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The *Drosophila* CCR4-NOT complex is required for cholesterol homeostasis and steroid hormone synthesis

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ABSTRACT

CCR4-NOT is a highly conserved protein complex that regulates gene expression at multiple levels. In yeast, CCR4-NOT functions in transcriptional initiation, heterochromatin formation, mRNA deadenylation and other processes. The range of functions for *Drosophila* CCR4-NOT is less clear, except for a well-established role as a deadenylase for maternal mRNAs during early embryogenesis. We report here that CCR4-NOT has an essential function in the *Drosophila* prothoracic gland (PG), a tissue that predominantly produces the steroid hormone ecdysone. Interfering with the expression of the CCR4-NOT components *twin*, *Pop2*, *Not1*, and *Not3* in a PG-specific manner resulted in larval arrest and a failure to initiate metamorphosis. Transcriptome analysis of PG-specific *Pop2*-RNAi samples revealed that *Pop2* is required for the normal expression of ecdysone biosynthetic gene *spookier* (*spok*) as well as cholesterol homeostasis genes of the NPC2 family. Interestingly, dietary supplementation with ecdysone and its various sterol precursors showed that 7-dehydrocholesterol and cholesterol completely rescued the larval arrest phenotype, allowing *Pop2*-RNAi animals to reach pupal stage, and, to a low degree, even survival to adulthood, while the biologically active hormone, 20-Hydroxyecdysone (20E), was significantly less effective. Also, we present genetic evidence that CCR4-NOT has a nuclear function where CCR4-NOT-depleted cells exhibit aberrant chromatin and nucleoli structures. In summary, our findings indicate that the *Drosophila* CCR4-NOT complex has essential roles in the PG, where it is required for *Drosophila* steroid hormone production and cholesterol homeostasis, and likely has functions beyond a mere mRNA deadenylase in *Drosophila*.

1. Introduction

The CCR4-NOT complex is an evolutionarily conserved protein complex found in eukaryotes. The functions of CCR4-NOT have been linked to the regulation of gene expression at nearly all levels, including histone modifications, transcriptional initiation, transcriptional elongation, heterochromatin formation, translational repression as well as the regulation of mRNA degradation (Goldstrohm and Wickens, 2008; Collart, 2016; Miller and Reese, 2012; Shirai et al., 2014; Cotobal et al., 2015). Given the versatility of the CCR4–NOT complex, many aspects of its functions remain unclear. Most studies on CCR4-NOT have been conducted in *Saccharomyces cerevisiae* or human cell culture systems, with only a few studies that have examined this complex in multicellular organisms. In *Drosophila melanogaster*, the main components of CCR4-NOT are conserved, which include the homologs of yeast carbon catabolite repression 4 (CCR4, encoded by *twin*), Pop2 (also known as CAF1), NOT1, NOT2 (encoded by *Regena*), NOT3 and CAF40 (encoded by *Rcd1*) (Temme et al., 2010; Temme et al., 2014; Temme et al., 2004; Sakai et al., 1992; Liu et al., 1997). Two of the components, CCR4/Twin and Pop2 hydrolyze mRNA poly(A) tails through their deadenylase activities (Temme et al., 2004; Niinuma et al., 2016), which leads to translational repression followed by mRNA decay. Two other *Drosophila* poly(A)-specific ribonucleases, the Pan2-Pan3 complex and the poly(A)-specific ribonuclease (PARN), have both specialized and redundant functions when compared to the CCR4-NOT complex, and together control overall mRNA stability (Virtanen et al., 2013; Boeck et al., 1996; Brown et al., 1996). Most studies on *Drosophila* CCR4-NOT have only focused on its role in regulating mRNA stability, especially during oogenesis and early embryonic development before the midblastula transition (Temme et al., 2014; Barckmann and Simonelig, 2013; Joly et al., 2013; Zaessinger et al., 2006; Chicoine et al., 2007; Morris et al., 2005). Before the midblastula transition, embryonic development relies exclusively on maternal mRNAs; hence there is almost no contribution from transcriptional

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regulation. Therefore, studying CCR4-NOT in the context of oogenesis and early embryogenesis provides only limited evidence as to whether the *Drosophila* CCR4-NOT complex has additional roles, such as transcriptional initiation or transcriptional elongation. While the CCR4-NOT complex can target a wide range of transcripts, a plethora of adapter proteins ensures context-specific degradation of select mRNA targets, allowing for tissue- and pathway-specific control (Joly et al., 2013; Chicoine et al., 2007; Chapat and Corbo, 2014). Genome-wide RNA interference (RNAi) screens conducted in two different biological contexts showed that CCR4-NOT complex function is essential for *Drosophila* neuroblast development where loss of *Pop2* or *NOT1* in neuroblasts resulted in animal lethality prior to the adult stage (Neumuller et al., 2011), while regulation of intestinal stem cell homeostasis is not dependent on CCR4-NOT function (Zeng et al., 2015). Thus, CCR4-NOT function is highly regulated and differs drastically depending on biological context, and as such does not act as a global mRNA deadenylase. We were interested in the specific roles of CCR4-NOT in the PG, which is a central endocrine tissue that coordinates multiple development events via the secretion of the steroid hormone ecdysone.

Like all holometabolous insects, the *Drosophila* life cycle has discrete developmental stages, and pulses of ecdysone trigger all developmental transitions such as molting and metamorphosis. In *Drosophila* larvae, ecdysone is synthesized in the principal steroidogenic tissue, the PG, which is part of the ring gland, an endocrine organ composed of three glands. In the PG, tissue-specific expression of ecdysone biosynthetic enzyme genes including *neverland (nvd), spookier (spok), shroud (sro), Cyp6t3, phantom (phm), disembodied (dib) and shadow (sad)* allow the conversion of suitable sterols (*e.g.*, cholesterol) to ecdysone (Ono et al., 2006; Yoshiyama et al., 2006; Ou et al., 2011; Niwa et al., 2010, 2005, 2004; Warren et al., 2004, 2002; Chavez et al., 2000a, 2000b). *spok, sro, Cyp6t3, phm, dib and sad* are commonly referred to as the Halloween genes. To ensure that these developmental transitions occur at the appropriate time, the PG acts as a decision-making center to integrate systemic and environmental cues into an ecdysone pulse that advances development when the proper conditions are met. Here, we report a requirement for the CCR4-NOT complex for the production of ecdysone and maintaining cholesterol homeostasis. PG-specific RNAi against five out of seven CCR4-NOT components resulted in larval arrest mainly in the third instar (L3), a phenotype consistent with a block in ecdysone production. Transcriptional profiling via RNA-Seq of PG-specific *Pop2*-RNAi ring gland samples revealed that ecdysone and cholesterol pathways were particularly affected, and that cholesterol feeding could rescue the larval arrest phenotype of *Pop2*-RNAi animals, strongly supporting the idea that CCR4-NOT has specialized tissue-specific functions.

2. Results & discussion

2.1. PG-specific disruption of CCR4-NOT function caused phenotypes consistent with ecdysone deficiencies

To determine the importance of the CCR4-NOT complex in the PG, we used a PG-specific Gal4 driver (*phm22*-Gal4, referred to as PG >) to express *UAS*-RNAi transgenes targeting all seven CCR4-NOT subunits individually. For each gene, we tested all available TRiP-RNAi lines from Bloomington Stock Center and also included the results from a previously published genome-wide PG-specific RNAi screen using VDRC RNAi lines (Table 1) (Danielsen et al., 2016; Dickson et al., 2007). For *Pop2*, two non-overlapping RNAi lines (PG > *Pop2*-RNAi^{1} and PG > *Pop2*-RNAi^{2}) caused comparable developmental arrest phenotypes, which validated the specificity of the RNAi constructs (Fig. 1A). 100% of the PG > *Pop2*-RNAi^{1} and ~93% of the PG > *Pop2*-RNAi^{2} animals failed to pupariate, and remained 3rd instar (L3) larvae for several weeks, which resulted in substantially increased body sizes. This L3 arrest phenotype is commonly observed when the major ecdysone pulse that triggers the onset of metamorphosis fails to occur (Fig. 1B). Of the ~7% PG > *Pop2*-RNAi^{2} larvae that attempted puparium formation, none completed metamorphosis and thus failed to eclose as adults (Fig. 1B). As for the other CCR4-NOT components, at least one RNAi line targeting *Twin*, *Not1*, *Not3*, or *Not4* was also found to cause L3 arrest (Table 1). Taken together, these results indicated that the CCR4-NOT complex is essential for PG function, possibly by contributing to regulating ecdysone production.

2.2. Transcriptome analysis shows that two ecdysone biosynthetic genes are downregulated

Given the potential requirement of the CCR4-NOT complex at nearly all levels of gene expression, we performed transcriptional profiling of hand-dissected ring glands via RNA-Sequencing (RNA-Seq). We collected ring glands from PG > *Pop2*-RNAi^{1} and PG > w^{1118} control animals at 24 h after the 2nd to 3rd instar molt. We chose this time point because ring glands from these larvae are large enough for dissection while larvae undergo minimal physiological changes compared to later time points closer to puparium formation. We used *Pop2*-RNAi^{1} to disrupt *CCR4-NOT* function because the RNAi phenotype was 100% penetrant and because the phenotypes were validated by a second independent RNAi line. For each sample, we dissected 50 ring glands, which should average out any individual timing differences between animals. Total RNA samples were enriched for mRNAs by selectively depleting rRNAs, followed by Illumina sequencing.

The L3 arrest phenotype in *Pop2*-RNAi animals suggested a defect in ecdysone biosynthesis, and we were thus curious as to whether the RNA-Seq results would reveal any reduction in the expression of the ecdysone biosynthetic enzyme genes. Interestingly, two genes critical for ecdysone biosynthesis were among the top 50 downregulated genes, namely *spookier* (*spok*) and *neverland* (*nvd*), ranked #31 (8.7-fold down) and #41 (7.7-fold down), respectively (Fig. 1C, Table S1, and Table S2). However, only *spok* was confirmed to be significant ($p < 0.05$) for both the original RNA-Seq result and the qPCR validation data (Fig. 1D). For validation via qPCR, we used the weaker *Pop2*-RNAi^{2} line at an earlier developmental time point (0 h L3). Next, we investigated whether these changes in mRNA levels translated into corresponding reductions of protein levels of Nvd and Spok. We found via immunostaining that Spok is strongly reduced in *Pop2*-RNAi^{1} PG cells, consistent with the mRNA expression levels (Fig. 1E). However, we did not observe a significant reduction of Nvd upon *Pop2* knockdown (Fig. 1E). A possible explanation is that the net effect of a (albeit non-significant) reduction in *nvd* transcript levels vs. increased *nvd* transcript stability (due to loss-of-*Pop2*, see next paragraph) results in overall similar protein levels. Alternatively, while the immunodetection and RNA-Seq experiments were conducted at the same developmental time point (96 h AEL = 24 h post L2/L3 molt), there may be a lag between reduction in transcript and protein levels due to protein perdurance. We also found significant transcriptional upregulation for *sro* and *dib* via qPCR, while *phm* was not significantly changed (Fig. 1D). To test whether this corresponded to elevated protein levels in the PG, we examined Sro and Phm via immunofluorescence. Of the two, only Sro appeared to have moderately increased protein levels, again mirroring the mRNA expression data. We conclude that the ecdysone deficiency phenotype caused by PG-specific RNAi of *Pop2* was likely caused by a reduction in *spok* function.

If Pop2 only functions as a mRNA deadenylase, one would expect a stabilization of direct target mRNAs in cells with impaired Pop2 function. Therefore, genes that register as downregulated in the RNA-Seq data, such as *nvd* and *spok*, are likely the result of indirect effects, or a combination of direct and indirect effects. Likewise, upregulated genes could be the result of direct stabilization via loss-of-Pop2 or again caused by direct and/or indirect effects. To distinguish these possibilities, we carried out poly(A) tail-length assays to test whether Pop2 depletion affects poly (A) tail lengths of six

Table 1
PG-specific RNAi of CCR4-NOT complex components resulted in ecdysone related phenotypes[a].

Gene name	PG-specific RNAi phenotype	Affecting Poly (A) length when disrupted	Tested RNAi lines with phenotype	Tested RNAi lines without phenotype
CCR4 (*twin*)	L3 arrest	Yes[b]	V104442	BL32490, BL32901
Pop2	L3 arrest	Yes[b,c]	BL52947[d], BL30492[d]	n.a.
NOT1	L3 arrest	Yes[c]	V106587, BL32836	BL31696, BL28681
NOT2 (*Regena*)	NOP	Weak[b,c]	n.a.	V20826, BL35460, BL57549
NOT3	L3 arrest	Yes[c]	V105990, BL34966[e]	BL33002, BL50517
NOT4	L3 arrest	No[c]	V110472	BL42513
CAF40 (*Rcd-1*)	NOP	No[c]	n.a.	V101462, BL67987

NOP: No obvious phenotype.
V: VDRC transgenic lines.
BL: transgenic lines from Bloomington Stock Center.
n.a: not applicable.
[a] *phm22-Gal4* was used for driving the expression of all RNAi constructs.
[b] according to Temme, 2004.
[c] according to Temme, 2010.
[d] only BL52947 and BL30492 are non-overlapping RNAi lines.
[e] Knocking down *NOT3* in the PG using the transgenic line BL34966 resulted in pupal lethality.

Halloween genes. For this, we tagged ring gland mRNAs with oligo-G/I bases followed by reverse-transcription into cDNA using the tag as the priming site. PCR was then performed by using a universal reverse primer complementary to the tag and a gene-specific primer, resulting in ~150–300 bp fragments (Table S3) (Fig. 1F). As controls, we used the same gene-specific primer coupled to a second downstream gene-specific primer that binds upstream of the poly (A) tail. This will give rise to an amplicon without the poly (A) tail, and allows estimation of the poly (A) tail length. We observed lengthening of the poly (A) tail for *spok*, *phm* and *dib* transcripts in a *Pop2*-RNAi[1] background relative to controls (Fig. 1F), indicating that these three Halloween transcripts are direct targets of Pop2. Since *spok* is showing a poly(A) upshift, but strongly downregulated both at the transcriptional and protein levels, we conclude that while *spok* is a direct target of Pop2, there might a superimposed transcriptional effect in a *Pop2* loss-of-function background. The results for *nvd* (repeated in two independents assays) were inconclusive. While *nvd* showed a clear upshift, a second band with little or no increased length was also present. In contrast, both *sro* and *sad* showed no lengthening of the poly(A) tail, indicating that the CCR4-NOT complex regulates only select Halloween transcripts.

Since *spok* transcript levels are downregulated, we see two possibilities that may explain this. First, it is conceivable that the CCR4-NOT complex acts directly as a transcriptional modulator of *spok* expression, in line with reported nuclear functions of CCR4-NOT in yeast (Cotobal et al., 2015; Grigull et al., 2004). However, it is equally possible that mRNAs encoding transcriptional regulators of the Halloween genes are regulated by CCR4-NOT, and the resulting stabilization of such transcriptional regulators upon *Pop2* knockdown would then indirectly alter Halloween gene expression. In fact, many such transcription factors have been identified (Ou and King-Jones, 2013), the most recent example being Kr-h1, which negatively regulates the transcription of the Halloween genes (Zhang et al., 2018). Interestingly both *nvd* and *spok* are strongly downregulated by Kr-h1 overexpression, suggesting that both genes are coordinately regulated by this and possibly other repressors. To conclude, regardless of the complex regulation and possible crosstalk between the CCR4-NOT and transcriptional regulators, the net outcome of PG > *Pop2*-RNAi is the specific reduction of Spok.

2.3. The developmental arrest caused by PG-specific Pop2 knockdown was partially rescued by 7DC and cholesterol feeding

Nvd catalyzes the first step of ecdysone biosynthesis where it converts cholesterol to 7-dehydrocholesterol (7DC) (Yoshiyama et al., 2006). Spok plays a crucial role in the “Black Box”, which consists of several not yet characterized conversion steps. However, the net result of the Black Box is that 5β-ketodiol (5βkd) is derived from 7DC (Ono et al., 2006). To test the idea that the ecdysone deficiency phenotype observed in PG > *Pop2*-RNAi animals was caused directly by reduced levels of *spok* and possibly *nvd*, we used fly media supplemented with either 7DC, 5βkd or the biologically active form of ecdysone (20E = 20-Hydroxyecdysone). Remarkably, 7DC alone rescued the L3 arrest phenotype and enabled nearly 80% of larvae to reach the pupal stage. The rescue of the L3 arrest phenotype by 7DC was observed in both PG > *Pop2*-RNAi lines (Figs. 2A and B), demonstrating the specificity of the results. Intriguingly, compared to 7DC, the rescue by 20E (the end product of ecdysone biosynthesis) was much less pronounced and not significant in PG > *Pop2*-RNAi[1] lines. Similarly, 20E did not rescue PG > *Pop2*-RNAi[2] animals (Figs. 2A and B). Moreover, combining 7DC and 20E significantly lowered pupal survival compared to 7DC alone (Figs. 2A and B), suggesting that 20E has some adverse effect or was neutralizing 7DC efficiency in a PG > *Pop2*-RNAi background. It should be noted that 20E-supplementation rescues other Halloween gene loss-of-function animals (including PG > *spok*-, *sro*-, *Cyp6t3*-, *phm*-, *dib*- and *sad*-RNAi lines) very effectively and is not considered toxic *per se* (Ou et al., 2011; Niwa et al., 2010; Ou et al., 2016). For instance, 20E can rescue the L2 arrest phenotype of PG > *sro*-RNAi larvae all the way to adulthood (~31% adult survival) (Fig. 2D). Interestingly, we recently reported that *séance* mutants selectively affect *nvd* expression, and can be effectively rescued with 7DC, but not 20E, consistent with our observation for *Pop2*-RNAi lines (Uryu et al., 2017). When we used 5βkd-supplementation, we saw no further improvement, as most of the PG > *Pop2*-RNAi[1] animals still could not develop beyond the larval stages (Fig. 2C). Thus, similar to 20E, rescue of PG > *Pop2*-RNAi animals via 5βkd was inferior to 7DC. A likely explanation for this is that 7DC feeds into pathways other than ecdysone production, and that Pop2 function is required for these undefined roles.

Given that 7DC is a cholesterol metabolite, it is of interest to note that three Niemann-Pick disease type C (NPC) genes, *npc2f*, *npc2d*, and *npc2c* were upregulated 6.8-fold (ranked #41), 5.1-fold and 3.9-fold respectively in *Pop2*-RNAi samples compared to control ring gland samples (Table S4 and Table S5). NPC proteins play critical roles in the intracellular transport of cholesterol and cholesterol metabolites, raising the idea that 7DC-supplementation compensates for this aberrant upregulation of NPC genes. Alternatively, NPC2 upregulation may compensate for an issue with cholesterol uptake or transport in *Pop2*-RNAi cells, an issue that could also be rescued by 7DC feeding since 7DC is the immediate metabolites of cholesterol. Consistent with the coordinated upregulation of three NPC2 genes, “intracellular cholesterol transport” was identified as one of the functional associa-

tion subgroups in the functional interaction analysis (see Section 2.4), where we used a subset of genes from our RNA-Seq data with > 3-fold upregulation in *Pop2*-RNAi ring glands compared to controls (Fig. 3A). To test whether cholesterol supplementation was as effective as 7DC, we carried out feeding experiments using fly media supplemented with cholesterol. Remarkably, cholesterol rescued ~33% of the *Pop2*-RNAi[1] animals to the pupal stage, and ~3% of the RNAi larvae were even rescued to adulthood, a result that could be confirmed with the second *Pop2*-RNAi line (Figs. 2A and B). Overall, cholesterol was as effective as 7DC in rescuing the larval arrest phenotypes, indicating that *nvd* is functioning normally in PG > *Pop2*-RNAi animals since Nvd is required for converting cholesterol to 7DC. In conclusion, three NPC2 genes

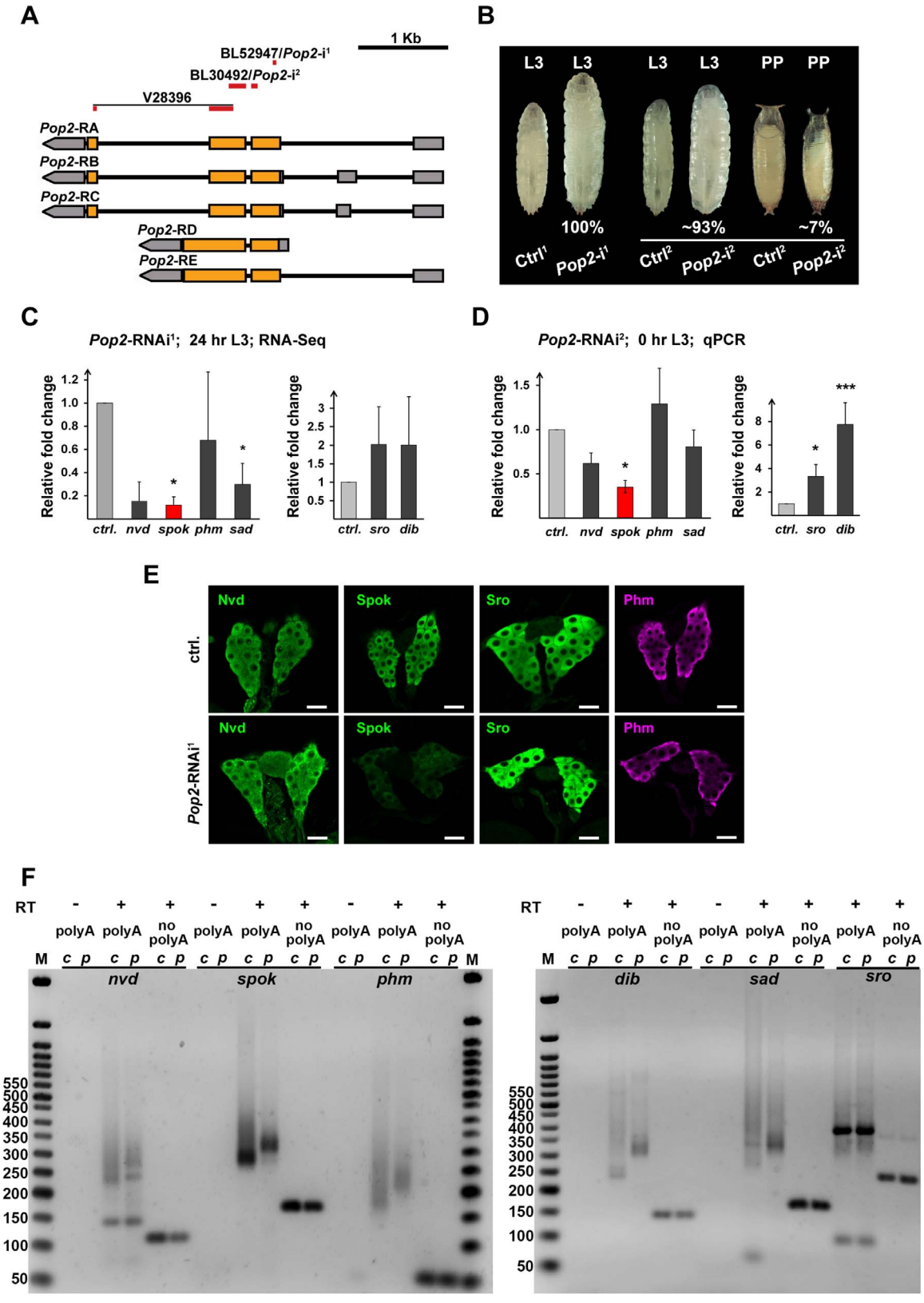

Fig. 1. *Pop2* function is required in the PG for ecdysone biosynthesis and metamorphosis. (A) *Pop2* gene structure (mRNA isoforms) and target sites of the RNAi lines. Orange indicates coding exons, and gray corresponds to non-coding exons. Lines represent introns. Red boxes indicate dsRNA coverage in RNAi lines. V28396: Vienna Drosophila Research Center line No. 28396. BL52947: TRiP (Transgenic RNAi Project, Bloomington Stock Center No. 52947). BL30492: TRiP line, Bloomington No. 30492. (B) Phenotypes caused by PG-specific *Pop2*-RNAi. Ctrl[1]: *phm22* > BL36304 (parental line for TRiP RNAi lines on 3rd chromosome). Ctrl[2]: *phm22* > BL36303 (parental line for TRiP RNAi lines on 2nd chromosome). *Pop2*-RNAi[1]: *phm22* > *Pop2*-RNAi (BL52947). *Pop2*-RNAi[2]: *phm22* > *Pop2*-RNAi (BL30492). L3: 3rd instar larvae; PP: prepupae. (C) Relative expression levels of six ecdysone biosynthetic genes in PG > *Pop2*-RNAi ring glands compared to controls (*phm22* > w^{1118}) based on the RNA-Seq analysis. Ring glands were collected at 24 h L3. *Pop2*-RNAi[1]: *phm22* > *Pop2*-RNAi (BL52947). Error bars represent standard deviation. $*p < 0.05$ (based on one-way ANOVA). (D) qPCR results showing the expression of the ecdysone biosynthetic genes. Ring glands were collected at 0 h L3. Relative fold change was determined by comparing the expression in the RNAi to that of the control for each gene tested. *Pop2*-RNAi[2]: *phm22* > *Pop2*-RNAi (BL30492). Error bars represent 95% confidence interval. $***p < 0.001$ and $*p < 0.05$ (based on Student's *t*-test). (C and D) Red bar indicates result that is confirmed by RNA-Seq, qPCR analysis as well as immunostaining. (E) Immunostaining of ring glands from control (*phm22* > BL36303) and *Pop2*-RNAi (*phm22* > BL52947) at 96 h after egg lay (AEL) with antibodies against Neverland (Nvd, green), Spookier (Spok, green), Shroud (Sro, green) and Phantom (Phm, magenta). Scale bar: 25 μm. (F) Comparison of poly(A) tail-lengths in six Halloween gene transcripts. C: *phm22* > BL36303 (ctrl.); P: *phm22* > *Pop2*-RNAi (BL52947). Reverse transcription (RT +); No RT negative control (-); poly(A) tail PCR amplifying the end of the transcript together with the Poly (A) tail (PolyA); gene-specific PCR amplifying only the end of the transcript (no polyA); M: 50 bp DNA Ladder (NEB N3236S), numbers indicate the size in base pair (bp).

linked to intracellular sterol transport appear to be misregulated upon *Pop2*-RNAi, indicating deregulation of cholesterol homeostasis, and consistent with this, both cholesterol and its immediate downstream metabolite, 7DC, are significantly more effective than 20E in rescuing PG-specific *Pop2*-depleted animals.

2.4. Term enrichment analysis of PG > Pop2-RNAi RNA-Seq data

When we analyzed significantly altered genes in our RNA-Seq datasets, we noticed that the number of upregulated genes in PG > *Pop2*-RNAi ring glands were consistently higher than the downregulated genes, regardless of which cut-offs we used (Table S6). For instance, the number of > 2.5-fold upregulated genes more than doubled the number of > 2.5-fold downregulated genes. This trend is consistent with the idea that Pop2 might primarily act to negatively regulate mRNA levels through deadenylation, and a decrease in RNA degradation would thus register as a higher percentage of upregulated genes.

Next, we generated functional association networks of differentially expressed genes using STRING database (Szklarczyk et al., 2015) to identify processes that are affected by the loss of Pop2 function. We then grouped genes within the interaction networks according to their gene ontology (GO) terms from DAVID GO and Flybase (Huang et al., 2009). For the 197 > 3.5-fold upregulated genes in the *Pop2*-RNAi ring gland

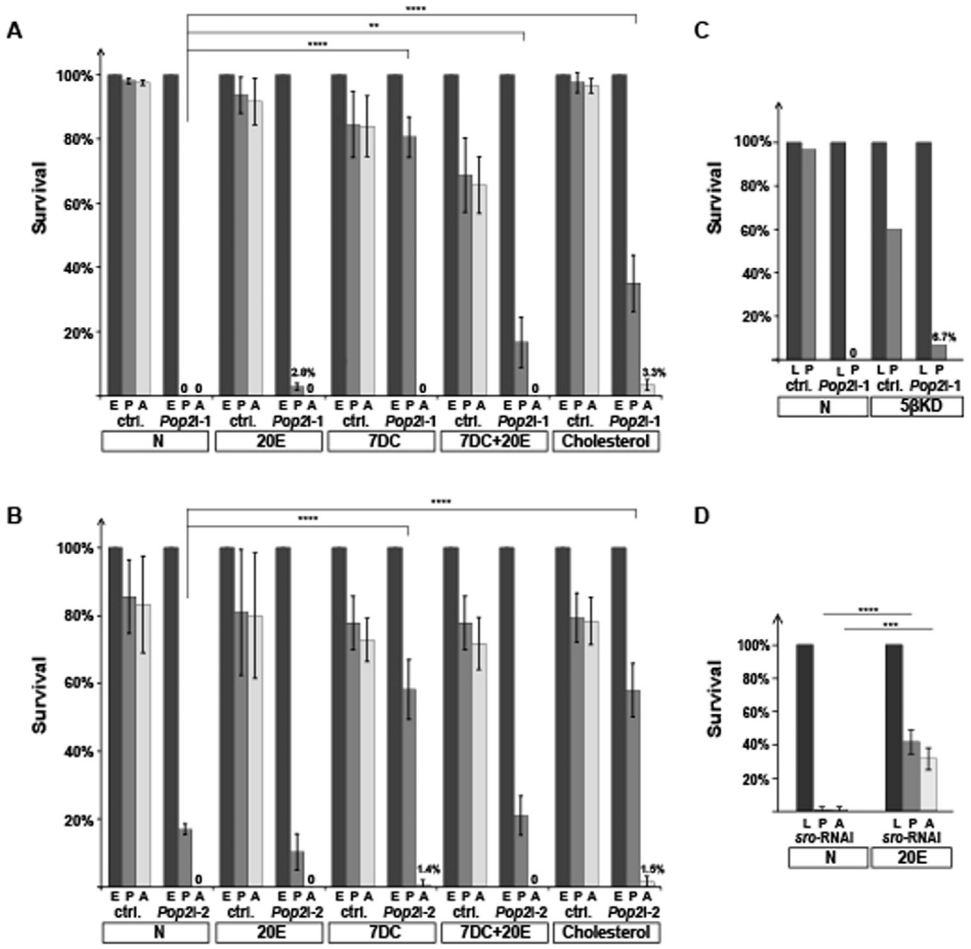

Fig. 2. Larval arrest phenotype caused by PG > *Pop2*-RNAi can be partially rescued by 7DC and cholesterol feeding. Percentage of animals survived to the indicated stages under different conditions and genotypes. E: embryo, L: 1st instar larvae, P: pupae, A: adult. N: Nutri-Fly food without any sterols, 20E: 20-Hydroxyecdysone, 7DC: 7-dehydrocholesterol and 5βKD: 5β-ketodiol. Error bars represent standard deviation. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$ (one-way ANOVA). (A-B) *Pop2i*-1: *phm22* > *Pop2*-RNAi (BL52947). ctrl.: *phm22-Gal4* crossed to Bloomington stock #36304 (the parental line for the BL52947). (C) *Pop2i*-2: *phm22* > *Pop2*-RNAi (BL30492). ctrl.: *phm22-Gal4* crossed to Bloomington stock #36303 (BL30492). (D) *sro*-RNAi: *phm22* > *shroud*-RNAi (VDRC #50111).

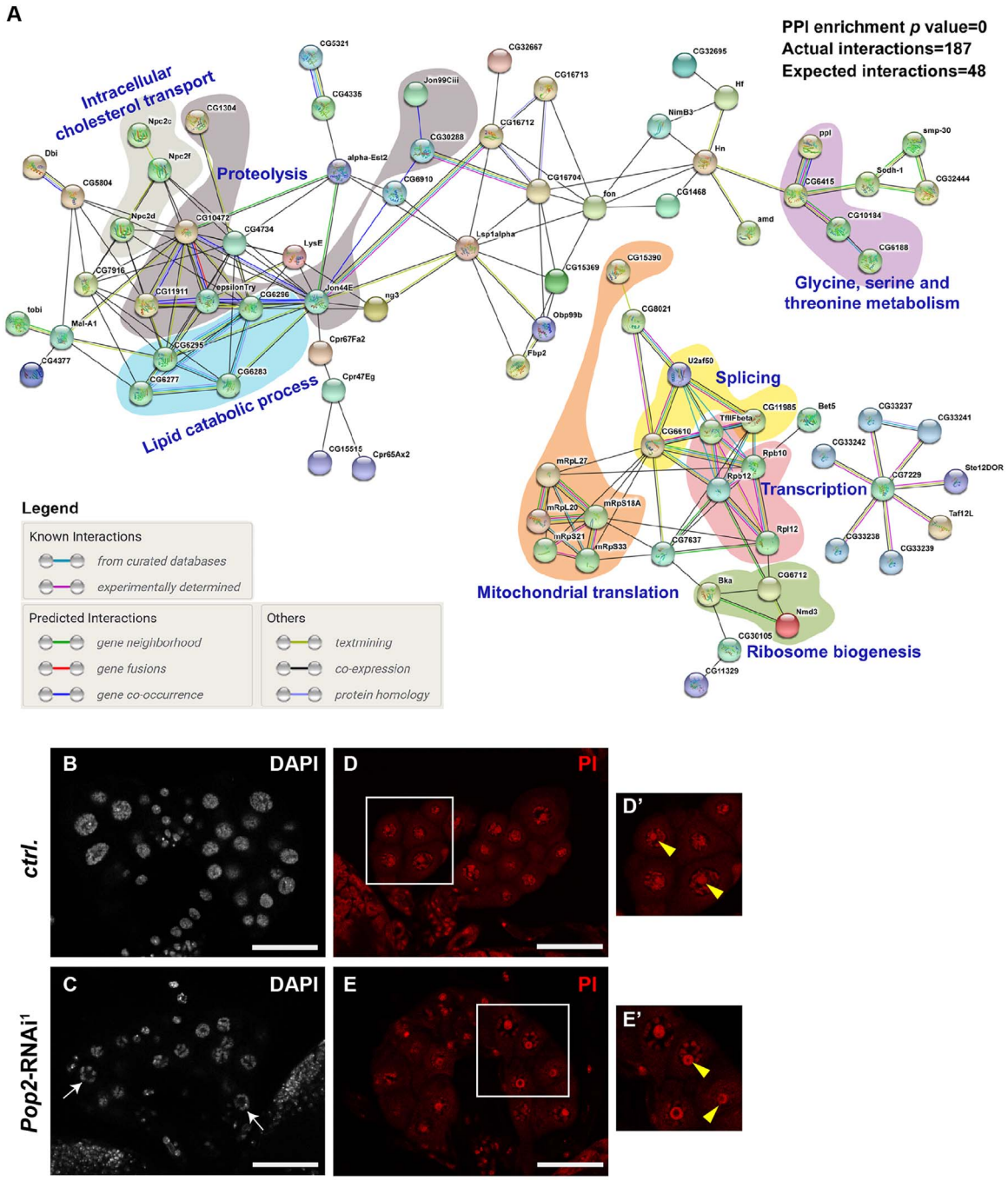

Fig. 3. Functional association analysis of the upregulated genes upon PG-specific *Pop2* knockdown suggests an alteration in global transcription and ribosome biogenesis. (A) Interaction network of the > 3.5-fold upregulated genes generated using STRING database. Several functional subgroups within the big network were highlighted using different color shading. The large node size indicates the availability of protein structure information. Node colors have no particular meaning. Line colors indicate different types of evidence for the interaction (see legend). Non-associated genes were not included in the figure. PPI enrichment *p* value: protein-protein interaction enrichment *p* value (the smaller, the more enriched). Actual interactions: number of protein interactions. Expected interactions: number of protein interactions that would be expected for a random set of proteins of similar size, drawn from the genome. (B-E) Nucleic acid staining reveals changes of chromatin architecture in *Pop2*-RNAi PG cells. Confocal microscopy of ring glands with DAPI (B and C) and PI (D and E) staining. White arrows indicate the petal-like arrangement of the chromatin. Yellow arrowheads point to the potential nucleoli. (D' and E') Zooms of white box area in (D) and (E). *ctrl.*: *phm22-Gal4* > BL36304. *Pop2*-RNAi[1]: *phm22* > *Pop2*-RNAi (BL52947). Scale bar: 50 μm.

compared to control samples, this analysis revealed genes associated with several functional groups: including "transcription" (as well as "splicing" and "ribosome biogenesis"), "mitochondrial translation", "intracellular cholesterol transport", "proteolysis", "lipid catabolic process" and "glycine/serine/threonine metabolism" (Fig. 3A). To complement this functional interaction analysis, we also carried out gene ontology (GO) term and KEGG pathway enrichment analysis via both DAVID GO and STRING database using a less stringent cut-off of > 2.5-fold upregulated genes (total 536 genes). Consistent with the results of the functional association study, genes that are involved in transcription as well as genes encoding mitochondrial ribosomal proteins and mitochondrial respiratory chain complex III component were significantly overrepresented (Table S7).

For the total of 196 > 3-fold downregulated genes, several functional groups were also identified, including: "translation", "mitochondria", "splice site/transcriptional start site selection", "actin filament-related" and "proteolysis" (Fig. 4). The term enrichment analysis using the > 2-fold (total 296 genes) downregulated genes confirmed

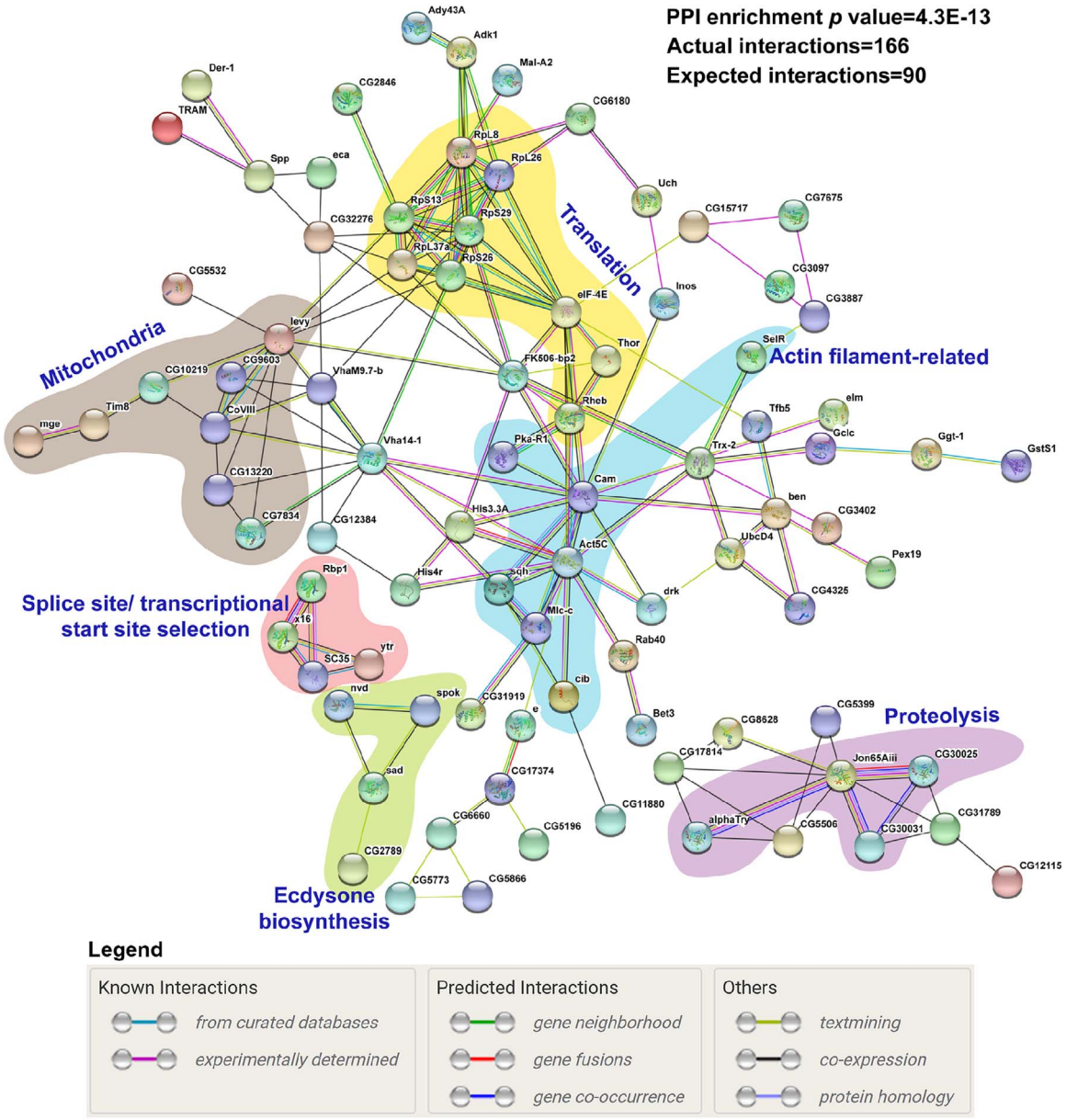

Fig. 4. Functional association networks of the downregulated genes in PG > *Pop2*-RNAi ring glands. Interaction network of > 3-fold downregulated genes generated using STRING database. Several functional subgroups within the big network were highlighted using different color shading. The large node size indicates the availability of protein structure information. Node colors have no particular meaning. Line colors indicate different types of evidence for the interaction (see legend). Non-associated genes were not included in the figure. PPI enrichment *p* value: protein-protein interaction enrichment *p* value (the smaller, the more enriched). Actual interactions: number of protein interactions. Expected interactions: number of protein interactions that would be expected for a random set of proteins of similar size, drawn from the genome.

that genes involved in “cytoplasmic translation” (5 out of 8 genes are ribosomal genes) and “mitochondrial protein complex” were significantly enriched among differentially downregulated genes (Table S7). Interestingly, two genes linked to “cytoplasmic translation” encode proteins acting in the target of rapamycin (TOR) signaling pathway, namely *Rheb* and the eukaryotic translation initiation factor (eIF) subunit 4E-binding protein (4E-BP) gene (*Thor* in *Drosophila*), which are both ~3.9-fold downregulated in *Pop2*-RNAi ring glands (Table S2 and Fig. 4). This is intriguing because recently it was shown that TOR not only acts in amino acid sensing but is also activated by cellular cholesterol (Zhang et al., 2000), consistent with the idea that CCR4-NOT has hitherto unknown links to cellular cholesterol homeostasis. In line with the misregulation in *4E-BP* and *Rheb*, *Pop2*-RNAi ring glands displayed growth defects in a nutrient-dependent manner (Figure S1). *Pop2*-RNAi ring glands were normal when larvae were reared on yeast but had a reduced size when we used the Nutri-Fly Bloomington formula or a standard cornmeal-based recipe. We would like to point out that larvae with smaller ring glands had equal (standard cornmeal) or better survival rates (Nutri-Fly) compared to those with normal-sized ring glands (yeast).

2.5. *Pop2* function is important for chromatin and nucleolus structure

When we analyzed PG nuclei via DAPI staining to assess the ring gland morphology, we found that *Pop2*-RNAi animals exhibited uneven and fragmented DAPI signals (Fig. 3B-C), resulting in a region with reduced DAPI staining. In some nuclei, the chromatin was arranged in a way that resulted in a petal-like appearance. In wild-type cells, nuclear zones that stain weakly for DAPI correspond to the nucleolus, where rRNA synthesis and ribosome biogenesis occurs. Since this observation was consistent with the functional association analysis (“ribosome biogenesis”, see Fig. 3A), we carried out propidium iodide

(PI) staining to visualize the nucleolus (because PI also binds RNA in addition to DNA) (Suzuki et al., 1997). Interestingly, PI staining of *Pop2*-RNAi PG cells revealed some nucleoli that had a distinct "hollow sphere" appearance (Fig. 3E, yellow arrowheads), a phenotype we never observed in controls. Chromatin fragmentation and compaction were also apparent in PI staining of *Pop2*-RNAi nuclei (Fig. 3C-E), consistent with the DAPI results. These nuclear phenotypes of *Pop2*-RNAi PG cells are consistent with finding in fission yeast that showed involvement of CCR4-NOT in heterochromatin formation (Cotobal et al., 2015). It, therefore, appears likely that Pop2 has a direct nuclear function that is critical for accessibility of the transcriptional machinery to DNA. We conclude that it is very likely that the *Drosophila* CCR4-NOT complex also has additional functions beyond its well-documented deadenylase activity.

3. Conclusion

Our study revealed that the function of the CCR4-NOT deadenylase complex is essential for ecdysone biosynthesis and a normal nuclear structure in the PG. Disrupting CCR4-NOT function specifically in the PG via *Pop2*-RNAi strongly reduced mRNA and protein levels of the ecdysone enzyme gene *spok*. We also present here the first transcriptome analysis of a *Drosophila* tissue with impaired CCR4-NOT function. Term enrichment analysis of these genomic data suggested a defect in cholesterol homeostasis, which is consistent with the finding that the L3 arrest phenotype in PG > *Pop2*-RNAi animals was rescued by cholesterol and 7DC feeding (Figs. 2 and 3). Moreover, *Pop2*-depletion in the PG appeared to affect the structure of both chromatin and the nucleolus, suggesting CCR4-NOT also has nuclear functions, consistent with previous reports in yeast. We conclude that CCR4-NOT has tissue-specific roles and is likely a multifunctional protein complex that has functions beyond the mRNA deadenylase in *Drosophila*.

4. Materials & methods

4.1. Fly stocks and fly crosses

Drosophila melanogaster stocks were maintained on standard agar-cornmeal medium at 25 °C. *phm22-Gal4* was obtained from Mike O'Connor's lab. All the UAS-RNAi lines used for this study were purchased from the Bloomington Stock Center (see Table 1).

4.2. Poly(A) Tail-Length Assay

100 ng RNA was extracted from ring glands using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The Poly (A) length assay was carried out using the Poly(A) Tail-Length Assay Kit (Thermo Fischer Scientific, #764551KT). Gene-specific primers amplifying the 3' end of each transcript were custom-designed and are listed in Table S3. The size of each amplicon exclusive of the poly (A) is also listed in Table S3, which can be used to compare its length to the amplicon with the poly (A) tail, thus allowing to assess the length of the poly (A) tail. PCR products were separated on a 2.5% agarose / 1X TAE gel at 120 V for 2.5 h. DNA was visualized with SYBR Safe DNA Gel Stain (Thermo Fischer Scientific #S33102).

4.3. Immunolocalization and nuclear stains

Immunofluorescence of ring glands for Halloween enzyme detection was performed as previously described (Komura-Kawa et al., 2015). Primary antibodies used were guinea pig anti-Nvd at 1:200 (Ohhara et al., 2015), rabbit anti-Phm at 1:200 (Parvy et al., 2005), guinea pig anti-Spok at 1:200 (Gibbens et al., 2011) and guinea pig anti-Sro at 1:1000 (Shimada-Niwa and Niwa, 2014). Secondary antibodies: goat anti-guinea pig Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555 (Life Technologies) were diluted at 1:500. Propidium Iodide (PI) or DAPI were used at 0.5 μg/ml and stained for 10 min.

4.4. RNA-Seq analysis

60 ring glands were used for each sample. Each condition was tested with two biological replicates. RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA integrity was analyzed using Agilent RNA 6000 Nano kit run on a 2100 Bioanalyzer Instrument. Isolated RNA was quantified using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific). 30 ng of total RNA was used for cDNA library construction using the Encore Complete RNA-Seq IL Multiplex System 1–8 (NuGEN, part no. 0312) and Encore Complete RNA-Seq IL Multiplex System 9–16 (NuGEN, part no. 0313). The final library for each sample was analyzed by an Agilent Bioanalyzer DNA 1000 kit and quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). Next-generation sequencing and raw data analysis were carried out as described before in Di Cara and King-Jones, 2016 (Di Cara and King-Jones, 2016). Reads Per Kilobase of transcript per Million mapped reads (RPKM) was used to represent the relative abundance of each transcript. Relative fold change of expression for each transcript was determined by the ratio of RPKM in PG > *Pop2*-RNAi samples to that in controls (PG > w^{1118}). RPKM > 1 was set as a cut off for a reliable read count for each transcript. The functional association networks analysis was performed using STRING database (Szklarczyk et al., 2015). Gene ontology statistics was performed with DAVID (Huang et al., 2009).

4.5. qPCR analysis

qPCR data are based on three biological samples each tested in triplicate. 40 ring glands were collected for each sample. Ring glands were dissected in pre-chilled PBS, immediately transferred to TRizol reagent (Ambion, Life Technologies), and then flash frozen in liquid nitrogen for short-term storage. RNA was extracted using the RNeasy MiniKit (Qiagen) following the manufacturer's instructions. 100–200 ng of total RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. qPCR analysis was carried out using KAPA SYBR Green PCR master mix (KAPA Biosystems) with a primer concentration of 400 nM each. qPCR reactions were performed on a StepOnePlus instrument (Applied Biosystems) using the ΔΔCT mode. Samples were normalized to *rp49*. All primer sequences can be found in Table S8.

4.6. Rescue by dietary sterol supplementation

Nutri-Fly™ Bloomington Formulation was used for the feeding experiments. The Nutri-Fly food was supplied with various sterols dissolved in ethanol to a final concentration of 75 μg/ml for cholesterol, 75 μg/ml for 7-dehydrocholesterol (7DC), 5 mg/ml for 5βKD and 200 μg/ml for 20E with a final concentration of 2% ethanol in the media. The control food was prepared similarly with only a final concentration of 2% ethanol. Sixty embryos were transferred to each vial, allowed to develop at 25 °C and the phenotypes of the larvae were scored.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:http://dx.doi.org/10.1016/j.ydbio.2018.08.012.

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