LETTERS

Drosophila TCTP is essential for growth and proliferation through regulation of dRheb GTPase

Ya-Chieh Hsu[1], Joshua J. Chern[2], Yi Cai[4], Mingyao Liu[4] & Kwang-Wook Choi[1,2,3]

Cellular growth and proliferation are coordinated during organogenesis. Misregulation of these processes leads to pathological conditions such as cancer. Tuberous sclerosis (TSC) is a benign tumour syndrome caused by mutations in either *TSC1* or *TSC2* tumour suppressor genes. Studies in *Drosophila* and other organisms have identified TSC signalling as a conserved pathway for growth control. Activation of the TSC pathway is mediated by Rheb (Ras homologue enriched in brain), a Ras superfamily GTPase[1,2]. Rheb is a direct target of TSC2 and is negatively regulated by its GTPase-activating protein activity[3–5]. However, molecules required for positive regulation of Rheb have not been identified. Here we show that a conserved protein, translationally controlled tumour protein (TCTP), is an essential new component of the TSC–Rheb pathway. Reducing *Drosophila* TCTP (dTCTP) levels reduces cell size, cell number and organ size, which mimics *Drosophila Rheb* (*dRheb*) mutant phenotypes. *dTCTP* is genetically epistatic to *Tsc1* and *dRheb*, but acts upstream of *dS6k*, a downstream target of *dRheb*. dTCTP directly associates with dRheb and displays guanine nucleotide exchange activity with it *in vivo* and *in vitro*. Human TCTP (hTCTP) shows similar biochemical properties compared to dTCTP and can rescue *dTCTP* mutant phenotypes, suggesting that the function of TCTP in the TSC pathway is evolutionarily conserved. Our studies identify TCTP as a direct regulator of *Rheb* and a potential therapeutic target for TSC disease.

TCTP is a highly conserved protein (Supplementary Fig. 1) upregulated in various tumours. Despite studies on the biochemical and structural properties of TCTP[6–9], the physiological significance of these findings has not been determined. Thus, we aimed to study the function of TCTP *in vivo* using *Drosophila* as a model organism.

We first knocked down dTCTP expression in developing flies by targeted expression of double-stranded RNA (dsRNA) for RNA interference (RNAi)[10] using the GAL4/UAS system[11]. Expression of *dTCTP* RNAi depleted endogenous dTCTP to nearly undetectable levels by different GAL4 drivers (Fig. 1a, b). Tissue-specific expression of *dTCTP* RNAi reduced the size of the eye, wing, notum, or a specific region in the wing pouch, corresponding to the expression domains of various *GAL4* lines (Fig. 1c–e; see also Supplementary Fig. 2a–d). The size reduction was caused by a decrease in both cell size and cell number (Fig. 1f; see also Supplementary Fig. 2e–g), a typical phenotype for mutations in the insulin or TSC pathways. Ubiquitous expression of *UAS-dTCTP* RNAi by *actin-GAL4* (*act>dTCTPi*) caused lethality around the third instar larval stage. A portion of these larvae was able to survive to the pupal stage with reduced body size (data not shown), consistent with the organ size reduction. The lethality and phenotypes caused by *dTCTP* RNAi were rescued by co-expression of a *dTCTP* complementary DNA, indicating that these defects were due to a reduction of dTCTP levels. We therefore concluded that *dTCTP* RNAi suppresses organ growth by affecting both cell size and number.

We further investigated the phenotypes of *dTCTP* loss-of-function mutants because RNAi may represent a hypomorphic situation. $dTCTP^{Ey09182}$ is a hypomorphic allele of *dTCTP* resulting from a P-element insertion in its 5′ untranslated region. Rare homozygous $dTCTP^{Ey09182}$ flies that escaped from lethality showed smaller body size compared with their heterozygous siblings (Fig. 2b and Supplementary Information). To create null alleles, we generated imprecise excisions from $dTCTP^{Ey09182}$ (see Methods). One imprecise

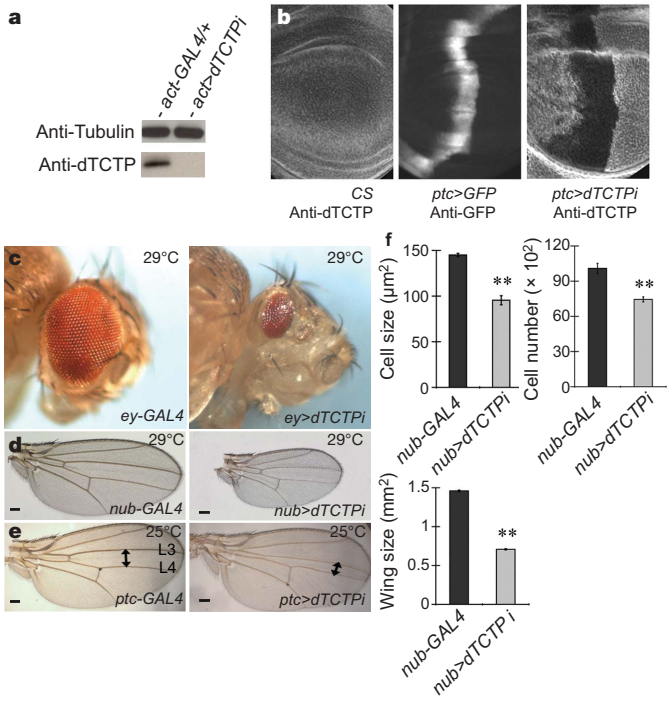

Figure 1 | *dTCTP* RNAi affects cell size, cell number and organ size. a, No dTCTP is detected in larval extract after *dTCTP* RNAi expression by *act-GAL4* at 29 °C. **b**, dTCTP is ubiquitously expressed in the wild-type (*CS*) wing disc, but is depleted by *ptc>dTCTPi* in the *ptc*-expressing region marked by GFP. **c**, Expression of *dTCTP* RNAi in eye disc (*ey>dTCTPi*) reduces the eye size. **d**, Expression of *dTCTP* RNAi in the entire wing pouch (*nub>dTCTPi*) reduces the wing size. **e**, Expression of *dTCTP* RNAi between L3 and L4 veins by *ptc-GAL4* reduces the distance between these two veins (double arrows). Scale bars in **d**, **e**, 100 μm. **f**, Quantification of defects in *nub>dTCTPi* wings ($n = 10$ for each genotype, error bars indicate s.d.; double asterisk, $P < 0.0001$). The small wing phenotype is due to smaller cell size (34% decrease) and a decreased cell number (26% decrease).

[1]Program in Developmental Biology, [2]Department of Molecular and Cellular Biology, and [3]Department of Ophthalmology, Baylor College of Medicine, and [4]Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, Texas 77030, USA.

excision line, *dTCTP*^{h59}, showed a 1.1-kilobase deletion downstream of the insertion site that removes the entire *dTCTP* coding sequence (Fig. 2a). Western blot analysis showed no detectable dTCTP protein in *dTCTP*^{h59} early first instar larvae (Fig. 2c). Both *dTCTP*^{h59} homozygotes and *dTCTP*^{h59} heterozygotes for a deficiency chromosome uncovering the *dTCTP* region (*dTCTP*^{h59}/*Df(3R)M-Kx1*) showed 100% lethality at the late first instar stage, indicating that this allele is a genetic null. Expression of dTCTP cDNA or a genomic DNA construct was able to rescue *dTCTP*^{h59} mutants, indicating that the lethality is due to deletion of the *dTCTP* gene (see Supplementary Information).

To examine the phenotypes of *dTCTP* null mutant cells, we generated *dTCTP* mutant clones using mitotic recombination.

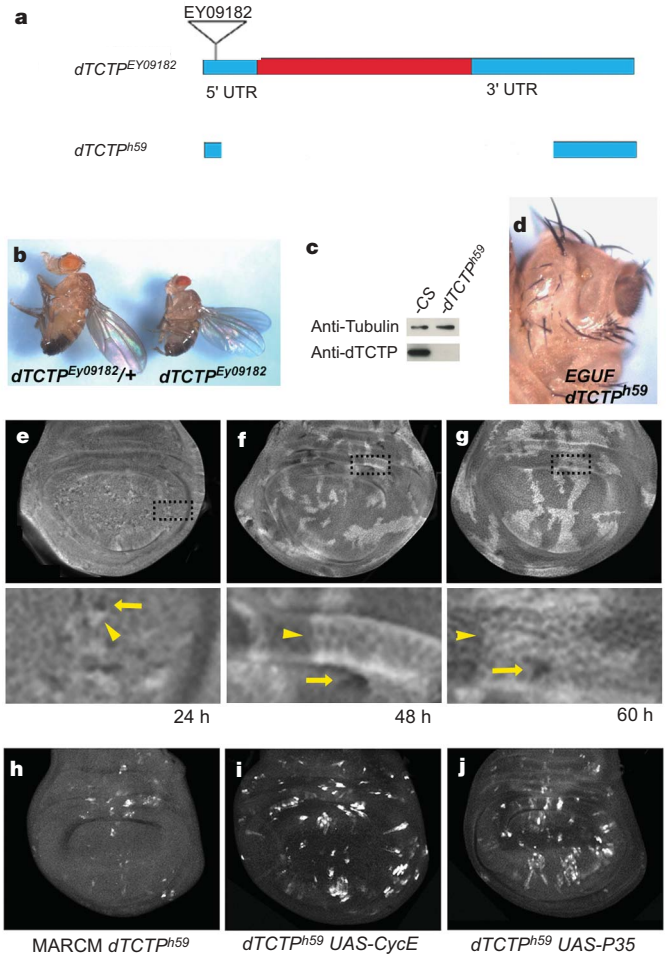

Figure 2 | *dTCTP* mutant phenotypes in cell proliferation and survival. **a**, Schematics of two *dTCTP* mutant alleles. **b**, An escaper of *dTCTP*^{EY09182} (right) and its heterozygous sibling (left). **c**, Western blot analysis detects no dTCTP protein in early first instar *dTCTP*^{h59} larvae. **d**, An eye composed of mainly *dTCTP*^{h59} cells (genotype: *w*; *ey-GAL4*, *UAS-FLP*/+; *FRT82 pGMR-hid cl3R*/*FRT82 dTCTP*^{h59}). **e**–**g**, The size of *dTCTP*^{h59} mutant clones. Discs are stained by dTCTP antibody. Clones are generated at the late second instar (**e**), the late first instar (**f**) and the mid first instar (**g**), and dissected at the late third instar stage. Bottom panels show an enlarged view of the boxed area in the top panels. Arrows and arrowheads mark *dTCTP*^{h59} homozygous mutant clones and the associated twin spots, respectively. **h**–**j**, Expression of *CycE* or *P35* within GFP$^+$ *dTCTP*^{h59} clones using the MARCM technique. The larvae are raised at 25 °C and the discs are dissected 72 h after heat shock. **h**, Most of the *dTCTP*^{h59} clones are eliminated (genotype: *hsflp, tub-GAL4, UAS-GFP*^{nls}; *FRT82 tub-GAL80/FRT82 dTCTP*^{h59}). **i**, Expression of *CycE* in *dTCTP*^{h59} clones (genotype: *hsflp, tub-GAL4, UAS-GFP*^{nls}; *UAS-CycE*/+; *FRT82 tub-GAL80/FRT82 dTCTP*^{h59}). **j**, Expression of *P35* in *dTCTP*^{h59} clones (genotype: *hsflp, tub-GAL4, UAS-GFP*^{nls}; *UAS-P35*/+; *FRT82 tub-GAL80/FRT82 dTCTP*^{h59}).

dTCTP^{h59} mutant clones showed growth disadvantage compared to their wild-type twin spots. The sizes of *dTCTP*^{h59} clones were similar to those of the twin spots 24 h after heat shock (Fig. 2e). However, the sizes of the twin spots were much larger than *dTCTP*^{h59} clones 48 h after heat shock (Fig. 2f), and most *dTCTP*^{h59} clones were eliminated by 60 h after heat shock (Fig. 2g). Similarly, using the EGUF/Hid technique[12] to remove most wild-type cells in *dTCTP*^{h59} mosaic eyes resulted in either a no-eye or small-eye phenotype (Fig. 2d). Therefore, the null phenotypes were qualitatively comparable to the *dTCTP* RNAi phenotype, but more severe.

The reduction in cell number caused by *dTCTP* RNAi and the behaviour of *dTCTP* mutant cells may result from a proliferation defect or abnormal cell death. We tested these possibilities using the MARCM (mosaic analysis with a repressible cell marker) technique[13]. Similar to clones generated by traditional mitotic recombination, numerous small *dTCTP*^{h59} green-fluorescent-protein-expressing (GFP$^+$) clones were observed at 24 h after heat shock (data not shown). By 72 h after heat shock, very few GFP$^+$ clones remained on the discs (Fig. 2h). In contrast, Cyclin E (CycE) overexpression, via the MARCM technique, within *dTCTP*^{h59} clones resulted in four times more *dTCTP*^{h59} cells at 72 h after heat shock (comparing Fig. 2i to h). Similarly, CycE overexpression also suppressed the *dTCTP* RNAi phenotypes (Supplementary Fig. 3). We next tested whether the *dTCTP*^{h59} phenotypes can be attributed to abnormal cell death. Expression of the P35 cell death inhibitor also significantly suppressed the *dTCTP*^{h59} phenotypes, leading to the presence of four times more GFP$^+$ cells at 72 h after heat shock (comparing Fig. 2j to h). These data indicate that loss of *dTCTP* causes defects in cell proliferation and triggers cell death.

Insulin and TSC signalling are two parallel but interacting pathways for growth control. Inactivation of positive regulatory components, such as *Insulin receptor* (*InR*), *dRheb* and *Tor*, leads to a decrease in organ size by affecting cell size and cell number[1,2,14–17]. In contrast, overexpression of *InR* and *dRheb*, as well as inactivation of negative regulatory components such as *Tsc1* (refs 18, 19), *Tsc2* (ref. 20) and *Pten* (refs 21–23), causes tissue overgrowth. Given the similar phenotypes between *dTCTP* mutants and mutants in the insulin/TSC pathways, we performed genetic epistasis experiments to test whether *dTCTP* has a role in these two pathways. Overexpression of *InR* by *patched* (*ptc*)*-GAL4* caused weak but consistent expansion of the distance between L3 and L4 wing veins (compare Fig. 3a, c and k). In contrast, co-expression of *InR* and *dTCTP* RNAi reduced the L3–L4 distance, resembling the *dTCTP* RNAi phenotype (compare Fig. 3b, d and k). Therefore, *dTCTP* is epistatic to *InR*.

Next, we examined the relationship between *Tsc1* and *dTCTP*. Mutations in *Tsc1* or *Tsc2* cause similar phenotypes because they function as a complex. Mosaic eyes and heads consisting primarily of *Tsc1* mutant cells were much larger than wild type (compare Fig. 3e and f). Expression of *dTCTP* RNAi in *Tsc1* mutant cells suppressed this overgrowth both in the eye and head (Fig. 3g). Furthermore, when the eye was composed of *Tsc1* and *dTCTP* double mutant cells, the flies displayed a small or no-eye phenotype indistinguishable from the *dTCTP* single mutant phenotype (compare Figs 3h and 2d), suggesting that dTCTP acts downstream or in parallel to Tsc1.

We next tested the relationship between *dTCTP* and *dRheb*. Ectopic expression of *dRheb* using *ptc–GAL4* resulted in a 15% increase in the L3–L4 distance compared with the *ptc>GFP* control (Fig. 3i, k). However, co-expression of *dTCTP* RNAi and *dRheb* in the *ptc* expression region showed the *dTCTP* RNAi phenotype (Fig. 3j, k), suggesting that *dTCTP* is epistatic to *dRheb*.

Finally, we tested whether activation of dS6k, a downstream effector of the insulin/TSC pathway, is dependent on *dTCTP*. The level of dS6k activation was measured using a phospho-specific antibody (dS6k p-Thr 398). Extracts from *act>dTCTPi* larvae showed a significantly lower amount of activated dS6k compared with the controls (Fig. 3l), indicating that *dTCTP* is required for dS6k activation. Consistent with this, the *eyeless* (*ey*)*>dTCTPi* phenotype was dominantly enhanced by heterozygosity for a null mutation of *dS6k* (*dS6k*$^{l-1}$) (Fig. 3m, n).

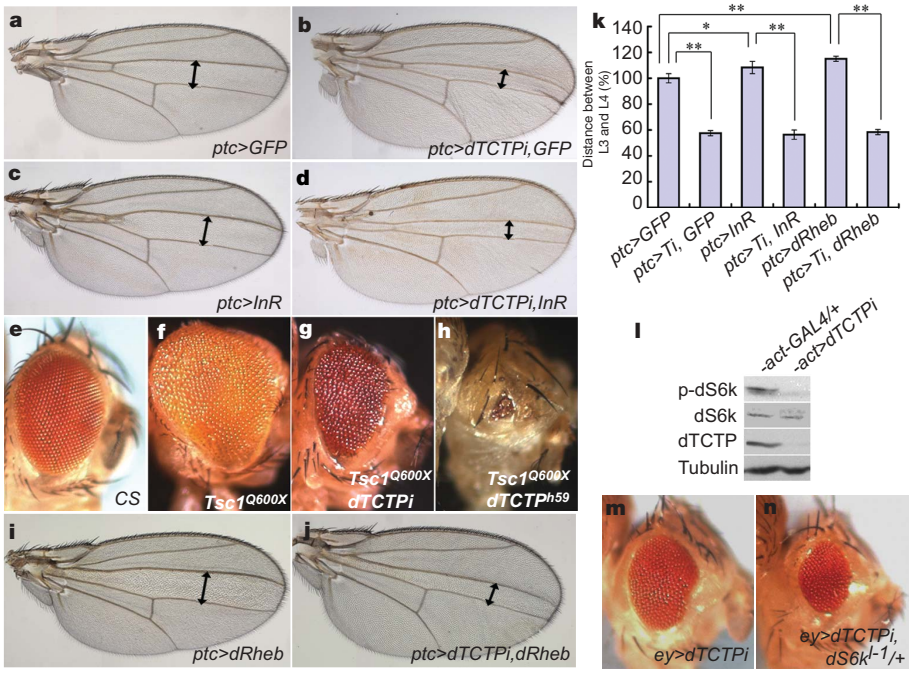

Figure 3 | Epistatic analysis between dTCTP and components of the insulin signalling and TSC pathways. Double arrows in the wings mark the L3–L4 region. **a**, A wing from a *ptc>GFP* fly as a wild-type control. **b**, *ptc>dTCTPi,GFP*. **c**, *ptc>InR*. **d**, *ptc>dTCTPi,InR*. **e**, A wild-type eye. **f**, An eye composed of only *Tsc1* null mutant cells (genotype: *w*; *ey-GAL4*, *UAS-FLP*/+; *FRT82 pGMR-hid cl3R*/*FRT82 Tsc1*^{Q600X}). **g**, Expression of *dTCTP* RNAi in the *Tsc1* mutant cells (genotype: *w*; *ey-GAL4*, *UAS-FLP*/*UAS-dTCTPi*; *FRT82 pGMR-hid cl3R*/*FRT82 Tsc1*^{Q600X}). **h**, An eye composed of *Tsc1* and *dTCTP* double mutant cells (genotype: *w*; *ey-GAL4*, *UAS-FLP*/+; *FRT82 pGMR-hid cl3R*/*FRT82 dTCTP*^{h59}, *Tsc1*^{Q600X}). **i**, *ptc>dRheb*. **j**, *ptc>dTCTPi,dRheb*. **k**, Quantification for the L3–L4 distance compared with the *ptc>GFP* control ($n = 8$ for each genotype, error bars indicate s.d.; asterisk, $P = 0.0023$; double asterisk, $P < 0.0001$). **l**, The phosphorylated dS6k level is reduced in *act>dTCTPi* flies at 18 °C. **m**, **n**, At 22 °C, *ey>dTCTPi* flies show a mild eye reduction (**m**). Removing a copy of *dS6k* enhances the phenotype (**n**).

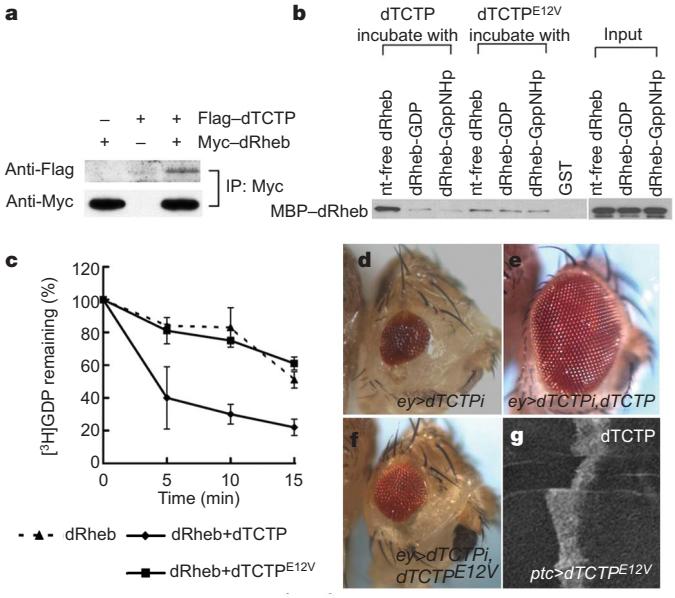

Figure 4 | dTCTP has GEF-like activity for dRheb. **a**, dTCTP and dRheb co-immunoprecipitate in 293T cells. **b**, GST–dTCTP interacts most strongly with nucleotide-free MBP–dRheb. GST– dTCTP^{E12V} can pull-down dRheb with a weaker affinity. **c**, *In vitro* GDP release assay using [^{3}H]GDP-loaded MBP–dRheb. dTCTP accelerates the GDP release from dRheb, whereas dTCTP^{E12V} shows no effect (error bars indicate s.d.; GST–dTCTP, GST–dTCTP^{E12V} or MBP–dRheb were used at 1 μM). **d**, *ey>dTCTPi* flies at 29 °C. **e**, Expression of *UAS-dTCTP* together with *dTCTP* RNAi rescues the growth defect. **f**, Expression of *UAS-dTCTP*^{E12V} fails to rescue the *dTCTP* RNAi phenotype, despite its high level of expression (**g**).

Removing a copy of *dS6k* caused an approximately 20% further reduction of eye size in *ey>dTCTPi* animals. Taken together, these data support a model wherein *dTCTP* functions either downstream or in parallel to *Tsc* and *dRheb*, but upstream of *dS6k*.

Epistatic analysis suggests that dTCTP may be a new component in the TSC pathway. Because TCTP structurally resembles a small GTPase regulator, Mss4 (ref. 9), we proposed that dTCTP might directly associate with dRheb and positively regulate its activity. To test this, we first performed co-immunoprecipitation experiments. Flag-tagged dTCTP immunoprecipitated together with Myc-tagged dRheb in 293T cell extracts (Fig. 4a), suggesting that dTCTP and dRheb form a complex *in vivo*. Furthermore, *in vitro* pull-down assays demonstrated direct binding of glutathione *S*-transferase (GST)–dTCTP to maltose binding protein (MBP)–dRheb. Notably, dTCTP showed preferential binding to nucleotide-free dRheb (Fig. 4b), a property shared among guanine nucleotide exchange factors (GEFs). To test whether dTCTP has GEF activity for dRheb, we carried out *in vitro* GDP release experiments. MBP–dRheb alone showed weak intrinsic GDP dissociation. In contrast, addition of GST–dTCTP stimulated the GTP/GDP exchange on dRheb rapidly even when low amounts of dTCTP were used (Fig. 4c; see also Supplementary Fig. 4a). The GEF-like activity is specific, as dTCTP did not accelerate the exchange reaction on dRas1, the closest GTPase to dRheb at sequence level (data not shown). Moreover, a glutamic acid to valine mutation in the putative GTPase binding groove[9] of dTCTP (dTCTP^{E12V}) abolished this GEF activity, even at a high concentration (Fig. 4c; see also Supplementary Fig. 4a). Because dTCTP^{E12V} still retained binding activity to dRheb (Fig. 4b), this residue seems to have a critical function in catalytic reactions rather than binding between the two proteins. To determine whether E12 is critical for the function of dTCTP *in vivo*, we performed genetic rescue experiments. Whereas wild-type *dTCTP* was able to rescue fully the *dTCTP* RNAi phenotype (Fig. 4d, e), mutant *dTCTP*^{E12V} failed to rescue the RNAi defects (Fig. 4f and data not shown) even

though the mutant protein was expressed at a high level (Fig. 4g). Therefore, the conserved E12 residue of dTCTP is essential for its normal function in development.

To test whether this *in vitro* GEF activity has a physiological relevance, we determined the *in vivo* level of GTP bound to dRheb in *Drosophila* S2 cells. S2 cells treated with dTCTP dsRNA or a control EGFP dsRNA were transfected with Myc-tagged dRheb. dTCTP-dsRNA-treated cells consistently showed a lower percentage of GTP-bound dRheb (Supplementary Fig. 4b), suggesting that dTCTP is required for dRheb activation *in vivo*. These GEF-like properties displayed by dTCTP towards dRheb are particularly intriguing, as no Rheb GEFs have been reported. Further kinetic and structural analysis will help to elucidate whether dTCTP is a bona fide GEF enzyme for dRheb.

Human TCTP (hTCTP) and dTCTP are roughly 50% identical in their protein sequence. We found that *dTCTP* RNAi and mutant phenotypes can be rescued by expression of *hTCTP* (Supplementary Fig. 5a–d). Furthermore, hTCTP interacted most strongly with the nucleotide-free hRheb and stimulated the GDP/GTP exchange of hRheb *in vitro* (Supplementary Fig. 5e, f). These data suggest that the function of TCTP in the TSC pathway is likely to be conserved throughout evolution.

dTCTP controls cell growth and proliferation by positively regulating dRheb activity. Our data suggest that dTCTP may function as a GEF or a related regulatory factor to activate dRheb. Given the strong epistatic effect of dTCTP to dRheb, it is also possible that dTCTP may have additional roles in the TSC pathway, acting downstream of dRheb but upstream of S6k (Supplementary Fig. S6).

TCTP has been implicated in a wide range of cancers. Nevertheless, we have not observed overgrowth phenotypes as a result of dTCTP overexpression (data not shown), suggesting that *dTCTP* is not sufficient to induce growth. Notably, reduction of TCTP levels is sufficient for suppression of malignancy in tumour reversion models[24,25]. Our study provides a possible explanation for this phenomenon. It will be intriguing to learn whether lowering the insulin/TSC signalling output can be a general mechanism for tumour reversion.

METHODS

Generation of the *dTCTP* null mutant. *dTCTP* deletion alleles were generated by imprecise mobilization of the $dTCTP^{Ey09182}$ insert. Potential excisions were identified by the loss of w^+ markers and were tested for complementation with *Df(3R)M-Kx1*, a deficiency line uncovering the *dTCTP* locus. Genomic DNA from lines that failed to complement the deficiency was used as polymerase chain reaction templates. Primers flanking the *Ey09182* insertion at position −33 and +967 relative to the insertion site were used in the reaction. A ~350-base-pair (bp) product was produced from the line $dTCTP^{h59}$. Sequencing results confirmed the left/right deletion break points at the 34- and 695-bp position of the *dTCTP* transcript, indicating a deletion of the entire *dTCTP* coding sequence. Other *Drosophila* strains and genetic crosses are detailed in Supplementary Information.

***In vitro* GST pull-down assays.** Removal of endogenous nucleotides and *in vitro* pull-down assays were performed similarly as described[26].

***In vitro* GDP release assays.** The guanine nucleotide exchange assay was performed essentially as described[27].

Measurements of the dRheb activation state *in vivo*. The percentage of activated (that is, GTP-bound) dRheb was determined based on the established methods for measuring Ras, Rap and Rheb GTPases[28–30].

Detailed information about methods used for molecular biology, immunohistochemistry, detection of phospho-dS6k levels, S2 cell RNAi, cell size and cell number measurements, and statistical analysis can be found in Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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