Divergent Functions Through Alternative Splicing: The *Drosophila* CRMP Gene in Pyrimidine Metabolism, Brain, and Behavior

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ABSTRACT DHP and CRMP proteins comprise a family of structurally similar proteins that perform divergent functions, DHP in pyrimidine catabolism in most organisms and CRMP in neuronal dynamics in animals. In vertebrates, one DHP and five CRMP proteins are products of six genes; however, *Drosophila melanogaster* has a single *CRMP* gene that encodes one DHP and one CRMP protein through tissue-specific, alternative splicing of a pair of paralogous exons. The proteins derived from the fly gene are identical over 90% of their lengths, suggesting that unique, novel functions of these proteins derive from the segment corresponding to the paralogous exons. Functional homologies of the *Drosophila* and mammalian CRMP proteins are revealed by several types of evidence. Loss-of-function *CRMP* mutation modifies both Ras and Rac misexpression phenotypes during fly eye development in a manner that is consistent with the roles of CRMP in Ras and Rac signaling pathways in mammalian neurons. In both mice and flies, CRMP mutation impairs learning and memory. CRMP mutant flies are defective in circadian activity rhythm. Thus, DHP and CRMP proteins are derived by different processes in flies (tissue-specific, alternative splicing of paralogous exons of a single gene) and vertebrates (tissue-specific expression of different genes), indicating that diverse genetic mechanisms have mediated the evolution of this protein family in animals.

BACTERIAL hydantoinases, dihydropyrimidinase (DHP), and collapsin response mediator proteins (CRMPs) comprise a family of proteins, which exhibit highly conserved structures, yet carry out divergent functions. DHP is found in most eukaryotes and catalyzes the second step of reductive pyrimidine catabolism (reviewed in Schnackerz and Dobritzsch 2008). On the other hand, CRMPs appear to be limited to nervous systems of metazoans, where they mediate a variety of processes: semaphorin signal transduction in axonal growth cones, cytoskeletal dynamics, neuronal polarity, and modulation of neurotransmitter release (reviewed in Schmidt and Strittmatter 2007; Hou *et al.* 2008; Chi *et al.* 2009; Yamashita and Goshima 2012).

All members of the DHP/CRMP family function *in vivo* as homotetramers and, possibly, heterotetramers (Wang and Strittmatter 1997; Deo *et al.* 2004); their resolved structures are extraordinarily similar (Abendroth *et al.* 2002a,b; Xu *et al.* 2003; Deo *et al.* 2004; Lohkamp *et al.* 2006; Stenmark *et al.* 2007). A key functional distinction among these proteins is that DHPs are zinc-coupled dihydropyrimidine hydrolases, whereas no comparable hydrolase activity has been shown for vertebrate CRMPs, which lack one or more essential zinc-binding site residues found in DHP proteins (Hamajima *et al.* 1998; Wang and Strittmatter 1997; Takemoto *et al.* 2000).

Vertebrate CRMPs mediate a variety of neuronal growth cone dynamics through SEMA3A/NP1/PlexA signal transduction and perhaps other signaling pathways (reviewed in Schmidt and Strittmatter 2007; Hou *et al.* 2008; Yamashita and Goshima 2012). The roles of CRMP in these pathways are regulated by phosphorylation of the protein, primarily within its C-terminal region (Amano *et al.* 2000; Mitsui *et al.* 2002; Brown *et al.* 2004; Cole *et al.* 2004; Arimura *et al.* 2005; Uchida *et al.* 2005; Yoshimura *et al.* 2005).

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Hyperphosphorylated CRMP-2 is a significant component of neurofibrillary tangles in Alzheimer's disease (Gu *et al.* 2000). At least one CRMP protein plays a role in learning and memory of mice (Su *et al.* 2007). During SEMA3 signal transduction, CRMP interacts with MICAL (Schmidt *et al.* 2008), a protein that mediates SEMA/PlexA signaling in *Drosophila* (Terman *et al.* 2002). The precise mechanism by which CRMP functions in PlexA/MICAL signaling is unknown.

In *Drosophila melanogaster*, the *CRMP* gene is the sole gene for the DHP/CRMP protein family and has been shown to encode DHP (Rawls 2006). To better understand DHP/CRMP proteins in *D. melanogaster*, we have carried out experiments to further characterize the fly *CRMP* gene and its products. We find that both DHP and CRMP are derived from this single gene through alternative splicing of paralogous exons: DHP in non-neuronal tissues throughout the animal and CRMP exclusively in the nervous system. We also show that Rac and Ras misexpression phenotypes are modified in *CRMP* mutants and that these animals exhibit behavioral defects: abnormal olfactory learning and memory defects and abnormal circadian locomotor rhythms.

Materials and Methods

Strains and transgenes

Most genes, markers, balancers, and strains are described at www.FlyBase.org (Tweedie *et al.* 2009). *CRMP^{supA4}* is a non-conservative missense mutation that abolishes DHP activity (Rawls 2006). The *CRMP^{supK1}* and *CRMP^{supla1}* mutations are intragenic deletions of the *CRMP* gene and are apparent null mutations; origins and properties of both mutations are described in [Supporting Information, File S1](#)). The *CRMP^{EP3238}* allele (obtained from Exelixis) contains an insertion of the *P{EPgy2}* enhancer trap transposon (Rørth *et al.* 1998) into the 5' end of the *CRMP* gene, upstream of the transcription start site (see FlyBase annotation). Strains containing the following transgenes were obtained from the Bloomington *Drosophila* Stock Center: GAL4 driver lines *P{GawB}elav^{C155}* (*P{elav-GAL}*), *P{GAL4-ey.H}3-8* (*P{ey-GAL}*), *P{ninaE-GAL4.GMR}12* (*P{GMR-GAL}*), and *P{sev-EP-GAL4}* (*P{sev-GAL}*); and UAS lines *P{UAS-Rac1.V12}1*, *P{UAS-Rac1.N17}1*, *P{UAS-Ras64B.V14}1*, *P{UAS-Ras85D}5-1*, *P{UAS-sggB}*, and *P{UAS-Rho1.V14}*. The *P{EP}Rac2^{EP3118}* strain, obtained from the Szeged *Drosophila* Stock Center, contains the *P{EPgy2}* enhancer trap transposon inserted into the 5' end of the *Rac2* gene, providing transcriptional control of *Rac2* by GAL4-responsive elements within the transposon (Rørth *et al.* 1998; Tseng and Hariharan 2002). The wild-type flies utilized in behavior experiments were Canton-S *w¹¹¹⁸* (iso CJ1) from the Dubnau lab; all mutations and transgenes analyzed in the behavioral experiments were introduced into the iso CJ1 genetic background by backcrossing at least six generations.

For this study, several derivatives were created of the *P{PYD2+}* transgene that contains a 10.9-kb wild-type genomic DNA fragment spanning the *CRMP* gene (Figure 1) inserted in the pCaSpeR4 vector (Rawls 2006). *P{PYD2GFP}*

contains an in-frame eGFP cassette inserted via *MluI* linkers, immediately upstream from the stop codon of the protein open reading frame within *CRMP* exon 12. Two modified forms of *P{PYD2GFP}* were created with frameshift mutations within exons E9a and E9b: *P{PYD2GFPfs9a}* was derived by deletion of a 26-bp *SacII* fragment within exon E9a; *P{PYD2GFPfs9b}* contains a 4-bp deletion within exon E9b that was created by *SacI* cleavage, exonuclease trimming, and blunt-end ligation. Lastly, the *P{PYD2GFPsupK1}* transgene was created by replacing a wild-type 2.7-kb *XhoI*–*EcoRV* fragment of the *P{PYD2GFP}* transgene, with the corresponding 2.55-kb genomic fragment of *CRMP^{supK1}* mutant DNA. For each of these transgene constructs, at least two independently derived strains were created and yielded the results reported here (*e.g.*, GFP fluorescence patterns, genetic complementation).

Flies were grown on standard medium at 25° or, where specified, at 29°.

Nucleic acids and analysis

cDNA clones (Rubin *et al.* 2000) were obtained from the *Drosophila* Genetics Resource Center and sequenced (Cincinnati Childrens Hospital Medical Center). The cDNA inserts of four clones revealed paralogous exons E9 (Figures 1 and 2): LP11064 (GU452674) and LP08604 (GU452675) derived from a larval/pupal library and contain E9a sequences (corresponding to FlyBase transcript CG1411-RE); GH07678 (GU452673); and GH22489 (GU452676), derive from an adult head library and contain E9b sequences (corresponding to FlyBase transcript CG1411-RA).

Extents of mutant deletions were determined by PCR analysis of genomic DNA from mutant animals, followed by sequencing gel-purified PCR fragments that contained the deletions (Rawls 2006). Methods for RNA isolation from adult flies and reverse transcriptase-coupled PCR were adapted from published protocols (Yang *et al.* 1995).

Purification and analysis of *Drosophila* DHP and CRMP proteins

Protein coding sequences of the *D. melanogaster* LP11064 and GH07678 cDNAs were PCR amplified and inserted between the *NdeI* and *BamHI* sites of pET16b (Novagen), permitting expression of DHP and CRMP proteins containing a 21-amino acid N terminus including a 10× His tag. Proteins were expressed in *Escherichia coli* BL21(DE3) cells and purified as described by Tanaka *et al.* 2007, with the following variations: small-scale preparations were performed using 50-ml aliquots of cell cultures and other volumes were proportionately reduced; buffers were supplemented with 0.1 mM zinc acetate; proteins were eluted from small columns of Ni-NTA Superflow (Qiagen) in 1 ml 0.5 M imidazole buffer, and then dialyzed overnight against 0.1 M sodium phosphate pH 7.0, 10% glycerol, 10 μM zinc acetate. Purification was monitored by analysis on 9% SDS–PAGE gels and Coomassie staining. DHP activity was spectrophotometrically measured as described by Kautz and

Figure 1 *CRMP* gene region in *Drosophila melanogaster*. Transcribed regions of the *CRMP* and neighboring *rheb* gene are represented above the double horizontal line. *CRMP* transcripts containing alternatively spliced exons E9a (red; corresponds to FlyBase transcript CG1411-RE) and E9b (blue; corresponds to FlyBase transcript CG1411-RA) are indicated. Below the double horizontal line are the extents of two *CRMP* deletion mutations *CRMP^{supla1}* (GU452678) and *CRMP^{supK1}* (GU452677) (see File S1), as well as transgene constructs used in this study.

Schnackerz (1989), except that reactions were carried out at 25°; a unit of DHP activity is the hydrolysis of 1 μ mol dihydrouracil per minute, based on triplicate assays of each sample. Protein concentrations were determined using the method of Bradford (1976), using bovine serum albumin as standard.

Microscopy and eye size comparisons

Live images of third instar larvae and dissected tissues were obtained using a Nikon SMZ microscope and a Leica TCS confocal microscope system (Ar laser). Images of adult heads were captured in Photoshop and the areas of eyes (pixels) were measured for 15–25 animals of each genotype using NIH ImageJ software. Eye areas of co-cultured sibling animals were compared using Student's *t* test analysis; *P*-values ≤ 0.05 were considered significant differences.

Aversive Pavlovian olfactory conditioning assay

Olfactory associative learning was assessed by training 2- to 3-day-old flies in a T-maze apparatus with a Pavlovian conditioning paradigm (Tully and Quinn 1985). In this assay, groups of 100 flies are trained by sequential exposure to one odor plus footshock (CS⁺), and a second odor with no footshock (CS⁻). Memory performance is measured by allowing trained animals to distribute themselves for a 2-min period within a T-maze in which odors were presented at opposite ends of the maze. To measure short-term memory performance, animals were tested within 2 min after training. Middle-term memory was assessed using the same single training paradigm, but testing occurred 3 hr

post-training (Dura *et al.* 1993). For long-term memory experiments, animals were subjected to 10 training sessions separated by 15-min rest intervals using robotic trainers (Tully *et al.* 1994); flies were tested 24 hr after training by placing them in the choice point of the T-maze apparatus. Odors used were 3-octanol (OCT, 180 μ l diluted in 10 ml mineral oil) and 4-methylcyclohexanol (MCH, 270 μ l diluted in 10 ml mineral oil). Each individual *n* consisted of ~ 200 flies, with half trained to one odor and the other half trained to the other odor. Odor preference was scored as the percentage of excess of animals at the CS⁻ end (*i.e.*, relative aversion to the CS⁺ end). A half performance index was calculated by subtracting the number of flies that chose correctly (odor that was unpaired with shock) minus the number of flies that chose incorrectly (odor that was paired with shock) and dividing that number by the total number of flies used in the experiment. A full performance index (PI) was calculated by averaging both reciprocal half performance indexes for the two odors. The final PI reported in each figure for each experiment was calculated by averaging the full performance indexes for all replicas or *n*'s that were performed for a given genotype. All genotypes were trained and tested in parallel.

Behavioral data collected using the odor discrimination paradigm are normally distributed. For short-term memory (STM) and middle-term memory (MTM), multiple genotypes were compared to wild type using one-way ANOVA with genotype as the main factor. For subsequent planned comparisons between each genotype and wild type, Dunnett's test was used. For long-term memory (LTM), only one

Figure 2 Alignment of DHP and CRMP protein segments. Protein segments derived from *D. melanogaster* exons E9a and E9b (Figure 1) are compared to corresponding segments of human DHP (Q14117) and human CRMP-1 (Q14194). Numbers in parentheses refer to the position of the first amino acid within the overall protein sequence. Identical amino acid residues among these sequences are colored: red (identical in CRMP and DHP proteins), green (identical in both DHP proteins), and blue (identical in both CRMP proteins). Underline and asterisk identify a DHP-critical histidinyl residue that is absent in CRMP proteins. Secondary structure features of the mouse CRMP-1 protein (Deo *et al.* 2004) are indicated below the alignment.

pairwise comparison was done so a Student's *t* test was used. Statistical significance in the figure represents a significant decrease in performance in comparison to the wild-type control levels (P -values ≤ 0.05). Error bars in the figures represent the standard error of the mean. All statistical analyses were performed using JMP 5.1 statistical discovery software.

Circadian locomotor activity assay

Locomotor activity rhythms of 1- to 3-day-old adult male flies were monitored at 25° using Trikinetic *Drosophila* activity monitors (Rosato and Kyriacou 2006). Locomotor activities of individual flies were monitored throughout a 3–4 day entrainment in 12 hr light/dark (12:12 LD) conditions, followed by 7–8 days in constant darkness (DD). The number of activity events, detected by infrared beam disruption, was recorded per half-hour bin for each fly. Data analysis was done with ClockLab software (Actimetrics). Dunnett's *post hoc* analysis, comparing all average values to the wild-type control strain, produced the following outcomes: $P < 0.01$ genotypes are significantly different, $P > 0.01$ genotypes are not significantly different.

Results

CRMP transcripts are alternatively spliced to produce DHP and CRMP-like proteins

To characterize RNA products of the *CRMP* gene, we sequenced six apparently full-length *CRMP* cDNA clones from the Berkeley *Drosophila* Genome Project (Rubin *et al.* 2000). Two distinct classes of transcripts were identified (Figure 1): cDNAs containing the exon designated E9a (corresponding to FlyBase transcript CG1411-RE); cDNAs containing an alternative exon designated E9b (corresponding to FlyBase transcript CG1411-RA). Otherwise, cDNAs of both classes are distinguished only by minor length variations at their 5' ends. The protein-coding regions of these transcripts are identical, except for the 75- to 80-amino-acid residue segment encoded by the alternative exons.

Polypeptide segments encoded by exons E9a and E9b are compared to the corresponding segments of human DHP and CRMP-1 proteins in Figure 2. Primary structures of the E9a segment and corresponding human DHP segment are identical at 42 of 80 residues. Both polypeptide segments

contain a histidinyl residue (H₂₆₁ in the E9a segment) that is critical to DHP catalysis (Schnackerz and Dobritzsch 2008), and that is absent in CRMP proteins (Schmidt and Stritmatter 2007). The fly E9b segment and the corresponding segment of human CRMP-1 are identical at 27 of 75 residues; the DHP-critical histidinyl residue is absent in both of these protein segments.

His-tagged forms of the two *CRMP* gene proteins were produced in *E. coli* and purified by Ni-NTA affinity chromatography (Figure 3). Final products (Ni-NTA bound lanes) contained two protein bands, the upper of which correspond closely to predictions for full-length polypeptides and the lower of which resemble the C-terminally truncated products that are found in preparations of bacteria-expressed, mammalian CRMP proteins (Deo *et al.* 2004). DHP assays of the purified proteins yielded very different results. The exon E9a-encoded protein (pETDHP) exhibited activity of 5.16 ± 0.09 μ mol dihydrouracil hydrolyzed/min/ μ g protein, very similar to the activity of purified human DHP (Kautz and Schnackerz 1989). Exon E9b-encoded protein (pETCRMP) displayed insignificant DHP activity. We conclude that E9a-containing transcripts encode DHP and that E9b-containing transcripts encode the presumptive *Drosophila* ortholog of vertebrate CRMP.

CRMP paralogous exons are expressed in mutually exclusive tissues

CRMP transcripts are expressed in a variety of embryonic tissues (Chintapalli *et al.* 2007). To distinguish the expression patterns of E9a- and E9b-containing transcripts and their proteins, we created several modified forms of the *P{PYD2+}* transgene (Figure 1), a genomic DNA segment that spans the coding sequences of the *CRMP* gene and that rescues the DHP deficiency in *CRMP* mutant animals (Rawls 2006). *P{PYD2GFP}* contains a GFP cassette inserted at the 3' end of the protein open reading frame, immediately 5' to the normal stop codon. Two derivatives of *P{PYD2GFP}* were also created: one containing a 26-bp frameshift deletion within exon E9a (*P{PYD2GFPs9a}*) and the other, a 4-bp frameshift deletion within exon E9b (*P{PYD2GFPs9b}*). Lines of transgenic animals were created and GFP fluorescence was observed in tissues of third instar larvae (Figure 4). *P{PYD2GFP}* larvae exhibit fluorescence in a variety of

Figure 3 Purification of alternative protein products of the *CRMP* gene. Protein open reading frame sequences from *CRMP* cDNAs were inserted into the pET bacterial expression vector, creating constructs pETDHP (includes E9a sequences) and pETCRMP (includes E9b sequences). Expression, purification, and SDS-PAGE analysis of His-tagged proteins are described in *Materials and Methods*. The upper protein bands in final products (Ni-NTA bound lanes) correspond closely to predictions for full-length polypeptides (67.6 kDa for His-tagged pETDHP product, 66.9 kDa for His-tagged pETCRMP product). Dihydrouracil hydrolase activity (units per microgram of protein) was 5.16 ± 0.09 for the pETDHP product and 0.002 ± 0.004 for the pETCRMP product. A different preparation of the proteins (results not shown) yielded similar results: activity values for pETDHP and pETCRMP products were 4.55 ± 0.17 and 0.13 ± 0.11 units/ μ g protein, respectively.

tissues, including somatic musculature and most intensely in brain and other neuronal tissues (Figure 4, A and B). Fluorescence in *P{PYD2GFPs9a}* animals (intact E9b) is limited to nervous system tissues, whereas fluorescence in *P{PYD2GFPs9b}* animals (intact E9a) is exclusively non-neuronal (Figure 4A). We conclude that E9a-containing transcripts are expressed in a variety of nonneuronal tissues, whereas E9b-containing transcripts are expressed exclusively in neuronal tissues.

In vivo DHP function of these transgene constructs was tested by a genetic epistatic interaction assay that detects pyrimidine catabolism (described in Rawls 2006). In *Drosophila*, uracil degradation is a primary source of β -alanine and low β -alanine levels in *black* (*b*) mutant animals results in dark cuticle pigmentation. The semidominant *r^{Su(b)}* mutation is a feedback-insensitive mutation of the first enzymatic step of *de novo* pyrimidine biosynthesis (Simmons *et al.* 1999), resulting in overproduction of pyrimidines. Thus, catabolism of overproduced pyrimidines results in suppression of the *black* body phenotype in *r^{Su(b)} b* animals; however, this suppression is blocked in DHP-deficient animals. That is, *r^{Su(b)} b CRMP⁺* adults have normalized (suppressed) body color, whereas *r^{Su(b)} b CRMP⁻* flies have *black* cuticle. We created *P{r^{Su(b)}} b CRMP⁻* animals that also received one of the GFP transgenes described above, and body color phenotypes were examined (Supporting Information, Figure S1). Flies receiving *P{PYD2GFP}* or

P{PYD2GFPs9b} displayed normalized pigmentation (*i.e.*, the transgene provided DHP), whereas animals receiving *P{PYD2GFPs9a}* were uniformly *black* body (*i.e.*, the transgene did not provide DHP). These results confirmed that exon E9a is required for *in vivo* DHP.

***CRMP* null mutant animals exhibit normal morphology and fertility**

Previously described *CRMP* mutations included small deletions of nonprotein coding DNA or nucleotide substitutions and all were selected by DHP-deficiency criteria; thus, it is possible that non-DHP functions of the gene are unaffected by those mutations (Rawls 2006). To ascertain unambiguous loss-of-function effects of *CRMP* (*i.e.*, knockouts of both DHP and CRMP), two new mutations were isolated (File S1). The *CRMP^{supla1}* mutation is a large intragenic deletion that removes all of exons E1 through E9a, removing the N-terminal 42% of the protein open reading frame (Figure 1). *CRMP^{supK1}* is a 153-bp deletion that removes 50 codons at the 3' end of exon E4 and extends into the downstream intron (I4). DNA sequences of these mutations, genetic epistasis assays, as well as transcript cDNA analysis indicate that they are *bona fide* null alleles (File S1). For both *CRMP^{supK1}* and *CRMP^{supla1}*, homozygous, hemizygous, or heteroallelic animals are viable, fertile, and morphologically normal when grown under standard laboratory conditions.

Figure 4 Alternative *CRMP* transcripts and proteins are expressed in mutually exclusive tissues in larvae. (A) GFP expression in larvae homozygous for transgene constructs: (1) *P{PYD2+}*, non-GFP control; (2) *P{PYD2GFP}*, GFP expression in a variety of tissues including brain and somatic musculature; (3) *P{PYD2GFPf9b}*, GFP only in nonneural tissue; and (4) *P{PYD2GFPf9a}*, GFP expression confined to neural tissue. (B) Confocal image of GFP expression in dissected tissues of a *P{PYD2GFP}* larva. Arrowheads denote central nervous system (solid) and somatic musculature (open).

***CRMP* mutation modifies multiple signal transduction pathways during eye morphogenesis**

To test functional homology of the nervous-system-limited fly *CRMP* protein and the vertebrate *CRMP* proteins, we investigated the effects of *CRMP* mutations on signal transduction pathways. The experimental system consisted of misexpressing components of various pathways during eye development using the UAS/GAL4 system (Brand and Perrimon 1993; Duffy 2002) and comparing the phenotypes of homozygous *CRMP^{supK1/supK1}* animals and their heterozygous wild-type siblings (*CRMP^{+/supK1}*).

In mammals, *CRMP* function is regulated by Ras/PI3-kinase/Akt/GSK-3 β signaling (Yoshimura *et al.* 2005). Misexpression of a normal form of the sole *D. melanogaster* Ras1 gene (*P{UAS-Ras85D}5-1*) with the *P{GMR-GAL4}* driver produces a rough eye phenotype in *CRMP^{+/supK1}* flies that results from fusion and irregular alignment of individual ommatidia (Figure 5A); however, this phenotype is normalized in *CRMP^{supK1/supK1}* siblings (Figure 5B). *CRMP* also affects misexpression phenotypes of the sole *D. melanogaster* GSK-3 β , Sgg. Under control of either the *P{GMR-GAL4}* or the *P{ey-GAL4}* drivers, expression of the normal form of Sgg (*P{UAS-sggB}*) in *CRMP^{+/supK1}* animals results in uniform pupal lethality; individuals that survive to the pharate adult stage lack significant eye or related neural head structures (Figure 5, C and E). *CRMP^{supK1/supK1}* homozygotes regularly survive to the pharate adult stage and frequently eclose as adults displaying reduced rough eyes (Figure 5, D and F). In summary, *CRMP^{supK1}* suppresses misexpression phenotypes of both Ras1 and GSK-3 β in eye morphogenesis, evidence that *Drosophila* *CRMP* mediates signaling through the Ras/PI3-kinase/Akt/GSK-3 β pathway.

In mammals, *CRMP* interacts with Rho GTPases of the Rac family to mediate neuronal growth cone collapse (Jin and

Strittmatter 1997; Arimura *et al.* 2000; Hall *et al.* 2001b). *Drosophila melanogaster* has two canonical Rac proteins, both of which mediate neuronal dynamics (Ng *et al.* 2002; Ng and Luo 2004). Flies expressing a constitutively active form of Rac1 were created by combining *P{UAS-Rac1.V12}* and the *P{sev-GAL4}* driver, resulting in a normal eye phenotype in *CRMP^{+/supK1}* animals (Fanto *et al.* 2000; Figure 5G). Severely reduced eyes are produced in *CRMP^{supK1/supK1}* animals (Figure 5H). Presence or absence of *CRMP* produced no discernible effect upon the misexpression of a dominant-negative form of Rac1 (*P{UAS-Rac1.N17}*)/*P{sev-GAL4}* animals; Figure 5, I and J).

Misexpression of Rac2 was studied using the *P{EP}* *Rac2^{EP3118}* enhancer trap that expresses a normal form of the protein under control of GAL4 (Rørth *et al.* 1998). In combination with the *P{GMR-GAL4}* driver, *P{EP}Rac2^{EP3118}* animals display rough but normal size eyes (Tseng and Hariharan 2002; Müller *et al.* 2005) (Figure 5K); this phenotype is enhanced in *CRMP^{supK1/supK1}* animals that have reduced eyes with extensively fused ommatidia (Figures 5L). The *P{ey-GAL4}* driver produces a normal eye phenotype in *P{EP}Rac2^{EP3118}* *CRMP^{+/supK1}* animals (Figure 5M), but irregular ommatidial arrangements and reduced eyes in *P{EP}Rac2^{EP3118}* *CRMP^{supK1/supK1}* animals (Figure 5N). Expression of the *P{EP}Rac2^{EP3118}* enhancer trap with the panneuronal *P{elav-GAL4}* driver results in normal eye development in *CRMP^{+/supK1}* animals (Figure 5O), but rough eyes in *CRMP^{supK1/supK1}* homozygotes (Figure 5P). Thus, the *CRMP^{supK1}* mutation enhances misexpression phenotypes of both Rac1 and Rac2 in eye morphogenesis, evidence that *Drosophila* *CRMP* mediates signaling through pathways utilizing these Rho GTPases.

In other experiments, *CRMP* mutation failed to modify eye phenotypes produced by misexpression of constitutively active forms of *Drosophila* Ras2 (Ras64B.V14; Brand and Perrimon 1993) and RhoA kinase (Rho1.V14; Fanto *et al.* 2000) (File S2). Thus, *CRMP* effects on Ras1, Rac1, and Rac2 signaling during eye development are not observed in animals misexpressing other signaling pathways.

The *CRMP* gene is necessary for normal olfactory memory

Memory capabilities of the *CRMP* mutant flies were assessed using a Pavlovian olfactory conditioning assay in which animals learn to associate a conditioned stimulus (odor) with an unconditioned stimulus (footshock) (Tully and Quinn 1985). *CRMP* mutant lines were outcrossed for at least six generations to the isogenic control strain *w¹¹¹⁸(isoCJ1)*, which shows normal levels of olfactory learning and memory (Tully *et al.* 1994) and served as the wild-type control strain in all experiments. Animals from all strains exhibited normal task relevant sensorimotor responses (odor avoidance and electric shock sensitivity) required for aversive olfactory learning and memory tasks (Figure S2).

When tested for STM performance 2 min after a single aversive Pavlovian training session, both *CRMP* mutant lines

Figure 5 *CRMP* modifies phenotypes produced by misexpression of cellular signal transduction proteins. Representative eye phenotypes are shown for a variety of misexpression genotypes: (A) *P{GMR-GAL4}/P{UAS-Ras85D}5-1; CRMP^{+/supK1}*, (B) *P{GMR-GAL4}/P{UAS-Ras85D}5-1; CRMP^{supK1/supK1}*, (C) *P{GMR-GAL4}/P{UAS-sggB}; CRMP^{+/supK1}*, (D) *P{GMR-GAL4}/P{UAS-sggB}; CRMP^{supK1/supK1}*, (E) *P{ey-GAL4}/P{UAS-sggB}; CRMP^{+/supK1}*, (F) *P{ey-GAL4}/P{UAS-sggB}; CRMP^{supK1/supK1}*, (G) *P{sev-EP-GAL4}/P{UAS-Rac1.V12}; CRMP^{+/supK1}*, (H) *P{sev-EP-GAL4}/P{UAS-Rac1.V12}; CRMP^{supK1/supK1}*, (I) *P{sev-EP-GAL4}/P{UAS-Rac1.N17}; CRMP^{+/supK1}*, (J) *P{sev-EP-GAL4}/P{UAS-Rac1.N17}; CRMP^{supK1/supK1}*, (K) *P{GMR-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{+/supK1}*, (L) *P{GMR-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{supK1/supK1}*, (M) *P{ey-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{+/supK1}*, (N) *P{ey-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{supK1/supK1}*, (O) *P{elav-GAL4}; P{EP}Rac2^{EP3118}/+; CRMP^{+/supK1}*, and (P) *P{elav-GAL4}; P{EP}Rac2^{EP3118}/+; CRMP^{supK1/supK1}*. Arrows in C and E point to residual head structure in dead imagos. Animals in A through N were grown at 25°; animals in O and P were grown at 29°. Detailed descriptions of these phenotypes may be found in File S2.

as well as heteroallelic animals (*CRMP^{supK1/supK1}*) exhibited significantly reduced performance compared to wild-type control flies (Figure 6A). Heterozygous animals displayed no significant learning impairment, demonstrating that the mutant phenotype is recessive. MTM performance measured 3 hr after a single aversive Pavlovian training session also was reduced in homozygous mutant and heteroallelic mutant animals, while MTM of heterozygous animals was in-

distinguishable from controls (Figure 6B). To test LTM, a spaced training procedure was used (Tully *et al.* 1994; see *Materials and Methods*) and performance was tested 24 hr after training. As with STM and MTM, *CRMP^{supK1}* mutant animals exhibited significantly reduced LTM performance compared to control animals (Figure 6C). Collectively, these results demonstrate that the *CRMP* gene is required for normal short-, medium-, and long-term memory performance in

Figure 6 Learning and memory performance is defective in CRMP mutant flies. Performance indices (PIs) are shown as means $\pm$ SEM. (A) Short-term memory of heterozygous *CRMP^{supK1/+}* and *CRMP^{supla1/+}* animals was indistinguishable from wild-type animals; however, PIs for *CRMP* mutants were significantly lower than wild type. (B) Middle-term memory of *CRMP* mutants was significantly lower than wild type. (C) Long-term memory of *CRMP^{supK1}* animals was significantly impaired relative to wild-type animals. $N = 8$ for tests for each genotype. Mutant genotypes differing significantly from wild type are indicated ($*P < 0.05$; see *Materials and Methods*).

this Pavlovian task, although it should be noted that only one allele was tested for the LTM task.

CRMP mutation can alter normal *Drosophila* circadian activity rhythm

Circadian rhythmic behavior was assessed using a locomotor activity assay, as described in *Materials and Methods*. Individual males were entrained 3 days in 12:12 LD cycles; then, free-running activity was monitored in constant darkness (DD) for an additional 8 days (*Materials and Methods*). Histograms in Figure S3 show the mean activities of flies of various genotypes during entrainment and during free-running periods. Normal flies exhibit two daily peaks of activity: a morning peak that corresponds to lights on and an evening peak around the time of lights off. After entrainment, these patterns are retained under DD conditions (Hamblen-Coyle *et al.* 1992; Renn *et al.* 1999; Bahn *et al.* 2009), providing a 23- to 24-hr circadian cycle period. Results obtained for a variety of genotypes are presented in Table 1.

Behaviors during entrainment were similar for all strains (Figure S3). Under free-running conditions (DD), wild-type and mutant animals exhibited similar overall activity (events/bin in Table 1), indicating that *CRMP* mutation does not alter overall activity of animals. Furthermore, period length was similar for all tested animals, indicating that length of the circadian clock is not affected by *CRMP* mutation. Relative strength of rhythmicity of free-running behaviors differs among genotypes, visualized graphically in Figure S3. Chi-square statistical analysis of the behaviors of individual animals resulted in power values for each genotype (Table 1). Compared with wild-type and heterozy-

gous animals, significantly reduced power values were observed among *CRMP^{supK1}* homozygotes and hemizygotes, *CRMP^{supla1}* hemizygotes, *CRMP^{supK1}/CRMP^{supla1}* heteroallelic animals, and *CRMP^{EP3238}* animals. Surprisingly, power values for *CRMP^{supla1}* homozygotes were not significantly reduced (Table 1). For most genotypes, some individual animals lacked significantly rhythmic behaviors (arrhythmic); such individuals were more frequently encountered among *CRMP^{supK1}* homozygotes and hemizygotes, and *CRMP^{EP3238}* genotypes.

Discussion

DHP and CRMP proteins are functionally divergent, yet their structures are highly conserved. Results presented here show that the *CRMP* gene of *D. melanogaster* encodes both DHP and CRMP through mutually exclusive, alternative splicing of paralogous exons. Evidence that DHP derives from transcripts containing the upstream exon includes dihydropyrimidine hydrolysis by bacterial-expressed protein, expression limited to nonneuronal tissues, and epistatic interactions with mutations in pyrimidine catabolism. CRMP orthology of the protein derived from downstream exon-containing transcripts is supported by several lines of evidence: lack of DHP activity, expression limited to nervous tissues, structural similarity to vertebrate CRMP protein segments. CRMP orthology is also supported by the ability of *CRMP^{supK1}* and *CRMP^{supla1}* mutations to modify phenotypes produced by misexpression of Ras (suppression) and Rac (enhancement) signaling, pathways known to be mediated by vertebrate CRMP proteins. However, it should be noted

Table 1 Free-running circadian activity behaviors of entrained animals

Genotype	N	Period	Events/bin	Power	Arrhythmic, (%)
<i>CRMP⁺</i> (isoCJ1)	59	23.7 ± 0.1	16.9 ± 1.1	83.7 ± 4.7	0
<i>CRMP⁺/supK1</i>	30	23.5 ± 0.2	14.6 ± 1.5	74.2 ± 6.8	2 (7)
<i>CRMP⁺/supla1</i>	23	23.6 ± 0.2	16.6 ± 1.7	67.9 ± 7.5	0
<i>CRMP^{supK1}/supK1</i>	67	23.9 ± 0.1	19.5 ± 1.0	9.4 ± 5.1*	18 (27)
<i>CRMP^Δ/supK1</i>	25	24.0 ± 0.3	15.5 ± 1.6	33.4 ± 6.8*	4 (16)
<i>CRMP^{supla1}/supla1</i>	43	23.6 ± 0.1	14.5 ± 1.2	77.0 ± 5.5	1 (2)
<i>CRMP^Δ/supla1</i>	21	23.6 ± 0.3	17.9 ± 1.7	33.4 ± 7.0*	1 (5)
<i>CRMP^{supK1}/supla1</i>	24	23.8 ± 0.3	17.3 ± 1.6	46.0 ± 6.6*	2 (8)
<i>CRMP^{EP3238/EP3238}</i>	23	23.1 ± 0.1	17.0 ± 1.7	40.2 ± 8.2*	4 (17)

Individual flies were monitored throughout a 3- to 4-day entrainment in 12 hr light/dark (12:12 LD) conditions, followed by 7–8 days in constant darkness (DD) (see Figure S3). N, number of individuals tested. Period, length of the observed behavioral rhythm in hours (mean ± SEM). Events/bin, the number of activity events (infrared beam disruption) per half-hour bin (mean ± SEM). Power (mean ± SEM), the power of rhythmicity exhibited by rhythmic animals of each genotype, derived as the amplitude of the peak above the significant line ($\alpha = 0.025$) in the chi-square periodogram of individual animals (Liu *et al.* 1991). Power values differing significantly ($P \leq 0.01$) from controls (*CRMP⁺*) are indicated with an asterisk (*). Arrhythmic, number (and percentage) of individual flies lacking significant rhythmic behavior (Clocklab analysis); computation and comparisons of power values excluded these animals. Hemizygous *CRMP* mutants were created using *Df(3R)noi-B* (Δ symbol), a deletion that removes a small chromosome segment including the *CRMP* gene.

that these intragenic deletion mutations are null for both protein forms and it is possible that at least some of the phenotypic effects observed in *CRMP* mutant animals could be due to DHP deficiency in nonneuronal cells.

During Sema3A-induced growth-cone collapse in mammalian neurons, phosphorylation of CRMP by GSK-3 β and other kinases inhibits association of CRMP with tubulin dimers and, possibly, with Sra-1/WAVE complex (Fukata *et al.* 2002; Kawano *et al.* 2005; Yoshimura *et al.* 2005), diminishing tubulin polymerization and Sra-1/WAVE-induced actin dynamics (see model of Schmidt and Strittmatter 2007). Our results that Ras1 and GSK-3 β misexpression phenotypes are suppressed in *CRMP^{supK1}* flies (Figure 5, A–F) are consistent with *Drosophila* CRMP functioning downstream of Ras1 and GSK-3 β within one or more signal transduction cascades. Furthermore, phosphorylated CRMP-2 interacts with α 2-chimaerin in mammals, thereby inhibiting Rac activation and consequently diminishing actin cytoskeleton dynamics (Hall *et al.* 2001a; Brown *et al.* 2004). We find that *CRMP^{supK1}* enhances misexpression phenotypes of constitutively active Rac1 (Figure 5, G and H), but not dominant-negative Rac1 (Figure 5, I and J); *CRMP^{supK1}* also enhances misexpression phenotypes of normal Rac2 (Figure 5, K–P). These results correlate with observations in mammalian cells, in which Rac1 misexpression phenotypes were suppressed by CRMP-2 (Hall *et al.* 2001b). Thus during eye development, *Drosophila* CRMP appears to interact with Rac pathways in a manner similar to mammalian CRMP.

Our results show that fly *CRMP* gene function is required for normal learning (STM), MTM, and LTM (Figure 6). In contrast with STM and MTM, LTM measured through the spaced training paradigm used in these studies requires acute protein synthesis under control of the CREB transcription factor (Tully *et al.* 1994; Yin *et al.* 1995; Qian *et al.* 2007; Wu *et al.* 2007). Given the reduced performance observed at earlier time points, we cannot distinguish whether the LTM phenotype is due to an active requirement for CRMP function during memory consolidation or rather if

the LTM defect is a consequence of reduced acquisition. Similar impairment of STM and LTM is observed in *rutabaga* mutant flies (Livingstone *et al.* 1984; Zars *et al.* 2000; Schwaerzel *et al.* 2002; Blum *et al.* 2009). Recent studies show that the STM and LTM defects of *rutabaga* mutant animals can be separately restored with expression of wild-type *rutabaga* protein in distinct subsets of mushroom body neurons (Blum *et al.* 2009). It is conceivable that *CRMP* mutant defects in olfactory learning and memory might be indirect consequences of defective neurodevelopment in mutant animals; however, the normal olfactory acuity and shock reactivity exhibited by *CRMP* animals argue that such neurodevelopmental defects are limited to circuits related to learning and memory, rather than general neurological function. Further studies will be required to understand circuit requirements of CRMP expression in learning and memory in flies.

Defective learning and memory performance is also exhibited by mice lacking the CRMP-1 protein (Su *et al.* 2007). A clue to molecular mechanisms of CRMP function in memory may come from recent studies showing that reductions in Rac signaling in flies leads to slower kinetics of memory decay (Shuai *et al.* 2010). Thus Rac would seem to be a potential target of CRMP signaling in the context of memory processing; impaired memory in *CRMP* mutants could result from enhanced Rac activity.

Results of circadian activity studies suggest a role of CRMP in another behavior that relies on synaptic plasticity—circadian activity rhythmicity in *Drosophila* adults. Following light/dark entrainment, most *CRMP* mutant genotypes exhibit abnormal free-running locomotor activity under constant darkness conditions (Table 1). Given that CRMP plays a role in neuronal differentiation in mammals, one possible cause of defective rhythmicity in flies could be aberrant development of neurons that provide clock function. The most important fly clock neurons are a cluster of s-LN ν neurons that produce pigment dispersing factor (PDF) neuropeptides (Renn *et al.* 1999; Park 2002). However,

PDF-containing neurons appear to be normally distributed in the brains of *CRMP* mutant animals (Figure S4). Thus, CRMP involvement in clock functions may be more indirect.

A surprising result was the essentially normal free-running locomotor activity of animals homozygous for the *CRMP^{supla1}* mutation, in which half of the protein coding sequence is removed (Table 1). However, hemizygous *CRMP^{supla}* animals as well as heteroallelic *CRMP^{supla1}/CRMP^{supK1}* animals were significantly arrhythmic, evidence that *CRMP^{supla1}* is indeed a loss-of-function allele for the circadian activity phenotype. The normal behavior of homozygous *CRMP^{supla}* animals may indicate that the *CRMP^{supla1}* chromosome retained a closely linked, recessive genetic suppressor of the arrhythmia phenotype, despite the extensive backcrossing to which these lines were subjected.

Another unanticipated result was the abnormal free-running activity of *CRMP^{EP3238}* animals (Table 1), because previous studies showed that the *CRMP^{EP3238}* mutation is wild type for DHP function (Rawls 2006 and J. Rawls and D. Morris, unpublished data). Thus, the *CRMP^{EP3238}* mutation appears to be a normal-function allele for DHP and a loss-of-function allele for CRMP. Effects of this mutation on circadian activity rhythm indicates that abnormalities derive from loss of CRMP rather than DHP. In the *CRMP^{EP3238}* strain, the *P{EP}* mobile element is inserted within the 5' end of the *CRMP* gene upstream of but near the transcription initiation site (Rawls 2006), a region that could contain sequences that differentially regulate *CRMP* gene transcription in nonneuronal and neuronal tissues. Future studies will be required to better understand *CRMP* gene regulation and the basis for differential effects of the *CRMP^{EP3238}* mutation.

In summary, results presented here show that the *Drosophila CRMP* gene of *Drosophila* encodes a DHP and a CRMP protein through mutually exclusive splicing of paralogous exons. DHP protein is expressed in nonneuronal tissues, whereas the CRMP protein is expressed in neuronal tissues, a distribution similar to humans (Hamajima *et al.* 1996; Van Kuilenberg *et al.* 2006). Presumably, mutually exclusive expression of these proteins prevents heteromultimerization or other interactions that might interfere with their function (Wang and Strittmatter 1996; Deo *et al.* 2004). In addition to structural similarities, fly and vertebrate CRMP proteins exhibit significant functional homologies: effects on Ras and Rac signaling pathways and loss-of-function consequences on behaviors (*i.e.*, learning and memory defects in mice and flies, circadian activity rhythm defects in flies). Further studies should elucidate the roles of these proteins as well as the diverse genetic mechanisms that have contributed to the evolution of the DHP/CRMP protein family in animals.

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Literature Cited

- Abendroth, J., K. Niefind, and D. Schomburg, 2002a X-ray structure of dihydropyrimidinase from a *Thermus* sp. at 1.3 Å resolution. *J. Mol. Biol.* 320: 143–156.
- Abendroth, J., K. Niefind, O. May, M. Siemann, C. C. Syltatk *et al.*, 2002b The structure of L-hydantoinase from *Arthobacter auriscens* leads to an understanding of dihydropyrimidinase substrate and enantio specificity. *Biochemistry* 9: 8589–8597.
- Amano, M., Y. Fukata, and K. Kaibuchi, 2000 Regulation and functions of Rho-associated kinase. *Exp. Cell Res.* 261: 44–51.
- Arimura, N., N. Inagaki, K. Chihara, C. Menager, N. Nakamura *et al.*, 2000 Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse. *J. Biol. Chem.* 275: 23973–23980.
- Arimura, N., C. Menager, Y. Kawano, T. Yoshimura, S. Kawabata *et al.*, 2005 Phosphorylation by Rho kinase regulates CRMP-2 activity in growth cones. *Mol. Cell. Biol.* 25: 9973–9984.
- Bahn, J., G. Li, and J. Park, 2009 Comparative analysis of Pdf-mediated circadian behaviors between *Drosophila melanogaster* and *Drosophila virilis*. *Genetics* 181: 965–975.
- Blum, A. L., W. Li, M. Cressy, and J. Dubnau, 2009 Short- and long-term memory in *Drosophila* require cAMP signaling in distinct neuron types. *Curr. Biol.* 19: 1–10.
- Bradford, M., 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.
- Brand, A., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118: 401–415.
- Brown, M., T. Jacobs, B. Eickholt, G. Ferrari, M. Teo *et al.*, 2004 Alpha2-chimaerin, cyclin-dependent Kinase 5/p35, and its target collapsin response mediator protein-2 are essential components in semaphorin 3A-induced growth-cone collapse. *J. Neurosci.* 24: 8994–9004.
- Chi, X., B. Schmutzler, J. Brittain, Y. Wang, C. Hingtgen *et al.*, 2009 Regulation of N-type voltage-gated calcium channels (Cav2.2) and transmitter release by collapsin response mediator protein-2 (CRMP-2) in sensory neurons. *J. Cell Sci.* 122: 4351–4362.
- Chintapalli, V., J. Wang, and J. Dow, 2007 Using FlyAtlas to identify better *Drosophila* models of human disease. *Nat. Genet.* 39: 715–720.
- Cole, A., A. Knebel, N. Morrice, L. Robertson, A. Irving *et al.*, 2004 GSK-3 phosphorylation of the Alzheimer epitope within

- collapsin response mediator proteins regulates axon elongation in primary neurons. *J. Biol. Chem.* 279: 50176–50180.
- Deo, R., E. Schmidt, A. Elhabazi, H. Togashi, S. Burley *et al.*, 2004 Structural bases for CRMP function in plexin-dependent semaphorin3A signaling. *EMBO J.* 23: 9–22.
- Duffy, J., 2002 GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* 34: 1–15.
- Dura, J., T. Preat, and T. Tully, 1993 Identification of *linotte*, a new gene affecting learning and memory in *Drosophila melanogaster*. *J. Neurogenet.* 9: 1–14.
- Fanto, M., U. Weber, S. Strutt, and M. Mlodzik, 2000 Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the *Drosophila* eye. *Curr. Biol.* 10: 979–988.
- Fukata, Y., T. Itoh, T. Kimura, C. Menager, T. Nishimura *et al.*, 2002 CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat. Cell Biol.* 4: 583–591.
- Gu, Y., N. Hamajima, and Y. Ihara, 2000 Neurofibrillary tangle-associated collapsin response mediator protein-2 (CRMP-2) is highly phosphorylated on Thr-509, Ser-518, and Ser-522. *Biochemistry* 39: 4267–4275.
- Hall, C., G. Michael, N. Cann, G. Ferrari, M. Teo *et al.*, 2001a α 2-chimaerin, a Cdc42/Rac1 regulator, is selectively expressed in the rat embryonic nervous system and is involved in neurite outgrowth in N1E-115 neuroblastoma cells. *J. Neurosci.* 21: 5191–5202.
- Hall, D., M. Brown, T. Jacobs, G. Ferrari, N. Cann *et al.*, 2001b Collapsin response mediator protein switches RhoA and Rac1 morphology in N1E-115 neuroblastoma cells and is regulated by Rho kinase. *J. Biol. Chem.* 276: 43482–43486.
- Hamajima, N., K. Matsuda, S. Sakata, N. Tamaki, M. Sasaki *et al.*, 1996 A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution. *Gene* 180: 157–163.
- Hamajima, N., M. Kouwaki, P. Vreken, K. Matsuda, S. Sumi *et al.*, 1998 Dihydropyrimidinase deficiency: structural organization, chromosomal localization, and mutation analysis of the human dihydropyrimidinase gene. *Am. J. Hum. Genet.* 63: 717–726.
- Hamblen-Coyle, M. J., D. A. Wheeler, J. E. Rutila, M. Rosbash, and J. C. Hall, 1992 Behavior of period-altered circadian rhythm mutants of *Drosophila* in light:dark cycles (Diptera: Drosophilidae). *J. Insect Behav.* 5: 417–446.
- Hou, S., S. Jiang, and R. Smith, 2008 Permissive and repulsive cues and signaling pathways of axonal outgrowth and regeneration. *Int. Rev. Cell Molec. Biol.* 267: 125–181.
- Jin, Z., and S. M. Strittmatter, 1997 Rac1 mediates collapsin-1-induced growth cone collapse. *J. Neurosci.* 17: 6256–6263.
- Kautz, J., and K. Schnackerz, 1989 Purification and properties of 5,6-dihydropyrimidine amidohydrolase from calf liver. *Eur. J. Biochem.* 181: 431–435.
- Kawano, Y., T. Yoshimura, D. Tsuboi, S. Kawabata, T. Kaneko-Kawano *et al.*, 2005 CRMP-2 is involved in kinesin-1-dependent transport of the Sra-1/WAVE1 complex and axon formation. *Mol. Cell. Biol.* 22: 9920–9935.
- Liu, X., Q. Yu, Z. Huang, L. Zwiebel, J. C. Hall *et al.*, 1991 The strength and periodicity of *D. melanogaster* circadian rhythms are differentially affected by alterations in *period* gene expression. *Neuron* 6: 753–766.
- Livingstone, M. S., P. P. Sziber, and W. G. Quinn, 1984 Loss of calcium/calmodulin responsiveness in adenylate cyclase of *rutabaga*, a *Drosophila* learning mutant. *Cell* 37: 205–215.
- Lohkamp, B., B. Andersen, J. Piškur, and D. Dobritzsch, 2006 The crystal structure of dihydropyrimidinases reaffirm the close relationship between cyclic amidohydrolases and explain their substrate specificity. *J. Biol. Chem.* 281: 13762–13776.
- Mitsui, N., R. Inatome, S. Takahashi, Y. Goshima, H. Yamamura *et al.*, 2002 Involvement of Fes/Fps tyrosine kinase in semaphorin3A signaling. *EMBO J.* 21: 3274–3285.
- Müller, D., S. Kugler, A. Preiss, D. Maier, and A. Nagel, 2005 Genetic modifier screens on hairless gain-of-function phenotypes reveal genes involved in cell differentiation, cell growth and apoptosis in *Drosophila melanogaster*. *Genetics* 171: 1137–1152.
- Ng, J., and L. Luo, 2004 Rho GTPases regulate axon growth through convergent and divergent signaling pathways. *Neuron* 44: 779–793.
- Ng, J., T. Nardine, M. Harms, J. Tzu, A. Goldstein *et al.*, 2002 GTPases control axon growth, guidance and branching. *Nature* 416: 442–447.
- Park, J. H., 2002 Downloading central clock information in *Drosophila*. *Mol. Neurobiol.* 26: 217–233.
- Qian, M., G. Pan, L. Sun, C. Feng, and Z. Xie *et al.*, 2007 Receptor-like tyrosine phosphatase PTP10D is required for long-term memory in *Drosophila*. *J. Neurosci.* 27: 4396–4402.
- Rawls, J., 2006 Analysis of pyrimidine catabolism in *Drosophila melanogaster* using epistatic interactions with mutations of pyrimidine biosynthesis and β -alanine metabolism. *Genetics* 172: 1665–1674.
- Renn, S. C. P., J. H. Park, M. Rosbash, J. C. Hall, and P. H. Taghert, 1999 A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* 99: 791–802.
- Rørth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm *et al.*, 1998 Systematic gain-of-function genetics in *Drosophila*. *Development* 125: 1049–1057.
- Rosato, E., and C. P. Kyriacou, 2006 Analysis of locomotor activity rhythms in *Drosophila*. *Nat. Protoc.* 1: 559–568.
- Rubin, G., L. Hong, P. Brokstein, M. Evans-Holm, E. Frise *et al.*, 2000 A *Drosophila* complementary DNA resource. *Science* 287: 2222–2224.
- Schmidt, E., and S. Strittmatter, 2007 The CRMP family of proteins and their role in Sema3A signaling. *Adv. Exp. Med. Biol.* 600: 1–11.
- Schmidt, E., S. Shim, and S. Strittmatter, 2008 Release of MICAL autoinhibition by Semaphorin-Plex signaling promotes interaction with collapsin response mediator protein. *J. Neurosci.* 28: 2287–2297.
- Schnackerz, K., and D. Dobritzsch, 2008 Amidohydrolases of the reductive pyrimidine catabolic pathway purification, characterization, structure, reaction mechanisms and enzyme deficiency. *Biochim. Biophys. Acta* 1784: 431–444.
- Schwaerzel, M., M. Heisenberg, and T. Zars, 2002 Extinction antagonizes olfactory memory at the subcellular level. *Neuron* 35: 951–960.
- Shuai, Y., B. Lu, L. Wang, K. Sun, and Y. Zhong, 2010 Forgetting is regulated through Rac activity in *Drosophila*. *Cell* 140: 579–589.
- Simmons, A., J. Rawls, J. Piškur, and J. Davidson, 1999 A mutation that uncouples uncouples allosteric regulation of carbamyl phosphate synthetase in *Drosophila*. *J. Mol. Biol.* 287: 277–285.
- Stenmark, P., D. Ogg, S. Flodin, A. Flores, T. Kotenyova *et al.*, 2007 The structure of human collapsin response mediator protein 2, a regulator of axonal growth. *J. Neurochem.* 101: 906–917.
- Su, K., W. Chien, W. Fu, I.-S. Yu, H. Huang *et al.*, 2007 Mice deficient in collapsin response mediator protein-1 exhibit impaired long-term potentiation and impaired spatial learning and memory. *J. Neurosci.* 27: 2513–2524.
- Takemoto, T., Y. Sasaki, N. Hamajima, Y. Goshima, M. Nonaka *et al.*, 2000 Cloning and characterization of the *Caenorhabditis elegans* CeCRMP/DHP-1 and -2; common ancestors of CRMP and dihydropyrimidinase? *Gene* 261: 259–267.
- Tanaka, N., A. Arnova, and B. Schwer, 2007 Ntr1 activates the Prp43 helicase to trigger release of lariat-intron from the spliceosome. *Genes Dev.* 21: 2312–2325.

- Terman, J., T. Mao, R. Pasterkamp, H. Yu, and A. Kolodkin, 2002 MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. *Cell* 109: 887–900.
- Tseng, A., and I. Hariharan, 2002 An overexpression screen in *Drosophila* for genes that restrict growth or cell-cycle progression in the developing eye. *Genetics* 162: 229–243.
- Tully, T., and W. G. Quinn, 1985 Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 157: 263–277.
- Tully, T., T. Preat, S. C. Boynton, and M. Del Vecchio, 1994 Genetic dissection of consolidated memory in *Drosophila*. *Cell* 79: 35–47.
- Tweedie, S., M. Ashburner, K. Falls, P. Leyland, P. McQuilton *et al.*, 2009 FlyBase: enhancing *Drosophila* gene ontology annotations. *Nucleic Acids Res.* 37: D555–D559.
- Uchida, Y., T. Ohshima, Y. Sasaki, H. Suzuki, S. Yanai *et al.*, 2005 Semaphorin3A signalling is mediated via sequential Cdk5 and GSK3 β phosphorylation of CRMP2: implication of common phosphorylating mechanism underlying axon guidance and Alzheimer's disease. *Genes Cells* 10: 165–179.
- Van Kuilenburg, A., H. Van Lenthe, and A. Van Gennip, 2006 Activity of pyrimidine degradation enzymes in normal tissues. *Nucleosides Nucleotides Nucleic Acids* 25: 1211–1214.
- Wang, L., and S. Strittmatter, 1996 A family of rat CRMP genes is differentially expressed in the nervous system. *J. Neurosci.* 16: 6197–6207.
- Wang, L., and S. Strittmatter, 1997 Brain CRMP forms heterotetramers similar to liver dihydropyrimidinase. *J. Neurochem.* 69: 2261–2269.
- Wu, C., S. Xia, T. Fu, H. Wang, Y. Chen *et al.*, 2007 Specific requirement of NMDA receptors for long-term memory consolidation in *Drosophila* ellipsoid body. *Nat. Neurosci.* 10: 1578–1586.
- Yamashita, N., and Y. Goshima, 2012 Collapsin response mediator proteins regulate neuronal development and plasticity by switching their phosphorylation status. *Mol. Neurobiol.* 45: 234–246.
- Yang, J., L. Porter, and J. Rawls, 1995 Expression of the dihydroorotate dehydrogenase gene, *dhod*, during spermatogenesis in *Drosophila melanogaster*. *Mol. Gen. Genet.* 246: 334–341.
- Yoshimura, T., Y. Kawano, N. Arimura, S. Kawabata, A. Kikuchi *et al.*, 2005 GSK-3 β regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 120: 137–149.
- Xu, S., Y. Liu, Y. Yang, W. Jiang, E. Arnold *et al.*, 2003 Crystal structure of D- hydantoinase from *Burkholderia pickettii* at a resolution of 2.7 Angstroms: insights into the molecular basis of enzyme thermostability. *J. Bacteriol.* 185: 4038–4049.
- Yin, J. C., J. S. Wallach, M. Del Vecchio, E. L. Wilder, H. Zhou *et al.*, 1994 Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* 79: 49–58.
- Yin, J. C., M. Del Vecchio, H. Zhou, and T. Tully, 1995 CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*. *Cell* 81: 107–115.
- Zars, T., M. Fischer, R. Schulz, and M. Heisenberg, 2000 Localization of a short-term memory in *Drosophila*. *Science* 288: 672–675.

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Divergent Functions Through Alternative Splicing: The *Drosophila* *CRMP* Gene in Pyrimidine Metabolism, Brain, and Behavior

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File S1 Isolation and characterization of *CRMP* null mutations

Isolation of new mutants: *CRMP^{supK1}* was recovered following hexamethylphosphoramide (HMPA) mutagenesis (NAIRZ *et al.* 2004) and selected by its ability to block suppression of the *black* body phenotype by the semidominant *r^{Su(b)}* mutation (RAWLS 2006). HMPA-treated *w/Y; b; +* males were mated with *w; b; ri CRMP^{supA4} p^p P{r^{Su(b).CSa}}* females. Among the progeny, the *CRMP^{supK1}* mutation was isolated from a rare *black* body animal. The *CRMP^{supla1}* mutation was isolated by imprecise excision of the *P{EP}* transposon within the *EP(3)3238* strain, using crosses and *r*-wing phenotypic suppression criteria described previously to obtain *CRMP* deletion mutations (RAWLS 2006). For both of these mutations, homozygous animals are viable, fertile and normal in overall morphology.

Characterization of the *CRMP^{supK1}* mutation: Genomic DNA spanning the lesions in mutant animals was isolated by PCR and sequenced. The *CRMP^{supK1}* mutation is a deletion of 153 bp of exon E4-intron I4 sequences (Figure 1), with an insertion of six nucleotides (Genbank GU452677). The predicted open reading frame created by the E4-I4 fusion terminates at an in-frame nonsense codon within intron I4, probably resulting in a severely truncated protein product consisting of only the N-terminal 10% of the CRMP/DHP protein.

Mutations of intron splice sites can result in use of novel splice sites and it is conceivable that unanticipated novel splicing might somehow restore the normal translational reading frame downstream from the *CRMP^{supK1}* mutant site, resulting in restoration of significant downstream portions of the protein. Therefore, several tests were performed to detect alternative splicing that might result in a partially functional product. First, the genetic epistasis assay described above failed to detect DHP activity in *CRMP^{supK1}* animals (*r^{Su(b)} b CRMP^{supK1}* animals have darkly pigmented, typically *black* cuticle). Second, a variety of primers were used in experiments to obtain reverse transcriptase-coupled PCR products from RNA isolated from *CRMP^{supK1}* adults, detecting only transcripts containing the predicted E4-I4 fusion sequence (data not shown). Lastly, the *P{PYD2GFPsupK1}* transgene was created in which the *CRMP^{supK1}* deletion was substituted into the *P{PYD2GFP}* construct; this construct should result in GFP expression in transgenic animals only if the protein open reading frame were somehow restored downstream from the *CRMP^{supK1}* lesion. In animals from two independently isolated *P{PYD2GFPsupK1}* strains, no GFP fluorescence was detected in larvae. In summary, the *CRMP^{supK1}* mutation appears to produce, at best, a severely truncated protein product.

Characterization of the *CRMP^{supla1}* mutation: Genomic DNA spanning the lesion in mutant animals was isolated by PCR and sequenced. The *CRMP^{supla1}* mutation is a deletion of 5380 bp (Genbank GU452678), removing all of exons E1 through E9a.

Nairz, K., P. Zipperlen, C. Dearolf, K. Basler, and E. Hafen, 2004 A reverse genetic screen in *Drosophila* using a deletion-inducing mutagen. *Genome Biol.* 5: R83.

Figure S1 Exon E9a is required for DHP. (A) The *black* body phenotype (dark gray cuticle) results from reduced levels of β -alanine (dark gray cuticle of a $w/Y; b; \square CRMP/TM3, CRMP^+$ male). (B) The $P\{r^{Su(b)}\}$ transgene enhances β -alanine levels, suppressing of the *black* body phenotype (brownish cuticle of a $w/Y; b; CRMP^{supA4} P\{r^{Su(b)}\}/TM3, CRMP^+$ male). (C) *CRMP* mutation blocks formation of β -alanine from pyrimidines, disrupting $P\{r^{Su(b)}\}$ suppression of the *black* body phenotype (dark gray cuticle of a $w/Y; b; CRMP^{supA4} P\{r^{Su(b)}\}/\square CRMP$ male). (D) An intact *CRMP* transgene supports the suppression of *black* by $P\{r^{Su(b)}\}$ (brownish cuticle of a $w/Y; P\{PYD2GFP\} b/b; \square CRMP P\{r^{Su(b)}\}/CRMP^{supK1}$ male). (E) A *CRMP* transgene with disrupted exon E9a does not support suppression of *black* by $P\{r^{Su(b)}\}$ (dark gray cuticle of a $w/Y; b; CRMP^{supA4} P\{r^{Su(b)}\}/CRMP^{supK1} P\{PYD2GFPfs9a\}$ male). (F) A *CRMP* transgene with disrupted exon E9b supports suppression of *black* by $P\{r^{Su(b)}\}$ (brownish cuticle of a $w P\{PYD2GFPfs9b\}/Y; b; CRMP^{supA4} P\{r^{Su(b)}\}/CRMP^{supK1}$ male). Cuticle phenotypes are most readily observed in pigmentation of the wing, leg, and dorsal thorax; all photographs were made with identical exposure settings. Phenotypic variations in eye pigmentation, bristle morphology, and wing morphology are due to transgene and chromosomal markers used in the crosses producing these flies.

File S2 Misexpression phenotypes observed in adult eyes

I. Descriptions and frequencies of the eye phenotypes represented in Figure 5 are provided below:

P{GMR-GAL4}/P{UAS-Ras85D}5-1; CRMP^{+/supK1} animals exhibit a rough eye phenotype that results from some enlarged ommatidia and irregular alignment of ommatidia (Fig. 5A); this phenotype was observed in all animals observed (47 total). All *P{GMR-GAL4}/P{UAS-Ras85D}5-1; CRMP^{supK1/supK1}* siblings (55 animals observed) exhibited normal ommatidial arrangement (Fig. 5B). Overall eye areas are similar among animals of these genotypes.

P{GMR-GAL4}/P{UAS-sggB}; CRMP^{+/supK1} animals die during various stages of pupal development. Dissection of pupae that survived to late pupal stages revealed severely undeveloped head structures (Fig. 5C). *P{GMR-GAL4}/P{UAS-sggB}; CRMP^{supK1/supK1}* siblings regularly survived as adults exhibiting a rough eye phenotype that resulted from some enlarged ommatidia and irregular alignment of ommatidia (Fig. 5D).

P{ey-GAL4}/P{UAS-sggB}; CRMP^{+/supK1} animals die during at various stages of pupal development. Dissection of pupae that survived to late pupal stages revealed severely undeveloped head structures (Fig. 5E). *P{ey-GAL4}/P{UAS-sggB}; CRMP^{supK1/supK1}* siblings regularly survived as adults exhibiting a rough eye phenotype that resulted from reduced number and irregular alignment of ommatidia (Fig. 5F).

The eyes of all *P{sev-EP-GAL4}/P{UAS-Rac1.V12}; CRMP^{+/supK1}* animals were morphologically normal (Fig. 5G; 37 animals observed). The eyes of all *P{sev-EP-GAL4}/P{UAS-Rac1.V12}; CRMP^{supK1/supK1}* siblings were severely reduced, with extensive fusion of ommatidia (Fig. 5H; 45 animals observed). Areas of eyes were measured (described in Materials & Methods); the two genotypes differed significantly ($p < 0.05$).

Both *P{sev-EP-GAL4}/P{UAS-Rac1.N17}; CRMP^{+/supK1}* and *P{sev-EP-GAL4}/P{UAS-Rac1.N17}; CRMP^{supK1/supK1}* siblings exhibited reduced eye size with irregular alignment of ommatidia (Figs. 5I and J). Phenotypes were very similar in all animals of both genotypes grown at 25° or 29°. Growth at 19° produced milder phenotypes that were similar for the two genotypes.

P{GMR-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{+/supK1} animals exhibited a rough eye phenotype that resulted from fusion and irregular alignment of ommatidia (Fig. 5K); this phenotype was observed in all animals observed (67 animals). All *P{GMR-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{supK1/supK1}* siblings (78 animals) exhibited severely reduced eyes with fused ommatidia (Fig. 5L). Areas of eyes were measured (described in Materials & Methods); the two genotypes differed significantly ($p < 0.05$).

The eyes of all *P{ey-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{+/supK1}* animals were uniformly normal (Fig. 5M; 48 animals observed). Considerable variation was found in severity of the eye phenotypes of *P{ey-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{supK1/supK1}* animals: normal eye, normal-size eye with misalignment of a few ommatidial rows, eyes with reduced number and misaligned

ommatidia (Fig. 5N), absence of all ommatidia. This variation is observed among eyes of the same animal. All *P{ey-GAL4}/P{EP}Rac2^{EP3118}; CRMP^{supK1/supK1}* animals exhibited at least one abnormal eye (49 animals observed).

Eyes of *P{elav-GAL4};P{EP}Rac2^{EP3118}/+; CRMP^{+/supK1}*, animals were uniformly normal (Fig. 5O; 154 animals observed), whereas all *P{elav-GAL4}; P{EP}Rac2^{EP3118};/+; CRMP^{supK1/supK1}* siblings exhibited a rough eye phenotype due to irregular alignment of ommatidia (Fig. 5P; 68 animals observed). Overall eye area is similar among these flies.

II. Additional misexpression phenotypes that are not affected by *CRMP* mutation (photos not shown):

Eyes of *P{sev-EP-GAL4}/P{UAS-Rho1.V14}; CRMP^{+/supK1}* animals and *P{sev-EP-GAL4}/P{UAS-Rho1.V14}; CRMP^{supK1/supK1}* siblings reared at 25° are reduced in size and rough, due to fewer and irregularly aligned ommatidia. No differences were detected in the eye phenotypes of these two genotypes.

The eyes of *P{sev-EP-GAL4}/P{UAS-Ras64B.V14}; CRMP^{+/supK1}* animals and *P{sev-EP-GAL4}/P{UAS-Ras64B.V14}; CRMP^{supK1/supK1}* siblings are phenotypically similar. When raised at 19°, both genotypes result in normal eye size and ommatidial arrangement. When raised at 25° or 29°, eyes produced by both genotypes are normal in size but rough, due to enlargement of some ommatidia and irregular alignment of ommatidia.

Figure S2 *CRMP* mutant flies display normal olfactory acuity and shock reactivity. Because the Pavlovian olfactory learning and memory assay is based upon association of two stimuli (odor discernment and electric shock stimulus, Dubnau and Tully 1998), *CRMP* mutant animals were tested to ensure that they are unaffected in their abilities to sense and respond to olfactory stimulation and electrical shock. For each trial to test olfactory acuity, one hundred untrained flies of each genotype were exposed to a two-minute test trial in the T-maze (Boynton & Tully 1992), in which they were offered a choice between either octanol (Oct) versus untainted room air (Panel A) , or methylcyclohexanol (MCH) versus untainted room air (Panel B). The performance index (PI) for each genotype (computed as described in Materials & Methods) is represented in the panels as mean PI $\pm$ standard error for eight trials. *CRMP^{supK1}* and *CRMP^{supla1}* animals avoided the odors of octanol and methylcyclohexanol to the same degree as the wild-type control animals, indicating that perception of these odors and the activation of relevant motor circuits are normal in mutant flies. Shock reactivity was examined by exposing one hundred untrained flies of each genotype to a two-minute test trail in the T-maze, but this time each arm of the T-maze contained an electric shock grid (Dura *et al.* 1993). The flies were given a choice between shock (60V) versus no shock. Panel C shows mean PI $\pm$ standard error (four trials) for shock reactivity; again, the performance of mutant animals was indistinguishable from that of wild-type control animals. We conclude that mutant animals exhibit normal sensorimotor responses required for the aversive Pavlovian olfactory conditioning assay.

Boynton, S., and T. Tully, 1992 *latheo*, a new gene involved in associative learning and memory in *Drosophila melanogaster*, identified from P-element mutagenesis. *Genetics* 131: 655–672.

Figure S3 Circadian locomotor activity of normal and *CRMP* mutant flies. Histograms show locomotion activity levels of various genotypes during successive 24 hr periods. The first three days of data collection occurred under a 12:12 hr LD cycle (entrainment); data collection during days four through eight occurred under 24 hr constant darkness (DD). Bar heights indicate mean activity events per 30 minute bin per fly. In order to visualize the overlaps of consecutive days, the histogram of the following day is duplicated to the right of each LD and DD day. These data are summarized in Table 1, which includes the number of flies tested for each genotype and results of statistical analysis of data.

Figure S4 PDF expression in the adult brain. The number of PDF-positive neurons, including both I-LNvs and s-LNvs neuronal groups in the brain, and their projection patterns appeared normal in the two mutant strains. Whole-mount anti-PDF-immunohistochemistry was performed in dissected adult brains, as described in BAHN *et al.* 2009. A) wildtype (*isoC11* strain) animals (10 brains examined); B) *CRMP^{supK1}* animals (11 brains examined); C) *CRMP^{supla1}* animals (10 brains examined). Right panels are magnified images of the red-linked boxes in the left panels. White arrowheads indicate s-LNvs. Red arrowheads indicate dorsal projections stemming from s-LNvs.