14-3-3 proteins regulate Tctp–Rheb interaction for organ growth in *Drosophila*

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14-3-3 family proteins regulate multiple signalling pathways. Understanding biological functions of 14-3-3 proteins has been limited by the functional redundancy of conserved isotypes. Here we provide evidence that 14-3-3 proteins regulate two interacting components of Tor signalling in *Drosophila*, translationally controlled tumour protein (Tctp) and Rheb GTPase. Single knockdown of 14-3-3e or 14-3-3z isoform does not show obvious defects in organ development but causes synergistic genetic interaction with Tctp and Rheb to impair tissue growth. 14-3-3 proteins physically interact with Tctp and Rheb. Knockdown of both 14-3-3 isoforms abolishes the binding between Tctp and Rheb, disrupting organ development. Depletion of 14-3-3s also reduces the level of phosphorylated S6 kinase, phosphorylated Thor/4E-BP and cyclin E (CycE). Growth defects from knockdown of 14-3-3 and Tctp are suppressed by CycE overexpression. This study suggests a novel mechanism of Tor regulation mediated by 14-3-3 interaction with Tctp and Rheb.
The Tor signalling pathway is important for the regulation of cell growth, proliferation, survival and metabolism in eukaryotes. Abnormal regulation of Tor signalling can cause various diseases including cancer and diabetes. Tor is the catalytic kinase subunit of the TORC1 complex that consists of several regulatory components such as Raptor and Pras40. The TORC1 complex integrates extracellular signalling and intracellular processes\(^1\). Growth factors like insulin activate PI3k, leading to Akt activation. Akt is known to phosphorylate tuberous sclerosis complex 2 (Tsc2) to inhibit its GTPase-activating protein (GAP) activity for Rheb GTase\(^2\,3\). Hence, inactivation of the Tsc1/2 complex promotes Rheb function to activate Tor kinase\(^4\). A key role of Tor kinase is to stimulate protein synthesis for cell growth by phosphorylating ribosomal S6 kinase (S6k) and Thor (Drosophila 4E-BP). Lack of S6k results in a reduction of the cell size but not the cell number\(^5\). In Drosophila, Rheb and Tor kinase mutants show cell cycle arrest\(^6\,7\). Loss of Tor in imaginal discs leads to a reduced cell proliferation and cyclin E (CycE), the S-phase regulator. Further, the cell cycle arrest for mutant can be rescued by overexpressing CycE\(^8\), indicating that Tor signalling is required for the regulation of the CycE level, thus affecting cell proliferation.

Recent studies have shown that translationally controlled tumour protein (Tctp) is involved in Tor signalling\(^8\,\,13\). Tctp is a family of evolutionarily conserved proteins involved in a number of fundamental processes, including cell proliferation, apoptosis and DNA damage control\(^14\,\,17\). Tctp is upregulated in cancer cells, and its reduction results in the reversion of tumour phenotypes, implicating its role in tumorigenesis and tumour regression\(^14\,\,18\,\,19\). In Drosophila, Tctp is required for organ growth by promoting RhoB function for Tor signalling as a guanine nucleotide exchange factor\(^2\). The role of Tctp in Tor signalling has also been reported in other organisms\(^9\,\,13\). The function of Tctp family proteins in growth regulation seems to be conserved since Drosophila growth defects by Tctp knockdown can be restored by human Tctp\(^8\). Remarkably, functional conservation has also been found between animals and plants. For instance, Drosophila Tctp rescues embryonic lethality and cell proliferation defects in Arabidopsis Tctp (AtTctp) loss-of-function mutant. Consistent with this interspecies complementation, Drosophila Rheb not only interacts with Tctp but also with the plant AtTctp\(^13\). Since Rheb is essential for Tor activation, it is important to understand how the interaction between Tctp and Rheb is regulated.

Interestingly, 14-3-3 proteins interact with multiple regulators of Tor signalling such as Tsc2, Pras40 and Raptor in mammalian cells\(^20\,\,24\). 14-3-3 proteins are conserved adaptor molecules that control diverse signalling pathways\(^25\,\,26\), but their function in vivo has not been well characterized. Loss-of-function studies in Drosophila indicated that Pras40 is only required in ovary but not in other tissues\(^27\). Thus, it is important to identify physiologically critical functions of 14-3-3s and their interacting proteins in animal models. In Drosophila, 14-3-3 proteins are encoded by two genes, 14-3-3\(\alpha\) and 14-3-3\(\varepsilon\) (also named leo for leonardo)\(^28\). Null mutations of either 14-3-3\(\alpha\) or 14-3-3\(\varepsilon\) lead to embryonic lethality. However, some 14-3-3\(\alpha\)-homoyzgous mutants are viable, because lack of 14-3-3\(\alpha\) is compensated by elevated level of 14-3-3\(\varepsilon\) protein\(^29\). Drosophila 14-3-3 genes participate in Ras/Mapk signalling and neuronal differentiation\(^30\,\,31\). They are also known to modulate FoxO-mediated apoptosis\(^32\), Hippo signalling\(^33\,\,34\), and cell cycle regulation in syncytial nuclear division during embryogenesis\(^35\), but their roles in Tor signalling have not been studied.

In this study, we identify 14-3-3 proteins as binding partners for Tctp and Rheb. 14-3-3 genes show strong genetic interaction with Tctp and Rheb. 14-3-3 proteins physically interact with Tctp and Rheb. We provide evidence that 14-3-3 proteins are required for promoting the interaction between Tctp and Rheb. Loss of both 14-3-3 isoforms critically impairs organ development, and such defects are strongly suppressed by CycE. Our data suggest that 14-3-3 proteins regulate the interaction between Tctp and Rheb for organ growth, providing novel insights into their functions in Tor signalling.

**Results**

**Tctp and Rheb interact genetically with 14-3-3 genes.** We have attempted to identify new genes interacting with Tctp by searching for genetic modifiers of the reduced eye phenotype caused by Tctp RNA interference (RNAi) using Gal4/UAS system\(^36\). UAS-Tctp RNAi and eyeless (ey)-Gal4 were used to induce Tctp silencing mainly in undifferentiated cells of eye imaginal disc\(^8\). Knockdown of either 14-3-3\(\alpha\) or 14-3-3\(\varepsilon\) alone did not show gross abnormalities in the eye (Fig. 1b,c). In contrast, 14-3-3\(\alpha\) RNAi strongly enhanced the effects of Tctp knockdown in the eye, resulting in greatly reduced eyes (Fig. 1g) compared with a mild reduction by Tctp RNAi alone (Fig. 1f). We tested whether 14-3-3\(\varepsilon\) also shows genetic interaction with Tctp. Similar to 14-3-3\(\alpha\) RNAi, knockdown of 14-3-3\(\varepsilon\) enhanced the eye phenotypes of Tctp RNAi (Fig. 1b), while 14-3-3\(\varepsilon\) RNAi alone did not show obvious defects in the eye (Fig. 1c). 14-3-3\(\alpha\) or 14-3-3\(\varepsilon\) mutant clones generated in eye and wing discs also appeared to be similar in the pattern of Elav (neuron-specific nuclear marker) and Discs-large (Dlg; cell membrane marker), respectively, compared with the surrounding wild-type tissues (Supplementary Fig. 1). To further confirm the synergistic genetic interaction between Tctp and 14-3-3 genes, we tested whether Tctp RNAi eye phenotypes can be enhanced by 14-3-3 mutations. 14-3-3\(\alpha\)/14-3-3\(\varepsilon\) RNAi alone did not reduce the wing size (Fig. 1m), although it showed partial loss of crossveins (Fig. 1l). However, 14-3-3\(\alpha\) RNAi strongly enhanced the effects of Tctp knockdown, causing reduction and severe wrinkling of wings (Fig. 1o,p). We performed similar genetic tests with two additional independent Tctp RNAi lines and obtained similar results, as shown in Supplementary Fig. 2. These RNAi lines driven by ey-Gal4 or nub-Gal4 resulted in reduced eye and wing, respectively (Supplementary Fig. 2d,g,m,p). Both 14-3-3\(\alpha\) RNAi and 14-3-3\(\varepsilon\) RNAi enhanced the eye/wing phenotypes caused by Tctp RNAi (Supplementary Fig. 2d–i,m–r). These data indicate that 14-3-3 and Tctp genetically interact in both eye and wing development.

In Tor signalling, Tsc1/2 complex functions as GAP towards Rheb\(^37\,\,38\). Thus, we tested whether 14-3-3 genes genetically interact with Tsc2 in the wing model. Control single knockdown of either isoform of 14-3-3 in the wing using nub-Gal4 did not affect the wing size (Fig. 2b,c), as also shown in Fig. 1. Knockdown of Tsc2 in the wing led to 19.5 ± 0.05% tissue enlargements by overgrowth (Fig. 2d,j, n = 10). Remarkably, silencing of either 14-3-3\(\alpha\) or 14-3-3\(\varepsilon\) strongly inhibited the wing growth caused by Tsc2 RNAi, even showing severe undergrowth of wings (Fig. 2e,f). Next, we examined whether 14-3-3 genes might also interact genetically with Rheb. Overexpression of Rheb by nub-Gal4 induced 23 ± 0.06% increase in wing size (Fig. 2g,j, n = 10). Knockdown of 14-3-3 inhibited the wing overgrowth...
caused by Rheb overexpression (Fig. 2h). Enlarged wing by Rheb overexpression was also suppressed by 14-3-3\(^{\gamma}\) RNAi (Fig. 2i). This suppression by either one of 14-3-3 RNAi resulted in mild curling at the wing margin, but the wing size was consistently reduced to that of the wild-type control (Fig. 2j). We also tested the effects of 14-3-3 RNAi on cell size. Because each wing cell generates a single hair, the hair density within a given area provides an approximate estimation of cell size. Knockdown of either 14-3-3\(\alpha\) or 14-3-3\(\zeta\) did not noticeably affect the wing hair density (Fig. 2k–m). The hair density was decreased by 13.1 ± 0.58% in nub\(>\)Tsc2 RNAi wings (Fig. 2n,t, n = 10) and by 14.5 ± 1.83% in nub\(>\)Rheb wings (Fig. 2q,t, n = 10), suggesting an increase in cell size. The decreased wing hair density by Tsc2 silencing or Rheb overexpression was suppressed by knockdown of either 14-3-3\(\gamma\) (Fig. 2o,r, n = 10) or 14-3-3\(\zeta\) (Fig. 2p,s, n = 10).

**Tctp and Rheb physically interact with 14-3-3 proteins.** Strong genetic interaction of 14-3-3\(\gamma\) with Tctp and Rheb suggests that they may function together to regulate organ development. Hence, we checked the expression of Tctp and Rheb in tissues (Supplementary Fig. 3a–f). Consistent with the physical interaction between Tctp and Rheb\(^{8}\), both proteins showed an overlapping expression pattern in eye disc (Supplementary Fig. 3g–i). Interestingly, loss of Tctp in mutant clones resulted in a reduction of the Rheb level in both eye and wing discs (Fig. 3c,g). We used an anti-14-3-3 antibody that specifically recognizes 14-3-3\(\gamma\) but does not distinguish 14-3-3\(\alpha\) and 14-3-3\(\zeta\) on western blot\(^{32}\). Immunostaining of wing discs with this antibody showed ubiquitous expression of 14-3-3\(\gamma\), but its level was reduced by 14-3-3\(\gamma\) RNAi (Supplementary Fig. 3j–l), indicating the specificity of this antibody. Unlike Rheb, loss of Tctp did not cause obvious changes in the level of 14-3-3\(\gamma\) (Fig. 3d,h).

Strong genetic interaction between Tctp and 14-3-3\(\gamma\), raised a possibility that they might physically interact. Co-immunoprecipitation (Co-IP) tests indicated that 14-3-3\(\gamma\) and 14-3-3\(\zeta\) are associated with Tctp in a protein complex in S2 cell extracts (Fig. 4a). GST pull-down assays using bacterially expressed fusion proteins indicate that both 14-3-3\(\gamma\) and 14-3-3\(\zeta\) directly interact with Tctp in a protein complex. As shown in Fig. 1, 14-3-3\(\gamma\) genes show genetic interaction with Rheb as well as Tctp. Because Tctp and Rheb are directly associated, it is plausible that Rheb might also form a complex with 14-3-3\(\gamma\). Co-IP assays using S2 cell extracts showed that Rheb co-immunoprecipitates with 14-3-3\(\gamma\) and 14-3-3\(\zeta\) (Fig. 4c). Remarkably, Rheb was also pulled down by GST-14-3-3\(\gamma\) and GST-14-3-3\(\zeta\), indicating their direct binding in vitro (Fig. 4d).
14-3-3s are required for Tctp–Rheb interaction and growth. The data that 14-3-3s bind to both Tctp and Rheb led to a question if 14-3-3s are required for the interaction between Tctp and Rheb to form a complex. To test this hypothesis, we knocked down 14-3-3s in S2 cell culture, and checked whether reduced 14-3-3s were depleted in day 6th after treating S2 cell with 14-3-3s RNAi (Fig. 5a). As shown above, single knockdown or loss-of-function mutation of either one of 14-3-3s did not affect organ growth. Since depletion of both 14-3-3s forms results in the disruption of physical interaction between Tctp and Rheb (Fig. 5b), we examined the effects of depleting both 14-3-3s isoforms in developing organs. In contrast to single knockdown that did not noticeably affect development (Fig. 5c–e), double knockdown of both 14-3-3s resulted in nearly complete loss of physical interaction between Tctp and Rheb (Fig. 5b). Levels of Tctp and Rheb were not altered by depleting 14-3-3s, indicating that the loss of Tctp–Rheb binding was not due to reduction of these proteins (Fig. 5a). As shown above, single knockdown or loss-of-function mutation of either one of 14-3-3s did not affect organ growth. Since depletion of both 14-3-3s forms results in the disruption of physical interaction between Tctp and Rheb (Fig. 5b), we examined the effects of depleting both 14-3-3s isoforms in developing organs. In contrast to single knockdown that did not noticeably affect development (Fig. 5c–e), double knockdown of both 14-3-3s resulted in nearly complete loss of physical interaction between Tctp and Rheb (Fig. 5b). Levels of Tctp and Rheb were not altered by depleting 14-3-3s, indicating that the loss of Tctp–Rheb binding was not due to reduction of these proteins (Fig. 5a).
proteins stained by anti-GST.

and GST-14-3-3 shows MBP–Rheb stained by anti-MBP. First lane indicates 5% input of GST-14-3-3 anti-Flag. (Flag-14-3-3 not by GST. Second blot shows GST and GST-fusion proteins stained by Flag-14-3-3 Flag-14-3-3 used for pull-down MBP–Tctp as indicated. First blot shows MBP–Tctp proteins

between 14-3-3s and T ctp. GST-14-3-3 and Flag-14-3-3 Flag-14-3-3 was used to pull-down MBP–Tctp as indicated. First blot shows MBP–Tctp proteins stained by anti-MBP. First lane indicates 5% input of MBP–Tctp used for pulldown. MBP–Tctp is pulled down by GST-14-3-3 and GST-14-3-3 but not by GST. Second blot shows GST and GST-fusion proteins stained by anti-GST. (c) Co-immunoprecipitation of 14-3-3s and Rheb. First blot shows 5% input of V5–Rheb. Second blot shows V5–Tctp co-immunoprecipitated by Flag-14-3-3c and Flag-14-3-3c but not by Flag-GFP. Third blot shows Flag-14-3-3c, Flag-GFP and Flag-14-3-3c immunoprecipitated with anti-Flag. (b) Direct binding between 14-3-3s and Tctp. GST-14-3-3c or GST-14-3-3c was used to pull-down MBP–Tctp as indicated. First blot shows MBP–Tctp proteins stained by anti-MBP. First lane indicates 5% input of MBP–Tctp used for pulldown. MBP–Tctp is pulled down by GST-14-3-3c and GST-14-3-3c but not by GST. Second blot shows GST and GST-fusion proteins stained by anti-GST.

Silencing 14-3-3s affects downstream targets of Tor. Rheb activates Tor kinase, which leads to cell growth and proliferation through S6 kinase (S6k), Thor and CycE, respectively.7,39 It has been shown that S6k phosphorylation is greatly reduced by Tctp silencing6. On the basis of genetic and physical interaction of 14-3-3s with Tctp and Rheb, we tested whether 14-3-3 knockdown can affect Tor activity by checking the levels of phosphorylated S6k and Thor in S2 cells. Either single or double knockdown of 14-3-3s resulted in similar reduction of pS6k detected by anti-pS6k (Thr 398) antibody, while there was little change in the total level of S6k protein. Phosphorylated Thor was strongly reduced by either single or double knockdown of 14-3-3s (Fig. 6a). Double knockdown of 14-3-3s resulted in some reduction of Thor protein levels while single knockdown did not.

To check the effects of 14-3-3 mutations on CycE, we generated 14-3-3c mutant clones in eye discs. These clones showed reduced levels of CycE (Fig. 6b–d). Similarly, 14-3-3c mutant clones also resulted in a reduction of CycE level (Fig. 6e–g). However, such partial reduction of CycE by loss of one 14-3-3 isoform had little effect on the growth of mutant clones in the eye disc (Supplementary Fig. 1a–c,g–i), although reduced 14-3-3 causes abnormal differentiation of photoreceptor cells30,31.

Defects by Tctp and 14-3-3 knockdown are suppressed by CycE. Because both Tctp and 14-3-3 affect Tor signalling, we tested whether double knockdown of Tctp and 14-3-3 could be suppressed by S6k and CycE. Growth defects from double knockdown of Tctp and 14-3-3 could not be suppressed by overexpressing S6k alone (Supplementary Fig. 4). Next, we tested the effects of overexpressing CycE. Single knockdown of either form of 14-3-3 strongly enhanced the effects of Tctp RNAi, as shown earlier (Fig. 1; Supplementary Fig. 2). Overexpression of CycE restored the small eye phenotype caused by knockdown of Tctp and one of 14-3-3s in ~80% of flies examined (Fig. 6k–n), while CycE overexpression in the wild-type background did not increase the eye size (Fig. 6i). The small-wing phenotype resulting from double knockdown of Tctp and one of 14-3-3 isoforms was
Figure 5 | 14-3-3s are required for the interaction between Tctp and Rheb and organ development. (a) Knockdown of 14-3-3s in S2 cells shows depletion of 14-3-3s levels after 6 days of RNAi treatment. α, ζ and α + ζ indicate RNAi for 14-3-3α, 14-3-3ζ, and both isoforms, respectively. Levels of Tctp and Rheb were not noticeably affected by 14-3-3 depletion. The treatment performed to detect Tctp, Rheb, and actin level was double knockdown of both isoforms of 14-3-3. (b) 14-3-3 depletion affects the interaction between V5-Tctp and V5myc-Rheb after 6 days of RNAi treatment. Anti-Myc antibody was used for IP. Tctp was detected by anti-Tctp antibody. Depletion of both isoforms abolished the Tctp–Rheb interaction. (c–j) Double knockdown of 14-3-3s causes loss of targeted tissues. ey-Gal4 control (c) 14-3-3α RNAi (d) and 14-3-3ζ RNAi (e) show normal pattern of eye-head in pupae. Knockdown of both 14-3-3 isoforms causes pupal lethality with loss of eye and head (f). Knockdown of both 14-3-3 isoforms causes loss of eye and head (g). Knockdown of both 14-3-3 isoforms results in strong activation of Caspase 3 (h). Knockdown of both 14-3-3 isoforms results in strong reduction of PH3 in wing disc (i). Knockdown of both 14-3-3 isoforms causes strong reduction of PH3 staining in wing disc. Knockdown of both 14-3-3 isoforms results in reduced proliferation. MS1096-Gal4 control (k) 14-3-3α RNAi (l) and 14-3-3ζ RNAi (m) show normal level of PH3 staining in wing disc. Knockdown of both 14-3-3 isoforms causes strong reduction of PH3 in the wing pouch (circled area) (n). (o–r) Caspase activation marked by anti-Caspase 3 (Casp-3) staining is not detected in MS1096-Gal4 control (o) 14-3-3α RNAi (p) and 14-3-3ζ RNAi (q). Double knockdown of both 14-3-3 isoforms results in strong activation of Casp-3 (r). Scale bar, 300 µm (c–f); 100 µm (g–j); 100 µm (k–r).

also partially rescued by CycE in nearly 100% flies examined (Fig. 6a–r).

We also examined whether CycE can suppress the effects of depleting both forms of 14-3-3 on wing development. Double knockdown of 14-3-3 isoforms in the wing pouch by MS1096-Gal4 led to more than 90% pupal lethality and severe reduction of wing tissues in the surviving adult flies (Supplementary Fig. 5c). CycE overexpression in the wild-type background did not affect the wing size or survival (Supplementary Fig. 5b). In contrast, CycE overexpression in the 14-3-3 double knockdown condition suppressed the wing growth defect in >80% of the flies examined (Supplementary Fig. 5d–g), as well as pupal lethality (90% survival). These results suggest that reduced 14-3-3s synergize with partial loss of Tctp to affect the Tctp–Rheb interaction and Tor signalling, leading to a reduction of the CycE level.
the role of 14-3-3s in Tor signalling in vivo has been limited due to their redundant roles. A clue for 14-3-3 knockdown of 14-3-3e of either one of these isoforms has little effect. Interestingly, our data indicate that double knockdown of 14-3-3s results in reduction of CycE level. (k–m) Overexpression of CycE rescues the small-eye phenotype resulting from double knockdown of Tctp and 14-3-3. The rescue is varied, ranging from strong (S, 81–100% of wild-type size) (k), intermediate (I, 41–80%) (l) to no rescue (N, 40% or lower) (m). (n) Quantification of rescue shown in k–m. More than 60 flies with the genotype of ey > Tctp RNAi/CycE GFP, 14-3-3 RNAI/ were scored. (o–r) CycE suppresses the wing phenotypes of double knockdown of Tctp and 14-3-3. Control with one copy of MS1096-Gal4 (o) or CycE overexpression (p) does not affect wing development. (q) Knockdown of both Tctp and 14-3-3 results in small and wrinkled wing. (r) Overexpression of CycE partially rescues the double-knockdown phenotype in 100% of tested flies. Scale bar, 100 μm (b–g); 200 μm (h–m); 400 μm (o–r).

Discussion

Rheb plays a key role in Tor signalling by activating Tor kinase. Thus, how the Rheb function is regulated is an important issue. In our study, we have shown that 14-3-3 proteins play important roles in organ growth by affecting Tctp and Rheb for positive regulation of Tor signalling. Functional analysis of 14-3-3 genes in vivo has been limited due to their redundant roles. A clue for the role of 14-3-3s in Tor signalling in vivo was obtained from our finding of strong genetic interaction between Tctp and 14-3-3. Our data indicate that double knockdown of 14-3-3s causes severe defects in organ growth, whereas silencing of either one of these isoforms has little effect. Interestingly, knockdown of 14-3-3s together with Tctp RNAi results in synergistic growth defects. 14-3-3 genes also show strong genetic interaction with Rheb and Tsc2 in which knockdown of both 14-3-3 isoforms suppresses the wing overgrowth caused by Rheb overexpression or Tsc2 silencing. Knockdown of either 14-3-3s or 14-3-3z has little effect on wing development. But together with Rheb overexpression, it results in smaller and curled-down wings in comparison with Rheb-overexpressed wing. Similarly, 14-3-3 RNAi did not simply suppress the overgrown wing phenotype of Tsc2 RNAi to normal wing size. Instead, wings with knockdown of both 14-3-3 and Tsc2 are much smaller than the normal size of 14-3-3 RNAi wings. Currently, it is unknown how 14-3-3 RNAi negatively affects wing tissues under Rheb overexpression or Tsc2 knockdown conditions while 14-3-3 RNAi alone does not affect wing growth in the wild-type condition. It has been reported that tissues from Tsc1 or Tsc2 mutant patients show abnormal expression in a number of genes. Therefore, it is possible that wing tissues with Rheb overexpression or Tsc2 RNAi may have alterations in many intracellular events. Under these conditions, such tissues might become more sensitive to a reduction of 14-3-3 than wild-type tissues, resulting in abnormal or reduced wings. Alternatively, activated Tor signalling might affect other linked signalling pathways to induce cell death under certain conditions such as reduced 14-3-3. Some oncogenic genetic changes are known to promote apoptosis. In case of Tor signalling, Tsc-deficient cells can constitutively activate Rheb-Tor signalling, which inhibits Akt signalling to promote cell death. Therefore, it is possible that Tsc2 depletion or Rheb overexpression might lead to downregulation of other 14-3-3-dependent growth signalling pathways, thus resulting in wing growth defects when 14-3-3 levels are compromised. Identification of the mechanism underlying the synergistic interaction between 14-3-3 and Rheb/Tsc2 needs further studies.

Figure 6 | Effects of 14-3-3 RNAi on Tor targets and the suppression by CycE. (a) Effects of 14-3-3 RNAi on S6k and Thor. Either one of 14-3-3 isoforms or both 14-3-3s were knocked down in S2 cells. Single or double knockdown of 14-3-3s reduced the level of pS6k (Thr 398) but does not affect the level of S6k protein. Knockdown of both 14-3-3s, but not one isoform, causes slight reduction of Thor. In contrast, pThor (Thr 37/46) is strongly reduced by single or double knockdown of 14-3-3s. (b–d) Loss of 14-3-3s results in reduction of CycE level. (b) 14-3-3s mutant clones induced by ey-flp are marked by the absence of GFP. Mutant clones show strong reduction of CycE (c). (e–g) 14-3-3– mutant clones induced by hs-flp are marked by the absence of Arm-lacZ. CycE is reduced in mutant clones (f). (h–m) Effects of CycE in eye. Control with one copy of ey-Gal4 (h) or overexpression of CycE (i) shows normal eye. (j) Double knockdown of Tctp and 14-3-3 results in small and rough eye phenotype (< 40% of wild-type eye size). (k–m) Overexpression of CycE rescues the small-eye phenotype resulting from double knockdown of Tctp and 14-3-3. The rescue is varied, ranging from strong (S, 81–100% of wild-type size) (k), intermediate (I, 41–80%) (l) to no rescue (N, 40% or lower) (m). (n) Quantification of rescue shown in k–m. More than 60 flies with the genotype of ey > Tctp RNAi/CycE GFP, 14-3-3 RNAI/ were scored. (o–r) CycE suppresses the wing phenotypes of double knockdown of Tctp and 14-3-3. Control with one copy of MS1096-Gal4 (o) or CycE overexpression (p) does not affect wing development. (q) Knockdown of both Tctp and 14-3-3 results in small and wrinkled wing. (r) Overexpression of CycE partially rescues the double-knockdown phenotype in 100% of tested flies. Scale bar, 100 μm (b–g); 200 μm (h–m); 400 μm (o–r).
In addition to genetic interaction, 14-3-3, Tctp, and Rheb physically interact with each other. Double knockdown of both 14-3-3 isoforms results in reduction of Tor downstream components such as phosphorylated S6k, phosphorylated Thr and CycE (Fig. 6a,b). In S2 culture cells, 14-3-3 RNAi did not affect the level of total S6k but it reduced the level of pS6k even by single knockdown of 14-3-3ε or 14-3-3ζ. Phosphorylated Rheb was strongly reduced by knockdown of a single 14-3-3 isoform (Fig. 6a). On the contrary, single knockdown of 14-3-3ε did not strongly affect the interaction between Tctp and Rheb (Fig. 5b). These data seem to suggest that 14-3-3s may not affect TORC1 activity by modulating Tctp–Rheb binding. However, it is worth noting that 14-3-3s may also affect other Tor components such as Tsc2. Different 14-3-3 isoforms are known to inhibit Tsc2 in mammalian cells by binding to Tsc2 phosphorylated by Akt. Therefore, although single knockdown of 14-3-3ε may not strongly affect Tctp–Rheb interaction, depletion of a 14-3-3 isoform could elevate Tsc2 function to inhibit Rheb activity, thus reducing pS6k and pThr as shown in Fig. 6a. Our data for genetic interactions between 14-3-3ε and Tctp (or Rheb) support the role of 14-3-3ε affecting Tor signalling by interacting with Tctp and Rheb, although we do not exclude the possibility that 14-3-3s may also affect Tor signalling independent of Tctp–Rheb interaction.

An intriguing question is how single knockdown of 14-3-3ε affects Tor signalling in S2 cells but not in animal tissues like eye/wing discs. Such difference might be due to cell- or tissue-specific function of Tor signalling. For example, Tsc2 phosphorylation by Akt increases Tor signalling in mammalian cells. Although Drosophila Tsc2 is also phosphorylated by Akt in cultured S2 cells, Tsc2 phosphorylation does not play a critical role during normal development. Thus, signalling events can vary between culture cells and intact organisms or even between different culture cell types. We noted that double knockdown of 14-3-3εs fails to completely eliminate pS6k and pThr. Low levels of residual 14-3-3ε (either 14-3-3ε or 14-3-3ζ) after RNAi treatment might be sufficient to keep some of TORC1 activity. It is also possible that Tor might be activated in part by Rheb-independent mechanisms in S2 cells as reported in mammalian cells.

Mammalian Tctp proteins are known to be phosphorylated at several sites by Polo-like kinase 1 (ref. 47) and insulin signalling. Mammalian Rheb is phosphorylated at Ser-130 by p38 regulated/activated protein kinase (Prak)49. Although Drosophila Tctp and Rheb have conserved Ser or Thr residues at the similar positions, no experimental evidence for phosphorylation has been reported. It seems that these serine or threonine sites do not belong to two conserved 14-3-3 binding motifs R[S/A]pSXP and RX[A/S]pSXP, where R is an aromatic amino acid. However, some 14-3-3 binding ligands have sequences diverged from these motifs or do not require phosphorylation for binding. Therefore, it remains to be determined whether 14-3-3 binding to Tctp is mediated through phosphorylation.

Importantly, our data show that the binding between Tctp and Rheb is almost completely disrupted by knockdown of both 14-3-3 isoforms but not by a single knockdown. This suggests that 14-3-3 isoforms might play a redundant role to promote direct interaction between Tctp and Rheb. Since 14-3-3s bind directly to both Tctp and Rheb, Tctp–Rheb binding might be promoted, possibly by using the known dimerization of 14-3-3 proteins. For such interactions to occur, these three proteins must be present in a stoichiometric ratio to form a proper complex. In our co-IP assays in S2 cells, the levels of endogenous 14-3-3 proteins were similar to the levels of exogenous Tctp and Rheb (Supplementary Fig. 6), suggesting that 14-3-3εs, Tctp and Rheb are present in a stoichiometric ratio to form a direct complex. However, it is also possible that 14-3-3s might interact separately with Tctp and Rheb to promote the binding between Tctp and Rheb without forming a direct complex coupled by 14-3-3 proteins. Further studies are necessary to identify the precise mechanism of molecular interactions among these proteins.

Our study reveals a novel function of 14-3-3ε for promoting the Tctp–Rheb interaction at the critical step of Tor activation. 14-3-3 proteins are also known to regulate Tor signalling by interacting with Tsc2, Pras40 and Raptor20–24, although in vivo functions of these interactions have not been studied in Drosophila. Thus, 14-3-3 isoforms may be involved in more steps than those known so far, both up and/or downstream of Tor kinase. It would be important to study how such diverse 14-3-3 interactions with multiple Tor components contribute to generate signalling outputs for cell growth and proliferation. Nonetheless, the new function of 14-3-3ε isoforms for the interaction between Tctp and Rheb is crucial for organ growth in Drosophila. It would be interesting to see whether the function of 14-3-3ε shown in this study is conserved in mammals and whether loss of a functionally redundant human 14-3-3 isoform can synergize with partial defects in Tor signalling components, leading to more severe pathological conditions.

**Methods**

Fly genetics. 14-3-3 RNAi fly lines were obtained from the Vienna Drosophila Resource Center (VDRC) and the National Institute of Genetics (NIG). 14-3-3ε RNAi lines were 31196R-4 and 31196R-3 (NIG). 14-3-3ε RNAi lines were 48724, 48725, 104496 (VDRC) and 17870R-2 (NIG). 14-3-3ζ loss-of-function mutant strain (w1188; y2, w2; fly; GMR-lacZ; FRT82B 14-3-3ζ/14-3-3ε1-TM6 Tb) was from the Drosophila Genomics Resource Center (DGRC). 14-3-3ζ insertion lethal mutant strain (w,Tet2D 14-3-3ζ/14-3-3ε;CyO) was from the Bloomington Drosophila Stock Center. Tctp RNAi and mutant line FRT82B TctpT69/TM6 Tb were as described5. Two other Tctp RNAi lines from VDRC were v45331 and v45532. Flies were grown at room temperature, unless stated otherwise.

For double knockdown of 14-3-3εs, MS1096-Gal4 and UAS-14-3-3ε RNAi were recombined on the first chromosome. MS1096> 14-3-3ε RNAi recombinant females were crossed with male UAS-14-3-3ε, RNAi.

For double knockdown of Tctp and 14-3-3ε, ey>Tctp RNAi/Cyo recombinants were crossed with 14-3-3ε RNAi or UAS-14-3-3ε RNAi. Flies were grown at room temperature, unless stated otherwise.

For rescue of double knockdown of 14-3-3εs by CycE, MS1094> 14-3-3ε RNAi recombinant flies were crossed with UAS-CycE GFP on the second chromosome. Progeny from this cross was mated with UAS-14-3-3ζ RNAi on the third chromosome. Larvae showing green fluorescent protein (GFP) expression with the genotype MS1096> 14-3-3ε RNAi/+; CycE GFP/+; 14-3-3ε RNAi/+ were selected to check genotypes.

For rescue of double knockdown of Tctp and 14-3-3ε by CycE, ey>Tctp RNAi/Cyo; 14-3-3ε RNAi/TM6 Tb or ey>Tctp RNAi/Cyo; 14-3-3ζ RNAi/TM6 Tb were selected based on eye phenotype. These recombinant flies were crossed with UAS-CycE GFP, and the progeny with GFP expression and without TM6 Tb balancer was kept for further analysis.

**Generation of mosaic clones in eye and wing discs.** Mutant clones were generated by FLP/FRT method31. Clones of 14-3-3ε mutation in eye were made using 14-3-3εstop/fl008 mutant fly line (w2, y1188; fly; GMR-lacZ; FRT82B 14-3-3ε/14-3-3ε1-TM6 Tb) from DGRC. 14-3-3ε mutant clones were crossed with FRT82B ubi-GFP/TM3 Sb fly (Bloomington). Eye discs from third instar larval progeny were dissected and immunostained with antibodies indicated in each figure. Mutant clones were identified by the absence of GFP.

To generate 14-3-3ε mutant clones in wing discs, we crossed hs-flp; FRT82B ubi-GFP/TM3 Sb with y2, w2; y1188; fly; GMR-lacZ; FRT82B 14-3-3εstop/TM6 Tb. First instar larvae from this cross were heat shocked at 37 °C for 1 h and incubated at 25 °C until third instar stage. Wing discs were dissected for immunostaining. Mutant clones were identified by the absence of GFP staining. To generate 14-3-3ε mutant clones, we; FRT42D 14-3-3ε0705/Cyo) flies were crossed with hs-flp; FRT42D arm-lacZ. First instar larvae were heat shocked as described above, and eye and wing discs from third instar larval progeny were dissected for immunostaining. Mutant clones were identified by the absence of LacZ staining. Tctp mutant clones were generated by crossing hs-flp; FRT82B arm-lacZ with w; FRT82B TctpT69/TM6B Tb. First instar larvae were heat shocked as described above, and imaginal discs from third instar larval progeny were dissected for immunostaining.

**Generation of anti-Rheb antibody.** Five-hundred forty-nine-base pair full-length Rheb complementary DNA (cDNA) including stop codon was cloned to pMAL-c2.

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vector. MBP–Rheb fusion protein was purified from bacteria and used for generating rat anti-Rheb antibody from Abclon. Anti-Rheb antibody was used at 1:500 for western blot and 1:100 for immunocytochemistry as described below.

**Immunocytochemistry.** Third instar eye and wing imaginal discs were dissected in 1× PBS, fixed in 10% paraformaldehyde, 0.01 M periodate, 0.075 M lysine and 0.035 M phosphate buffer) for 30 min, and incubated with primary antibodies at 4°C overnight. After washing, imaginal discs were incubated with secondary antibodies for 2 h at room temperature. First, antibodies were used as following: rat anti-Rheb at 1:100, rabbit anti-Tctp8 at 1:1200, rabbit anti-14-3-3 (Invitrogen 51-0700) at 1:1200, mouse anti-β-gal (DSHB 40-1a) at 1:50, rat anti-Elav (DSHB 7E8A10) at 1:100, rabbit anti-PH3 (Milipore) at 1:200, rabbit anti-Cas-3 (Cell Signaling 9661) at 1:300, sheep anti-GFP (Ab-life Direct Sorretes 475-1051) at 1:100, rabbit anti-Cyclin (Santa Cruz) at 1:100 and rabbit anti-Dlg (from Dr Kung-Oko Chao) at 1:500. Secondary antibodies was conjugated with Cy3, Cy5 or FITC (Molecular Probes) were: anti-rabbit Cy3, anti-rat FITC, anti-mouse Cy5, and anti-sheep FITC. Mounting solution was Vectorshield with 4,6-diamidino-2-phenylindole (Vector Laboratories H-1200). Fluorescent images were acquired using Carl Zeiss confocal microscope.

**In vitro GST pull-down assays.** 14-3-3c (cDNA (LD27992) and 14-3-3x (cDNA (RE61958) were from DGRC. Five hundred eighty eight base pair of 14-3-3c coding sequence (including stop codon) was cloned into PGEX-4T1 vector. Fusion tagged proteins were expressed in E.coli. For GST pull-down assay, 20 μg of each MBP and GST-tagged protein were added to 500 μl pull-down buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM diethiotheniol, 1 mM Na3VO4, 10 mM NaF and a protease inhibitor cocktail (Roche 11 873 580 001)), 0.5 mM EDTA, 10% glycerol, 0.1% Triton X-1 and mouse anti-β-actin (Abcam Ab8824) at 1:5,000. For detecting S6k and phospho-S6k, mouse anti-S6k (ref. 8) and rabbit anti-pS6k (Thr(398) (Cell signaling 9209S) were used at the same 1:1000 dilution, respectively. For detecting Thr protein and phosphorylated Thr, rabbit anti-Thr antibody and rabbit anti-pThr (Thr (37/46) (Cell Signaling 2855S) were used at 1:5,000 and 1:1,000, respectively. All uncropped western blots can be found in Supplementary Fig. 7.

**Co-immunoprecipitation assays.** Coding sequences of 14-3-3s were cloned into Flag-tagged pRHa3-3 vector (DGRC). Tctp- and Rheb-coding sequences were cloned into pMAL-c2 vector. Fusion tagged proteins were expressed in E.coli (BL2). For GST pull-down assay, 20 μg of each MBP and GST-tagged protein were added to 500 μl pull-down buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM diethiotheniol, 1 mM Na3VO4, 10 mM NaF and a protease inhibitor cocktail (Roche 11 873 580 001)), followed by incubation overnight at 4°C with Glutathione Excellose resin (Bio-rad). The full-length Rheb was washed and incubated with 2 μl of anti-Flag antibody (Sigma 1,804) in 500 μl of CHAPS-based lysis buffer, 20 μl of lysis buffer was mixed with 5 μl of S6 loading buffer and was added and samples were boiled at 94°C for 5 min, then centrifuged at 12,000 r.p.m for 5 min and loaded. For immunostaining of western blots, rabbit anti-MBP antibody (Santa Cruz) and secondary anti-rabbit antibody (Jackson) were used at 1:5,000 and 1:1,000, respectively.

**References.**


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Author contributions
Y.-C.H. and K.-W.C. made the initial observation of genetic interaction; T.P.L. and K.-W.C. designed the experiments. L.T.V. performed co-IP assays. A.-R.K. and T.P.L. generated anti-Thor antibody. T.P.L. performed all the other experiments; T.P.L. and K.-W.C. analysed the data and wrote the paper.

Additional information
Supplementary Information accompanies this paper at www.nature.com/nature.

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