anterior—RNA residing at anterior, Diffused—RNA not tightly associated with ON and/or cortex, Defective ON position—ON not positioned at the anterior cortex, *n* — total number of eggs counted. The total may exceed 100% as chambers displaying defective oocyte positioning, could have an additional phenotype.

the localization of *bcd* and *grk* to the anterior appeared quite insensitive to the significant reduction in *ddlc1* expression levels, estimated to be ~25–40% that of wt (data not shown). Thus, if Dynein was required for transport of these mRNAs from the NCs (were they are synthesizes) into the oocyte and for their permanent (*bcd*) or transient (*grk*) anterior localization, as previously suggested (reviewed in [3]), the levels needed must be quite low. Alternatively, a different subunit of Dynein could be involved in these transport and localization events. Two additional *Drosophila* DLCs; Dtctex-1 and Roadblock involved in sperm production [46] and axonal transport [47], respectively, have been reported, however, there is no indication for their involvement in oogenesis.

Close examination of *grk* RNA localization patterns revealed that in a significant fraction of egg chambers from *ddlc1* mutants (20–25%) (some with an apparently normal localization pattern), *grk* RNA was not as tightly localized as in wt and appeared more diffused away from the anterior margin or from the oocyte nucleus (Table 2, Fig. 4G, H, I, K). Such extensive diffusion of *grk* RNA has not been observed with any other mutation that causes mislocalization of *grk* RNA to the anterior margin. In the case of *sqd*, *fs(1)K10*, *vasa* and *kinesin heavy chain* (*Khc*) mutants, for instance, mislocalized *grk* RNA was confined to the anterior [9,25,48]. This *ddlc1* specific phenotype raises the possibility that *ddlc1* may function in tethering *grk* RNA to the cortex and maybe also to the oocyte nucleus**.** Support for such a notion is offered by a previous observation indicating that disruption of the Dynein–Dynactin complex in egg chambers with already localized *grk* mRNA, resulted in the loss of *grk* [25]. Additionally, recent work suggests that Dynein may function in the apical anchoring of pair-rule transcripts in the *Drosophila* embryo [49]. Intriguingly, unlike in wt, where GRK protein was tightly associated with both the cortex and the ON (Fig. 4D), in some of the mutant chambers GRK appeared to be association only with the cortex but mot with the nucleus (Fig. 4M, N). An interesting possibility that could arise from this observation would be that cortical anchoring of the *grk* RNA may be essential for alleviating translational repression and initiating translation, whereas association of *grk* with the ON may not be crucial for this process. Further work will be required to address this issue.

In addition to the RNA localization defects observed with the *ddlc1* mutants, incorrect positioning of the ON was apparent. Unlike in wt where the ON becomes anchored at the AD starting at mid-oogenesis, in a fraction of egg chambers from *ddlc1* mutants (3–7%) the ON resided at various positions along the AP axis (Table 2, Fig. 4I, J, L). Thus, DDLC1 may play a role in the localization and/or anchoring of the ON which is in accordance with previous data demonstrating that the positioning of the ON depended on MT integrity [50] and on a functional Dynein–dynactin complex [25,26]. Similar observations were reported also for nuclear positioning in other systems (Reviewed in [51]). Although the direct interaction of *grk* RNA and DDLC1 supports a role for DDLC1 in the germline, we could not rule out the possibility that function of DDLC1 in the soma may also have some (indirect) effect on localization and nuclear positioning.

How could DDLC1 participate both in transport and in anchoring? Recent data support the idea that under certain conditions Dynein, Kinesin-like proteins and Myosins may function as static anchors [49,52,53]. Recently, studies with *Drosophila* embryos have suggested that apical anchoring of pair-rule transcripts may involve Dynein as a static anchor that was independent of its motor activity [49]. In other systems, actin-associated-motor-proteins of the unconventional Myosin family could, under certain conditions, anchor to actin (Myosin-VI) [53,54], or function in coupling actin to MTs (Myosin-V and Myosin-X) [55,56]. For example, disrupting the function of Myosin-X was shown to interfere with nuclear anchoring in *Xenopus* [56]. Noteworthy, both Myosin-Va and Myosin-X directly bind to LC8 [57,58]. It would have been tempting then to speculate that both the ON and *grk* RNA could anchor to the cortex via association of DDLC1 with members of the unconventional Myosins. Nevertheless, in contrast to the obvious role for the actin cytoskeleton in *osk* RNA anchoring [59], involvement of actin in *grk m*RNA and/or ON anchoring has never been demonstrated. Our results would still support a role for DLC1 in *grk* RNA anchoring and ON positioning, but the exact mechanisms involved are currently elusive.

In conclusion, whereas the importance of the dynein motor complex in *grk* mRNA localization has previously been demonstrated in a number of studies [25,26], our observations suggest, for the first time, a specific requirement for the DLC subunit in the process. Particularly, the results demonstrated that DDLC1 may directly bind to *grk* RNA and suggested a role for DDLC1 in the differential transport of *grk* to the AD. Our observations also raised the possibility that DDLC1 may be required for the anchoring of *grk* RNA to the cortex and to the ON, as well as for correct positioning of the ON.

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