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EXTRA VIEW

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A triangular connection between Cyclin G, PP2A and Akt1 in the regulation of growth and metabolism in *Drosophila*

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ABSTRACT

Size and weight control is a tightly regulated process, involving the highly conserved Insulin receptor/target of rapamycin (InR/TOR) signaling cascade. We recently identified Cyclin G (CycG) as an important modulator of InR/TOR signaling activity in *Drosophila*. *cycG* mutant flies are underweight and show a disturbed fat metabolism resembling TOR mutants. In fact, InR/TOR signaling activity is disturbed in *cycG* mutants at the level of Akt1, the central kinase linking InR and TORC1. Akt1 is negatively regulated by protein phosphatase PP2A. Notably the binding of the PP2A B′-regulatory subunit Widerborst (Wdb) to Akt1 is differentially regulated in *cycG* mutants, presumably by a direct interaction of CycG and Wdb. Since the metabolic defects of *cycG* mutant animals are abrogated by a concomitant loss of Wdb, CycG presumably influences Akt1 activity at the PP2A nexus. Here we show that Well rounded (Wrd), another B′ subunit of PP2A in *Drosophila*, binds CycG similar to Wdb, and that its loss ameliorates some, but not all, of the metabolic defects of *cycG* mutants. We propose a model, whereby the binding of CycG to a particular B′-regulatory subunit influences the tissue specific activity of PP2A, required for the fine tuning of the InR/TOR signaling cascade in *Drosophila*.

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The co-ordination of organismal growth during development involves a tight network of regulating factors. In recent years, research notably in *Drosophila* has uncovered that both, extrinsic and intrinsic signals are engaged in the complex regulation of this process. A constant and well balanced nutrient supply is key to organismal growth and development, which needs to be sensed and recognized by intrinsic signaling pathways. The Insulin receptor (InR)/target of rapamycin (TOR) signaling cascade is the unchallenged first address to assure a well balanced answer toward nutrient conditions, thereby coordinating the proper regulation of growth and energy storage. Dysfunction of this 'nutrient sensor' significantly increases the risk of diseases like cancer, cardiovascular disease, obesity or diabetes in humans.[1-5] Although failure of InR/Tor activity might be especially interesting from a medical point of view, many details on the modus operandi concerning the InR/Tor signaling network was gained in the model organism *Drosophila*. Due to the fact that the core signaling components are highly conserved in metazoans, insights gained from the genetically tractable model *Drosophila* help to improve our understanding of InR/Tor signaling in general.

In the center of this signaling cascade, a relay of kinases is activated by phosphorylation events, thereby ensuring a rapid and consequent response to InR activation. Upon binding to one of several *Drosophila* insulin like peptides (Dilps), the single InR of *Drosophila* initiates the intracellular kinase cascade by recruiting the phosphatidyl-inositol 3-kinase (PI3K) to the plasma membrane thereby increasing membrane tethered phosphoinositol (3,4,5) triphosphate (PIP3). As a further consequence, the protein kinase Akt1 is recruited to the membrane and activated through phosphorylation by PDK1.[6-9] The kinase Akt1 represents one of the central players in the pathway influencing different processes, such as metabolism, cell survival, gene transcription and cell cycle control.[10] The path leading to the regulation of metabolism goes on with the Ser/Thr kinase TORC1. TORC1 is activated by an Akt1-dependent derepression mechanism:

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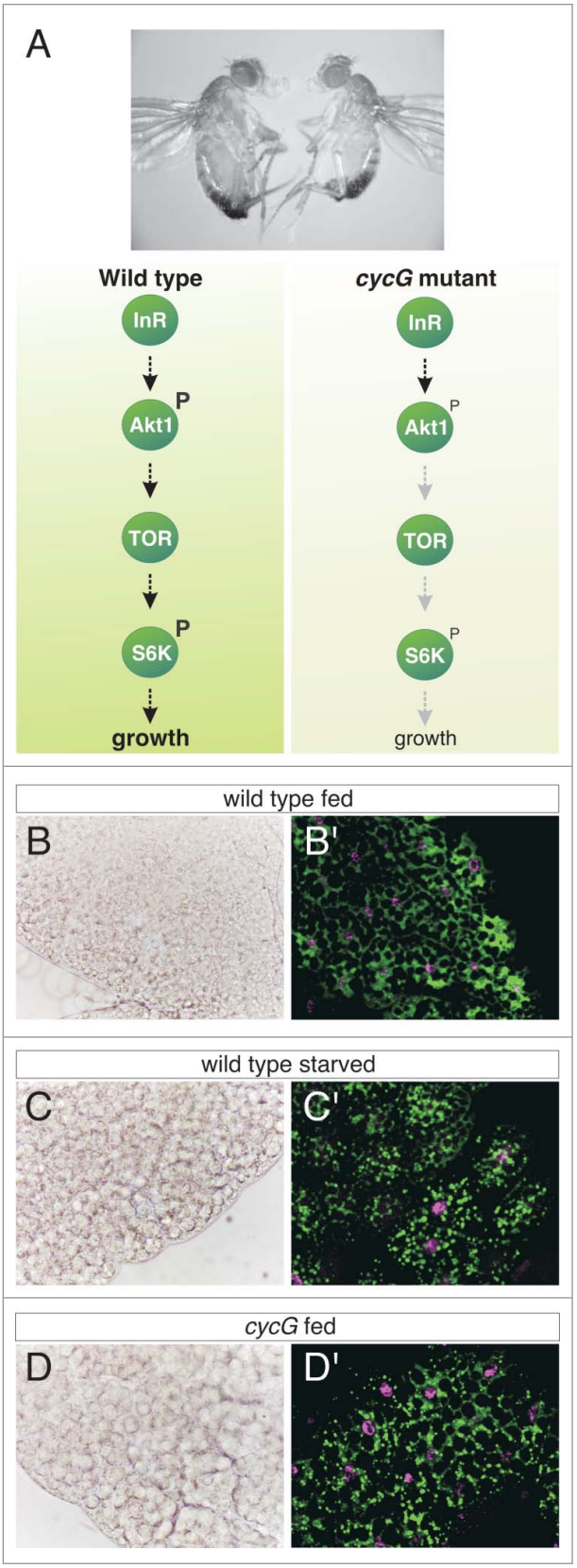
Through phosphorylation of the (TORC1) negative regulator Tsc2 by Akt1, the GTPase Ras homolog enriched in brain (Rheb) and consequently TORC1 are activated.[11-13] TORC1 activity is central to the control of growth and metabolism, acting as a nutrient sensor for growth regulation.[3,14,15] Hence, Akt1 serves as a link between InR and TOR pathways. Although the core components of InR/TOR signaling are well known, the spectrum of modulators is still expanding and the discovery of new regulators/mediators is ongoing.



cyclin G (cycG) is a positive regulator of InR/TOR activity in Drosophila

We recently established a positive role for *cyclin G (cycG)* in the regulation of growth and metabolism in *Drosophila* (Fig. 1A).[16] In order to analyze the function of *Drosophila cycG* during development, we generated 2 independent *cycG* null mutant alleles using both, ends-in and ends-out homologous recombination techniques for the knock out.[17,18] The resulting mutant flies are homozygous viable, although developmentally delayed and underrepresented when compared to the number of heterozygous siblings.[16] Moreover, females are sterile and lay ventralized eggs, as a result from a defect in recombination repair.[19,20] Notably, *cycG* mutant flies and larvae are underweight, show signs of starvation and have a disturbed fat metabolism (Fig. 1A). The latter is reflected by a reduced specific weight as measured in a buoyancy assay, by an increase in triacylgycerol levels relative to total protein content and by an accumulation of lipid droplets in larval oenocytes.[16] These phenotypes are typical of a downregulation of TOR signaling activity.[21] Accordingly, the TORC1 kinase targets S6K and 4E-BP show lower phosphorylation levels in the homozygous mutants when compared to the control flies (Fig. 1A).[16]

Figure 1. *Cyclin G* mutants affect InR/TOR signaling. (A) The upper panel shows the size reduction of $cycG^{HR7}$ mutants (right) compared to wild type (left). Underneath, a scheme of the InR/TOR signaling cascade is shown. (B-D) Small lipid droplets are seen in the fat body of a well-fed wild type larva (B). Droplet size increases in the case of amino acid deprivation (C). Similar sized droplets are seen in a $cycG^{HR7}$ mutant larva under fed conditions (D). (B'-D') Double staining of lysosomes (green) and nuclei (magenta) in larval fat body is shown. In well-fed wild type larvae no signs of autophagy are seen (B'), whereas starved larvae reveal a conspicuous enrichment of a punctate lysosomal staining (C'), which is likewise detected in well-fed $cycG^{HR7}$ mutant larvae (D').

TOR activity regulates growth during development by coupling growth factor signaling and nutrient availability. Starvation, and likewise loss of TOR, causes an aggregation of lipid vesicles in the larval fat body.[22] This can be interpreted as evidence of early fat mobilization for energy consumption, an observation never made in well-fed wild type larvae. Similarly, in *cycG* mutant larvae, lipid vesicles are increased in size compared to well fed wild type larvae, matching those of starved controls (Fig. 1B-D).

Autophagy is a more drastic nutritional deprivation response regulated by TOR. This lysosomal degradation process of cytoplasmic cell contents ensures survival of the organism in the absence of food, and also occurs in the late third instar larval stage and during metamorphosis.[21,23] Under starvation, cells within the larval fat body undergo autophagy to provide nutrients to the developing imaginal tissues.[24,25] In agreement with the other findings, autophagy was observed in *cycG* mutant larvae under feeding conditions: cells in the fat body showed a punctate staining with a lysosome-specific marker, which was also seen in starved but not in well-fed wild type animals (Fig. 1B'-D').

CycG regulates InR/TOR activity at the Akt1/PP2A nexus

Addressing the role of CycG in the InR/TOR regulatory network, we noted a reduced phospho-status at Serine 505 of the central kinase Akt1 (Fig. 1).[16] In the absence of CycG, no changes in the activity of the upstream PI3K kinase were observed, however, indicating that CycG presumably acts at the level of Akt1. Accordingly, elevated levels of Akt1 ameliorated *cycG* mutant defects.[16] It is well known that the protein phosphatase PP2A counteracts Akt1 by dephosphorylation, and hence may serve as a regulatory target of CycG.[26,27] PP2A consists of 3 subunits, a constant catalytic subunit C (Microtubule star [Mts] in *Drosophila*; corresponding to mammalian PP2Ac), a scaffolding subunit A (PP2A-29B in *Drosophila*; corresponding to mammalian PP2A-A or PR65) and a context-dependent regulatory subunit B (Twins [Tws] in *Drosophila*, corresponding to mammalian PP2A-B, B55 or PR55) or B′ (Widerborst [Wdb] and Well rounded [Wrd] in *Drosophila*, corresponding to mammalian PP2A-B′, B56 or PR61 and PR72) (Fig. 2A).[28] The regulatory B′-subunit Widerborst (Wdb) inhibits InR/TOR signaling by directing the PP2A to dephosphorylate Akt1.[27] CycG protein directly binds to Wdb in vitro and in vivo, suggesting a mechanistic link in the regulation of PP2A.[16] In fact, CycG binds to Wdb protein while in the PP2A protein complex, as we could co-precipitate the catalytic subunit Mts, the scaffolding subunit PP2A-29B as well as Wdb using antibodies directed against CycG protein (Fig. 2B). Interestingly, CycG binding to Wdb may not simply inhibit PP2A activity but rather seems to modify its specificity. Activated Akt1 kinase appears to exist in different subcellular pools that control different cellular processes in *Drosophila*. For example, Akt1 is mostly cytoplasmic in ovarian nurse cells, but rather membrane localized in eye imaginal discs.[27,29] Lipid metabolism in the ovary is regulated by Akt1 under the control of Wdb, involving a direct molecular interaction of the 2 proteins not seen under normal conditions in imaginal tissues.[27,29] This specific interaction appears to require CycG, since in *cycG* mutants, Akt1 and Wdb can be co-precipitated from extracts of fly heads.[16] This result suggests that in the absence of CycG, Wdb binding to Akt1 is facilitated, resulting in an increased dephosphorylation, and hence inactivation of Akt1 and downstream TOR signaling activity.

CycG interacts with the PP2A B′ subunit Well rounded (Wrd) molecularly as well as genetically

There is a second B′ subunit of PP2A in *Drosophila*, called Well rounded (Wrd) or PP2A-B′, and like Wdb, Wrd is also involved in the regulation of InR/TOR signaling activity. However, this interaction occurs at the level of the kinase S6K, a direct TORC1 target (Fig. 1A).[30] Similar to the role of Wdb in the inhibition of Akt1, Wrd targets PP2A to dephosphorylate and inhibit S6K, based on a direct molecular interaction between Wrd and S6K proteins.[30] S6K is required for ribosome biogenesis and enhances protein synthesis. It acts as pivotal effector of the InR/TOR signaling pathway, promoting cell growth and proliferation.[31] Potential interactions between CycG and the various PP2A subunits were tested, and strong binding of CycG to Wdb was observed, and, to a lesser degree, to Wrd (Fig. 2C).[16] Both Wdb and Wrd bind to the cyclin domains of CycG (Fig. 2D).[16] These data illustrate the potential of CycG to modulate PP2A complexes containing the B′ regulatory subunit Wrd, and hence its specific targets.

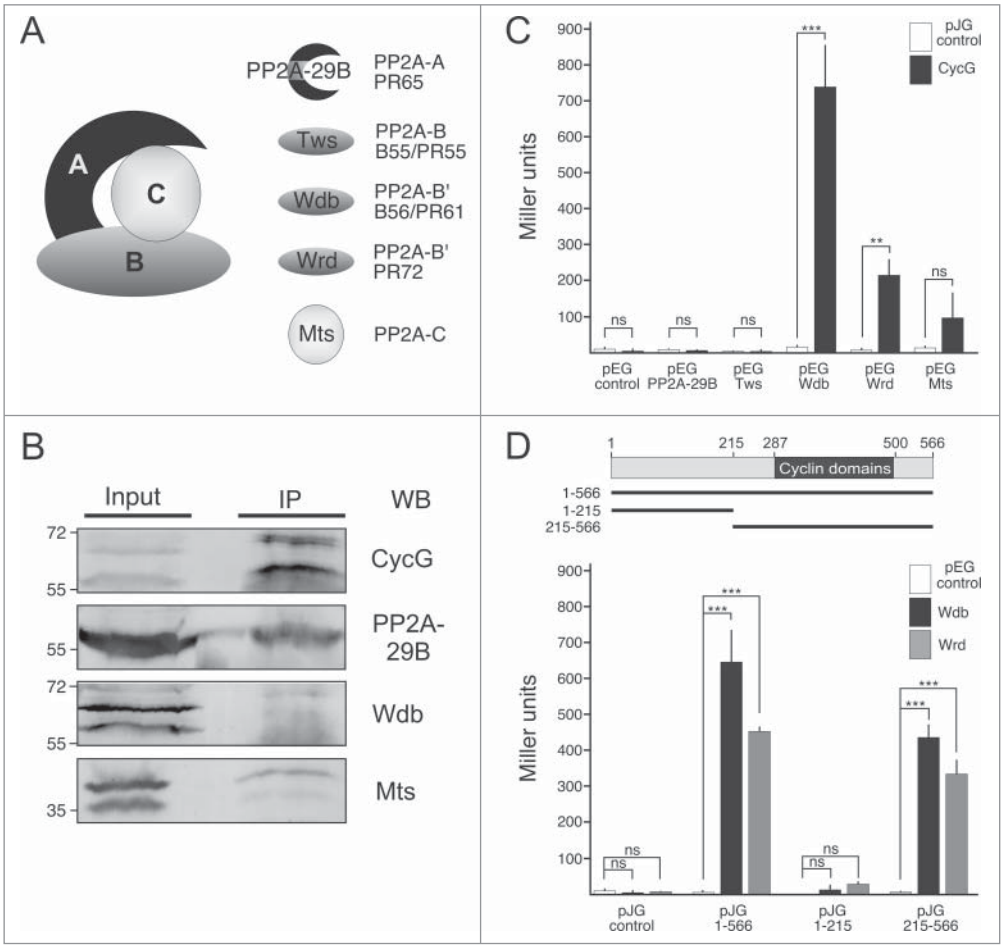


Figure 2. CycG interacts with the Wdb-PP2A complex and with Wrd. (A) Illustration of the heterotrimeric PP2A complex in *Drosophila*: The catalytic subunit Microtubule stars/Mts (PP2A-C) binds to the scaffold protein PP2A-29B (PP2A-A, called PR65 in mammals). Three regulatory subunits are known, PP2A-B called Twins/Tws (mammalian B55 aka PR55), and 2 PP2A-B′ subunits called Widerborst/Wdb (homolog of mammalian B56 aka PR61) and Well rounded/Wrd (homolog of mammalian PR72). (B) CycG binds the Wdb-PP2A complex. CycG proteins were immunoprecipitated (IP) from *Drosophila* Schneider (S2) cells using CycG antibodies, and the precipitates were probed with antibodies directed against the different subunits of PP2A (WB). The input lane contains 25% of the protein extracts used for the IPs and was used for a size comparison of the precipitated proteins. Size of proteins is given in kDa. (C–D) Yeast 2-hybrid assays were conducted for detection of direct protein interactions. Quantitative assays (measured in Miller Units, ordinate) were performed with full length CycG in pJG vector (1-566 in D) and the 5 subunits of PP2A in pEG vector (C). pEG-Wdb and pEG-Wrd were tested for binding to full length CycG (1-566, D), the N-terminal part (1-215, D) and the C-terminal part (215-566, D) of the CycG protein cloned into pJG vector. A scheme of CycG including the Cyclin domains is given, numbers correspond to amino acids. Empty vectors served as negative controls. Error bars denote standard deviation (n = 3 independent experiments). $^{***}p < 0.001$, $^{**}p < 0.01$, ns: not significant according to Student's T-test.

If CycG regulates Wrd in a manner similar to Wdb, we would expect a likewise rescue of the metabolic defects specific to *cycG* mutants resulting from a loss of Wrd. Double mutants of *cycG* and *wrd* were generated, and their weight compared to control, homozygous *cycG, wrd, wdb* transheterozygous combinations, as well as to double mutant *wdb cycG* animals. As shown in Figure 3A, the double mutants *wdb cycG* were indistinguishable from controls, i.e. fully rescued, whereas the *wrd cycG* animals exhibited a phenotype just as the homozygous *cycG* or *wrd* mutants. We next examined the weight of the mutants using a buoyancy assay: here, loss of either B′ subunits had a rescue effect, leading double mutants to be similar to control animals (Fig. 3B). Accordingly, the accumulation of lipid droplets in the larval oenocytes of the double mutants matched that of controls, whereas the *cycG* homozygotes showed increased droplet levels (Fig. 3C). These results show that regulation of body weight depends on CycG and Wdb but not Wrd, whereas regulation of lipid metabolism requires both B′ subunits Wdb and Wrd in a similar manner.

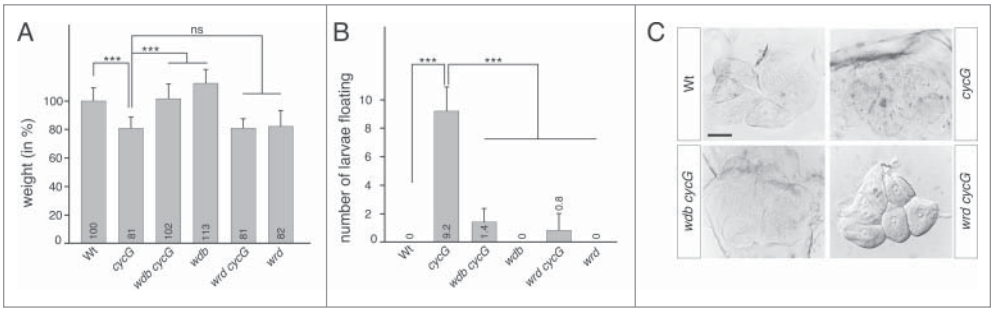


Figure 3. Analysis of *wrd cycG* double mutant phenotypes. (A) The weight reduction observed in $cycG^{HR7}$ mutants compared to Oregon R wild type control was not significantly ameliorated in wrd^{ko2} $cycG^{HR7}$ doubly mutant males, in contrast to *wdb cycG* double mutants (wdb^{14} $cycG^{HR7}$ / wdb^{dw} $cycG^{HR7}$). [16] Weight measurements of adult males are delineated in comparison to wild type flies which were set to 100%. The trans-heterozygous wdb^{14}/wdb^{dw} and the homozygous wrd^{ko2} were included for comparison. Error bars denote standard deviation (n = 100 per genotype). $^{***}p < 0.001$, ns: not significant according to Student's T-test. (B) Statistical analyses of buoyancy tests repeated 5 times with 10 larvae each. Whereas Oregon R wild type, wdb^{14}/wdb^{dw} and wrd^{ko2} larvae sink to the floor in 10% sucrose solution, $cycG^{HR7}$ mutants float due to their increased fat content. This effect was rescued in an either wdb^{14} $cycG^{HR7}$ / wdb^{dw} $cycG^{HR7}$ or wrd^{ko2} $cycG^{HR7}$ double mutant background, with only a small fraction of larvae still floating. [16] $^{***}p < 0.001$ according to Student's T-test. (C) Oil Red O staining reveals lipid droplet accumulation in larval oenocytes of $cycG^{HR7}$ mutants compared to wild type control. [16] No accumulation of lipid droplet was observed in the oenocytes from the double mutant combinations wdb^{14} $cycG^{HR7}$ / wdb^{dw} $cycG^{HR7}$ as well as wrd^{ko2} $cycG^{HR7}$ that match the control. Scale bar: 20μm.

CycG may modulate PP2A activity by influencing B′ subunit recruitment

PP2A is one of the few serine/threonine phosphatases that control the phospho-status of a large number of cellular phosphoproteins, including various kinases and other signaling molecules. PP2A regulates a plethora of cellular processes including cell proliferation and death, cell cycle control, cell motility as well as organismal growth and metabolism.[28] Through the recruitment of a regulatory B-type subunit, the heterodimeric core enzyme forms the heterotrimeric holoenzyme. The exquisite substrate specificity of PP2A holoenzyme is achieved through combinatorial interactions with a diverse array of regulatory B-type subunits.[32,33] Hence, assembly with the appropriate B-type subunit is pivotal to the specificity of PP2A. In mammals, 4 distinct classes of regulatory B-type subunits exist, each comprising several members.[28,32] In *Drosophila*, only 3 B-type subunits are known: a single B (Tws) and 2 B′ subunits (Wdb, Wrd) are sufficient to regulate substrate specificity of PP2A. The B′ subunits make extensive contacts to both, the scaffold and the catalytic subunits A and C of PP2A, and their acidic surface faces the active site of PP2A-C, allowing for substrate recognition.[33] Based on the structure of the PP2A holoenzyme, the major role of the regulatory B-type subunits is to target substrate proteins to the phosphatase.[33]

We have shown that CycG is an important regulator of growth and lipid metabolism in *Drosophila*, acting as a positive modulator of InR/TOR signaling activity at the PP2A/Akt1 nexus.[16] Since a loss of PP2A activity, or more specifically, of either regulatory B′ subunit Wdb or Wrd, ameliorates the loss of CycG

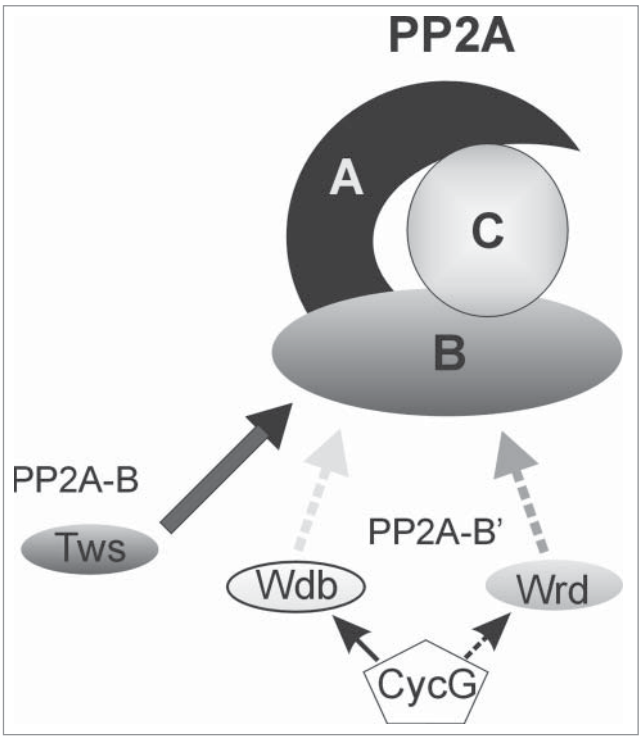


Figure 4. Model of CycG activity. By binding differentially to B′ subunits, CycG may influence their recruitment into PP2A trimeric complexes, thereby affecting PP2A specificity and activity in a context-dependent manner.

activity, formally CycG acts as a negative regulator of PP2A, i.e., *cycG* mutant phenotypes may be interpreted as a gain of PP2A activity in the metabolic context. CycG binds directly to the B′ subunit Wdb, and to a lesser extent to Wrd. Most likely this direct molecular interaction negatively modulates PP2A activity (Fig. 4). We do not think, however, that the binding per se interferes with PP2A phosphatase activity. We rather propose that CycG influences B′ subunit recruitment, and thereby PP2A specificity. Prerequisite for this model is the binding of CycG to the B′ subunit present in the PP2A trimeric complex, which occurs in vivo, as we have demonstrated here for Wdb (Fig. 2B). Moreover, the binding specificity of Wdb – and possibly of PP2A complexed to Wdb – is dependent on CycG: Wdb binding to Akt1 in imaginal tissues, but not in ovarian nurse cells, is only possible in the absence of CycG protein.[16,29] Perhaps PP2A is less likely to recruit a B′ subunit bound to CycG, which thereby influences PP2A subunit assembly, changing PP2A specificity for its targets (Fig. 4).

Cells have to be able to rapidly adapt to various environmental cues, for example to changing energy supply. B-type subunit assembly into PP2A holoenzyme is a dynamic process, representing a mechanism by which PP2A holoenzyme can rapidly alter specificity allowing adaptation to changing cellular demands.[32] The availability of a certain B′ regulatory subunit is a means to define the pool of specific PP2A holoenzymes present in the cell at any given time. If CycG is to determine B-type regulatory subunit availability, it is directly influencing PP2A specificity, and hence a vast array of cellular processes. In fact, CycG has been involved in a variety of cellular processes with little apparent commonalities.[16,19,30,34,35] Perhaps involvement of CycG in so many different contexts can be reconciled on the grounds of PP2A regulation in a cell type and tissue specific manner. The better we understand the various activities of CycG, the more likely will we be able to make sense of its pleiotropic roles in *Drosophila*.

Experimental procedures

Analysis of fly phenotypes

For this work $cycG^{HR7}$ was used.[19] The *cycG wdb* double mutant was a heteroallelic combination of wdb^{14} $cycG^{HR7}$ / wdb^{dw} $cycG^{HR7}$; details can be found in Fischer et al.[16] The *PP2A-B′*^{KO2}/TM6B (*wrd*) null mutant allele was obtained from A. Teleman;[30] it was recombined with $cycG^{HR7}$ to obtain a *wrd cycG* double mutant strain. The *wrd cycG* double mutant was molecularly verified by PCR reactions straddling the missing exon 5 in wrd^{ko2} and verifying the additional 350 bp deletion existing in the distal gene copy of $cycG^{HR7}$.[19,30] The following primer pairs were used for the PCR reaction: *Wrd KO* UP 5′ CCT GAG GTC CTC CGG CTA CTT GAT CCT T 3′ and *Wrd KO* LP 5′ TCC AAT AGT TGT AAT ACA AAT TGA TGG TCG ATA A 3′ as well as *HR7* UP 5′ ACT CGC CAT GGA CGC CCA CAG CT C 3′ and *HR7* LP 5′ TGG AAC GAG GCC ACG CTC ATG CAG G 3′. Weight measurements, buoyancy assays and Oil Red O stainings were performed exactly as described previously.[16]

Analysis of the fat body

For analyzing autophagy, third instar larvae were dissected in ice-cold PBS and stained with the nuclear dye Hoechst 33342 (1:2000) for thirty minutes at room temperature before the fat body was removed and incubated in the dark for two minutes with LysoTracker® (1:100 in PBS) (Invitrogen; Carlsbad CA, USA). The staining solution was removed, and the tissue was mounted in Vectashield (Vector lab; Burlingame CA, USA) and immediately documented with a Zeiss ApoTome using Axio-Imager Software (Carl Zeiss AG; Oberkochen, Germany). For amino acid deprivation, third instar larvae were starved on apple-juice plates [25% apple-juice, 2.4% saccharose, 2.4% agar] without any further food source for 14 hours. The larvae were dissected, and after fixation for 10 minutes in 4% paraformaldehyde the fat body was mounted in glycerol (80%) and directly examined with a Zeiss Axioskop coupled to an ES120 camera (Optronics, Goleta, CA), using Pixera (Santa Clara, CA) Viewfinder software, version 2.0. Pictures were assembled with Corel (Mountain View, CA) Photo-Paint and CorelDRAW software, version 9.0.

Analysis of protein-protein interactions

CycG protein was immuno-precipitated from Schneider S2 cells using guinea pig anti-CycG antibodies as described by Fischer et al. and references therein.[16] For detection either rat anti-CycG, rat anti-PP2A-29B or rat anti-Mts (kind gift of T. Uemura) or rat anti-Wdb was used.[16,34,36] Quantitative yeast 2 hybrid studies were performed according to Guarente.[37] Miller units are calculated

from the ratio of substrate turnover to cell density (1000x OD_{420}/time [min] x volume [ml] x OD_{600}). The pEG Wdb and pJG CycG constructs are described previously.[16] cDNA clones from PP2A-29B (LD10247), Tws (LD12394), Wrd (LD29902) and Mts (LD26077) were all obtained from DGRC, Bloomington IN, USA. Tws, Wrd and Mts were full length clones, whereas the cDNA from PP2A-29B lacks the first 88 aminoacids. For cloning in pEG vector cDNAs were PCR amplified and cloned as *Eco* RI / *Xho* I (Mts), *Bam* HI / *Not* I (PP2A-29B) or *Bgl* II (*Bam* HI) / *Not* I (Tws and Wrd) fragment into the pEG vector.[38]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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