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The target of rapamycin complex 2 controls dendritic tiling of *Drosophila* sensory neurons through the Tricornered kinase signalling pathway

Makiko Koike-Kumagai[1], Kei-ichiro Yasunaga[1], Rei Morikawa[1], Takahiro Kanamori[1] and Kazuo Emoto[1,2,3,*]

[1]Neural Morphogenesis Laboratory, National Institute of Genetics, Mishima, Japan, [2]Department of Genetics, SOKENDAI, Mishima, Japan, and [3]PRESTO, Japan Science and Technology Agency, Saitama, Japan

To cover the receptive field completely and non-redundantly, neurons of certain functional groups arrange tiling of their dendrites. In *Drosophila* class IV dendrite arborization (da) neurons, the NDR family kinase Tricornered (Trc) is required for homotypic repulsion of dendrites that facilitates dendritic tiling. We here report that Sin1, Rictor, and target of rapamycin (TOR), components of the TOR complex 2 (TORC2), are required for dendritic tiling of class IV da neurons. Similar to *trc* mutants, dendrites of *sin1* and *rictor* mutants show inappropriate overlap of the dendritic fields. TORC2 components physically and genetically interact with Trc, consistent with a shared role in regulating dendritic tiling. Moreover, TORC2 is essential for Trc phosphorylation on a residue that is critical for Trc activity *in vivo* and *in vitro*. Remarkably, neuronal expression of a dominant active form of Trc rescues the tiling defects in *sin1* and *rictor* mutants. These findings suggest that TORC2 likely acts together with the Trc signalling pathway to regulate the dendritic tiling of class IV da neurons, and thus uncover the first neuronal function of TORC2 *in vivo*.

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Introduction

The target of rapamycin (TOR) is an evolutionarily conserved Ser/Thr protein kinase that functions in two distinct multiprotein complexes referred as TOR complex 1 (TORC1) and complex 2 (TORC2). TORC1 is composed of TOR, Raptor, and LST8 (also know as GβL), whereas TORC2 contains TOR, Rictor, LST8, and Sin1 (Sarbassov *et al*, 2005a; Wullschleger *et al*, 2006; Bhaskar and Hay, 2007). TORC1 regulates cell growth by phosphorylating ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E-binding protein (4E-BP) in a rapamycin-sensitive manner. The function of TORC2 is less well-defined than that of TORC1, but some studies suggest that TORC2 is involved in actin cytoskeleton reorganization (Jacinto *et al*, 2004; Sarbassov *et al*, 2004). Both TORC1 and TORC2 are evolutionarily conserved in the functions and the compositions. Indeed, recent studies in both mammalian and *Drosophila* cell cultures have indicated that TORC2 can directly phosphorylate the serine residue (Ser473 and Ser505 in humans and *Drosophila* Akt, respectively) in the hydrophobic motif of Akt/PKB (Hresko and Mueckler, 2005; Sarbassov *et al*, 2005b; Jacinto *et al*, 2006).

In addition to the growth control in proliferating cells, TOR has critical functions in non-proliferating cells. In particular, recent genetic and pharmacological studies have shown that mammalian TOR (mTOR) is involved in various processes in the nervous system, including cell size control (Kwon *et al*, 2003), local protein synthesis in dendrites (Takei *et al*, 2004; Raab-Graham *et al*, 2006), synaptic plasticity (Tang *et al*, 2002; Cammalleri *et al*, 2003; Hou and Klann, 2004), and dendrite arborization (da) (Jaworski *et al*, 2005). These mTOR functions in neurons are believed to be mediated by TORC1 because rapamycin, a potential TORC1-specific inhibitor, can mimic the neuronal defects induced by mTOR ablation. In contrast, much less is known regarding the function of TORC2 in neurons, although Sin1 and Rictor are enriched in the developing neurons (Makino *et al*, 2006; Shiota *et al*, 2006).

Neurons in the same functional class are often organized in characteristic spatial patterns throughout the nervous system (Wassle and Boycott, 1991; Jan and Jan, 2003; Parrish *et al*, 2007). In many sensory circuits, a complete and non-redundant representation of sensory information is attained by a tiling arrangement of the dendrites, such that the dendritic arbors of the same cell type show little or no overlap. For example, the mammalian retina contains more than 20 distinct functional classes of retinal ganglion cells (RGCs), and the dendritic fields of some RGCs of the same subclass typically cover the retina with little overlap between neighbouring neurons, whereas RGCs of different subtypes have extensively overlapping arbors (Wassle and Boycott, 1991; Rockhill *et al*, 2002). Tiling of dendritic fields has also been observed in the sensory neurons of the leech *Hirudu medicinalis*, *Manduca*, *Drosophila*, and *Caenorhabditis elegans* (Gan and Macagno, 1995; Grueber *et al*, 2001, 2003; Gallegos and Bargmann, 2004), suggesting that tiling is a general mechanism that organizes the dendritic fields.

The *Drosophila* peripheral nervous system contains identifiable neurons with cell-type-specific dendritic morphologies, including da neurons (Bodmer and Jan, 1987). Dendrites of class IV da neurons tile the body wall in a cell-type-specific manner (Grueber *et al*, 2003; Parrish *et al*, 2007). Time-lapse analysis has indicated that terminal

*Corresponding author. Neural Morphogenesis Laboratory, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan. Tel.: +81 55 981 5860; Fax: +81 55 981 5860;
E-mail: kemoto@lab.nig.ac.jp

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dendrites of these class IV neurons often stop growing or turn when they encounter dendrites of the same type (Grueber *et al*, 2003; Sugimura *et al*, 2003; Emoto *et al*, 2004). In addition, laser ablation of class IV neurons causes an invasion of the vacated dendritic territories by neighbouring class IV neurons (Grueber *et al*, 2003; Sugimura *et al*, 2003). Conversely, duplication of class IV neurons results in a partitioning of the receptive field. These observations indicate that dendritic tiling in class IV neurons arises from homotypic repulsive interactions between dendrites of neighbouring cells. This tiling mechanism functions in class IV neurons to avoid crossing of homotypic branches in the same neurons (iso-neuronal tiling) as well as between neighbouring neurons (hetero-neuronal tiling). Recent studies indicate that in addition to the tiling mechanism in class IV neurons, the self-avoidance mechanism functions in all da neurons to ensure proper spacing of dendritic branches (Gao, 2007).

From the results of a genetic screen for genes that regulate dendritic tiling, the NDR family kinase Tricornered (Trc) and its activator Furry (Fry) were identified as important components of the intracellular signalling cascade that regulates homotypic repulsion in class IV da neurons (Emoto *et al*, 2004). Dendrites of *trc* and *fry* mutants fail to avoid homologous dendritic branches, resulting in a significant overlap of dendritic fields. The Trc kinase signalling is required for the homotypic repulsion between neighbouring dendrites (hetero-neuronal tiling) and also between dendritic branches within single neurons (iso-neuronal tiling) (Emoto *et al*, 2004; Gao, 2007; Parrish *et al*, 2007). The *C. elegans* Trc (Sax-1) and Fry (Sax-2) homologues have also been found to serve a similar function in mechano-sensory neurons (Gallegos and Bargmann, 2004), indicating an evolutionarily conserved function for the Trc signalling in dendritic tiling. Hence, a more detailed understanding of Trc signalling may provide new insights into dendritic tiling. The NDR family of kinases including Trc is activated by the phosphorylation of a conserved serine in the kinase domain (Ser292 in Trc) and a conserved threonine within the hydrophobic motif (Thr449 in Trc). Recent genetic and biochemical studies have indicated that the Ste20 family of MST kinases can contribute to phosphorylation of this conserved threonine (Mah *et al*, 2001; Stegert *et al*, 2005; Emoto *et al*, 2006; Seiler *et al*, 2006), whereas the serine residue appears to be phosphorylated by NDR kinases themselves. In *Drosophila*, for example, the Ste20 kinase Hippo (Hpo) directly phosphorylates Trc on Thr449 *in vivo* and *in vitro* (Emoto *et al*, 2006), yet the regulatory mechanism for Trc activation in neurons still remains elusive.

In this study, we report that TORC2 genes function cell-autonomously to regulate the dendritic tiling of *Drosophila* class IV da neurons. Mutations in the TORC2 genes cause significant defects in dendritic tiling of class IV da neurons, which are similar to those observed in *trc* and *fry* mutants. TORC2 mutations genetically interact with *trc* mutations to affect dendritic tiling, and both Trc and its human homologue NDR1 can form a complex with TORC2 in neurons and cultured cells. Furthermore, we provide genetic and biochemical evidence that TORC2 is required for Trc activation both *in vitro* and *in vivo*. These findings establish TORC2 as a critical regulator of dendritic tiling in *Drosophila* sensory neurons through the Trc signalling pathway.

Results

Sin1 and Rictor are required cell-autonomously to control dendritic tiling

To isolate the genes required for dendritic tiling of class IV neurons, we carried out a genetic screen using the *pickpocket*-EGFP (*ppk*-EGFP) reporter, which specifically labels class IV da neurons (Grueber *et al*, 2003). From ~300 mutant lines carrying PiggyBac transposon (PBc) insertions on the second chromosome (Thibault *et al*, 2004), we isolated one PBc insertion line with a robust dendritic tiling defect in class IV neurons (Figure 1A and B). This PBc is inserted into the single coding exon of *sin1* (Hietakangas and Cohen, 2007), and is therefore likely to eliminate the Sin1 activity (hereafter this PBac insertion line is referred as $sin1^{PBac}$). Homozygosity of $sin1^{PBac}$ or trans-heterozygous combinations of $sin1^{PBac}$ and a chromosomal deficiency (Df) that uncovers *sin1* showed identical dendritic tiling defects (Figure 1F). In contrast, a heterozygosity of $sin1^{PBac}$ or hemizygosity of *sin1* caused no such defects, indicating that the tiling defects we observed in $sin1^{PBac}$ result from the loss of *sin1* functions. Quantification of the crossing points between dendritic branches indicated that ~10% of dendritic branches crossed one another in both $sin1^{PBac}$ homozygotes ($11.8 \pm 2.8\%$, $n = 25$) and $sin1^{PBac}$/Df heterozygotes ($12.6 \pm 2.1\%$, $n = 25$), compared with ~1% of crossing in wild-type (WT) dendrites ($1.2 \pm 0.2\%$, $n = 15$) (Figure 1F). The excessive overlap of mutant dendrites is unlikely to result from abnormal stratification of terminal branches, as the terminal branches were sandwiched between the epidermis and muscles, which were typically ~1 μm apart in both mutant and WT larvae. In addition to the dendritic tiling defect, the total number of dendrite branches in *sin1* mutants was reduced to ~80% of WT (146.0 ± 11.4; $sin1^{PBac}/sin1^{PBac}$, 101.5 ± 10.7; and $sin1^{PBac}$/Df, $102.3 \pm 12.2/4 \times 10^4\ \mu m^2$) (Figure 1A, B, D, and E). Thus, in addition to the dendritic tiling, Sin1 may have a function in dendritic branching of class IV da neurons.

Sin1 is implicated in various signalling processes through its formation of a complex with different partners, including stress-activating protein kinase (Wilkinson *et al*, 1999; Schroder *et al*, 2005), Ras small GTPase (Lee *et al*, 1999), and the components of TORC2 Rictor and TOR (Jacinto *et al*, 2006; Yang *et al*, 2008). To determine whether Sin1 functions together with any of these known interactors to control dendritic tiling, we examined dendrite phenotypes in mutants for the potential Sin-binding partners and found a prominent tiling defect of class IV dendrites in mutants for *rictor*, which encodes a component unique to TORC2 (Figure 1C). The phenotypes observed in *rictor* dendrites were quantitatively similar to those observed in *sin1* mutants: the number of dendritic crossings was significantly higher ($8.1 \pm 1.7\%$, $n = 25$) than that in WT, whereas the terminal branch number was decreased to ~80% ($114.2 \pm 13.4/4 \times 10^4\ \mu m^2$) of that in WT (Figure 1D–F). Consistent with the earlier finding that Sin1 and Rictor function together to regulate tiling, trans-heterozygous combinations of *sin1* and *rictor* alleles caused significant dendritic defects that were qualitatively similar to *sin1* and *rictor* null mutants, whereas heterozygosity of *sin1* or *rictor* had no obvious dendritic phenotype on its own (Figure 1D–F). Finally, *sin1 rictor* double mutants showed dendritic tiling defects that were indistinguishable from the single mutants (Figure 1D–F). Hence, Sin1 and Rictor most

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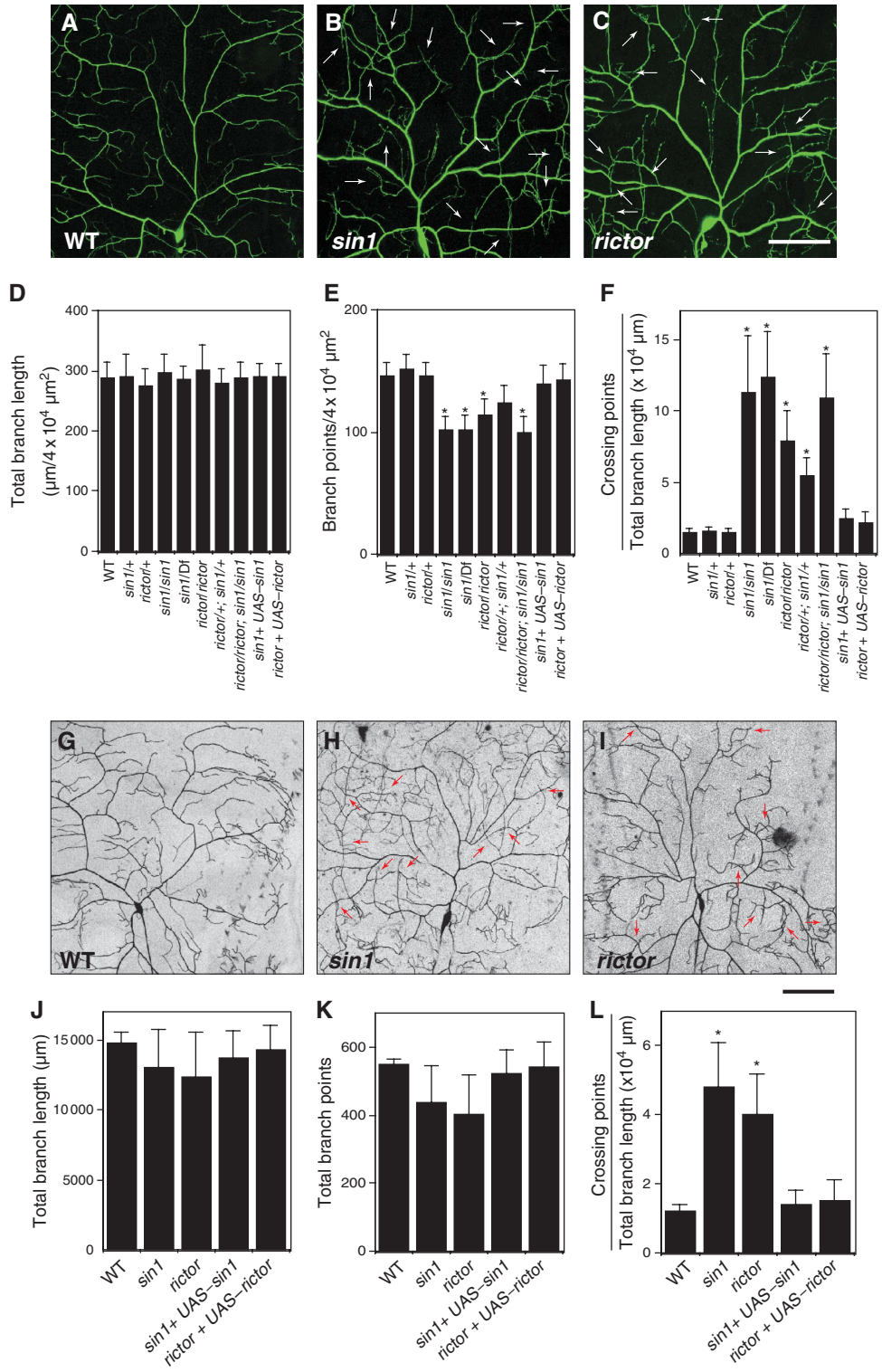

Figure 1 *sin1* and *rictor* function cell-autonomously in regulation of dendritic tiling in class IV neurons. (**A–C**) Live images of ddaC dendrites visualized by the *pickpocket*-EGFP (*ppk*-EGFP) reporter in wild-type (WT) (A), $sin1^{PBac}$ homozygote (B), $rictor^{\Delta 2}$ homozygote (C). Anterior is left and dorsal is up. Arrows indicate crossing points of dendritic branches. Scale bar = 50 μm. (**D–F**) Quantification of the total branch length (D), the branch number (E), and the crossing points (F) of WT and mutant ddaC dendrites. Error bars indicate mean ± s.d. (WT, $n = 15$; others, $n = 25$), $^*P < 0.01$ (Student's *t*-test). Note that larvae heterozygous for $sin1^{PBac}$ over a deletion [Df(2R)BSC11] uncovering the *sin1* gene show dendritic tiling defects identical to those of *sin1* homozygotes. Genotypes: (A) *yw; +/+; ppk*-EGFP/*ppk*-EGFP, (B) *yw;* $sin1^{PBac}/sin1^{PBac}$*; ppk*-EGFP/*ppk*-EGFP, and (C) *yw,* $rictor^{\Delta 2}/yw$, $rictor^{\Delta 2}$*; +/+; ppk*-EGFP/*ppk*-EGFP. (**G–I**) MARCM clones of WT (G), *sin1* (H), and *rictor* (J) are shown. Arrows indicate the crossing points of the dendrites. Scale bar = 50 μm. (**J–L**) Quantification of the branch length (J), the branch points (K), and the crossing points (L) of MARCM clones. (WT, $n = 5$; *sin1*, $n = 11$; *rictor*, $n = 9$) Clone genotypes: (G) *hsFLP, elav-Gal4, UAS-mCD8-GFP/+; FRT42D*, (H) *hsFLP, elav-Gal4, UAS-mCD8-GFP/+; FRT42D,* $sin1^{PBac}$, AND (I) *FRT19A,* $rictor^{\Delta 2}$*; UAS-Gal4[109(2)80], UAS-mCD8GFP/hsFLP.* Error bars indicate mean ± s.d., $^*P < 0.01$ (Student's *t*-test).

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probably function in the same signalling pathway to regulate dendritic tiling.

As *sin1* appears to be expressed ubiquitously (Supplementary Figure S1), the dendritic phenotypes in *sin1* and *rictor* mutants may reflect a cell-autonomous requirement of these genes in neurons or could be a consequence of non-autonomous functions of these genes in surrounding tissues such as the epidermis and muscles. To distinguish between these two possibilities, we carried out MARCM (mosaic analysis with a repressible cell marker) analysis (Lee and Luo, 1999) to generate single-cell clones that are homozygous for null mutations in *sin1* or *rictor* in a heterozygous background and analysed the effects on dendritic tiling. Similar to the *sin1* and *rictor* homozygous mutants, dorsal class IV neuron MARCM clones of *sin1* or *rictor* mutants showed defects in dendritic tiling, indicating that TORC2 genes are cell-autonomously required for dendritic tiling (Figure 1G–I). In contrast, dendrites of class I MARCM clones were not significantly affected in *sin1* or *rictor* mutants (Supplementary Fugure S2). Class IV neuron-specific expression of *sin1* and *rictor* largely rescued the dendritic phenotypes of *sin1* and *rictor* MARCM clones, respectively (Figure 1J–L), further confirming the cell-autonomous functions of Sin1 and Rictor in class IV da neurons. In general, the tiling defects in *sin1* or *rictor* clones were less severe than those observed in the *sin1* or *rictor* homologous mutant larvae. This could be an effect of protein perdurance in MARCM clones (Lee and Luo, 1999). Alternatively, there may be cell-nonautonomous functions of the TORC2 genes in dendrite development.

Sin1 and Rictor are required for dendritic tiling between neighbouring class IV neurons

Given the essential roles of Sin1 and Rictor in tiling of terminal branches from the same neuron (iso-neural tiling), we next tested for the requirement in tiling of dendrites from different class IV neurons (hetero-neural tiling). The dendrites of the three class IV neurons found in each hemisegment normally cover the whole epidermis with very little overlap (Grueber *et al*, 2003; Emoto *et al*, 2004; Parrish *et al*, 2007). For example, the adjacent v'ada and vdaB neurons appeared to restrict themselves to their respective dendritic territories and rarely branched into dendritic fields of their neighbours (Figure 2A, B and H). However, in *sin1* and *rictor* null mutants, the v'ada and vdaB dendrites often invaded the neighbouring fields (Figure 2C–F). Furthermore, the hetero-neuronal tiling defects in *sin1* and *rictor* mutants were largely rescued by the neuronal expression of *sin1* or *rictor*, respectively (Figure 2G). These observations suggest that Sin1 and Rictor regulate both iso-neuronal and hetero-neuronal tiling, presumably through the same mechanisms.

TOR controls dendritic arborization and tiling through distinct complexes

Sin1 and Rictor form a complex together with the TOR kinase referred as the TORC2 (Sarbassov *et al*, 2005a; Wullschleger *et al*, 2006; Bhaskar and Hay, 2007). We thus next examined *Tor* mutant MARCM clones for defects in dendritic tiling and found that unlike *sin1* and *rictor* mutant MARCM clones, *Tor* MARCM clones showed a severe and highly penetrant simplification of dendritic arbors, with significant reductions in the number and length of dendritic branches, and hence in the overall size of the receptive field (Figure 3B, G and H). In addition to TORC2, TOR is also found in the functionally distinct TORC1, which is composed of TOR, Raptor, and LST8 (Sarbassov *et al*, 2005a; Wullschleger *et al*, 2006) and has recently been reported to regulate the elaboration of dendritic arbors by phosphorylating ribosomal S6K and 4E-BP in cultured hippocampal neurons (Jaworski *et al*, 2005). We thus next examined *S6K* null mutant MARCM clones and observed dendritic defects similar to *Tor* MARCM clones (Figure 3C, G, and H). Furthermore, *Tor* and *S6K* trans-heterozygotes showed simplified dendrites qualitatively similar to *Tor* and *S6K* mutant MARCM clones (Figure 3D, I, and J). Thus, as observed in cultured neurons, the TORC1-S6K signalling seems to have a critical function in dendrite growth and branching in post-mitotic class IV neurons. In contrast to the *Tor*/*S6K* trans-heterozygotes, a significant tiling defect was observed in larvae trans-heterozygous for mutations in *Tor* and either *sin1* or *rictor* (Figure 3E, F, I and J), supporting the model in which TORC2 composed of TOR, Sin1, and Rictor together regulates the dendritic tiling of class IV neurons. Collectively, our data indicate that TORC1 and TORC2 have distinct functions in the dendrite developments of class IV neurons: TORC1 for dendritic growth and branching, and TORC2 for dendritic tiling.

TORC2 interacts with the Trc kinase signalling pathway to control dendritic tiling

Previous studies have shown that the NDR family kinase Trc/Sax-1 and its activator Fry/Sax-2 control both the iso-neuronal and hetero-neuronal dendritic tiling of sensory neurons in *Drosophila* and *C. elegans* (Emoto *et al*, 2004, 2006; Gallegos and Bargmann, 2004). To examine whether TORC2 genes and *trc* might function in the same genetic pathway to regulate dendritic tiling, we examined genetic interactions between *trc* and the TORC2 genes. As mentioned above, heterozygosity for null mutations in the TORC2 genes *sin1*, *rictor*, or *Tor* caused no significant defects in dendritic arborization including tiling (Figure 1D–F). Similarly, heterozygosity for null alleles of *trc* caused no discernable defects in dendrite development (Figure 4B; Emoto *et al*, 2004). However, trans-heterozygous combinations of mutations in *trc* together with *sin1* caused a significant tiling defect that was comparable to trans-heterozygous combinations of TORC2 mutants (Figure 4C and F). Similarly, trans-heterozygous combinations of *trc* together with *rictor* or *Tor* caused similar tiling defects (Figure 4D–F). Thus, Trc and the TORC2 genes genetically interact to regulate dendritic tiling.

Given the genetic interactions between Trc and TORC2 components in dendritic tiling control, we next tested whether Trc could physically associate with TORC2 proteins. We expressed an epitope-tagged version of Sin1 (Sin1-Flag) in larval neurons using a nervous-system-specific Gal4 driver and found that Trc could be co-immunoprecipitated with Sin1-Flag (Figure 4G). This co-immunoprecipitation appeared to be specific, because Warts, another NDR kinase present in neurons, did not co-immunoprecipitate with Sin1-Flag (Figure 4G). These results suggest that Trc might associate with TORC2 in the *Drosophila* nervous system.

To further examine the physical interaction between TORC2 and Trc, we evaluated whether endogenous TOR complexes can be immunoprecipitated with Trc (Figure 4H). As no reliable antibodies are available for *Drosophila* TORC2 components, we carried out co-immunoprecipitations using

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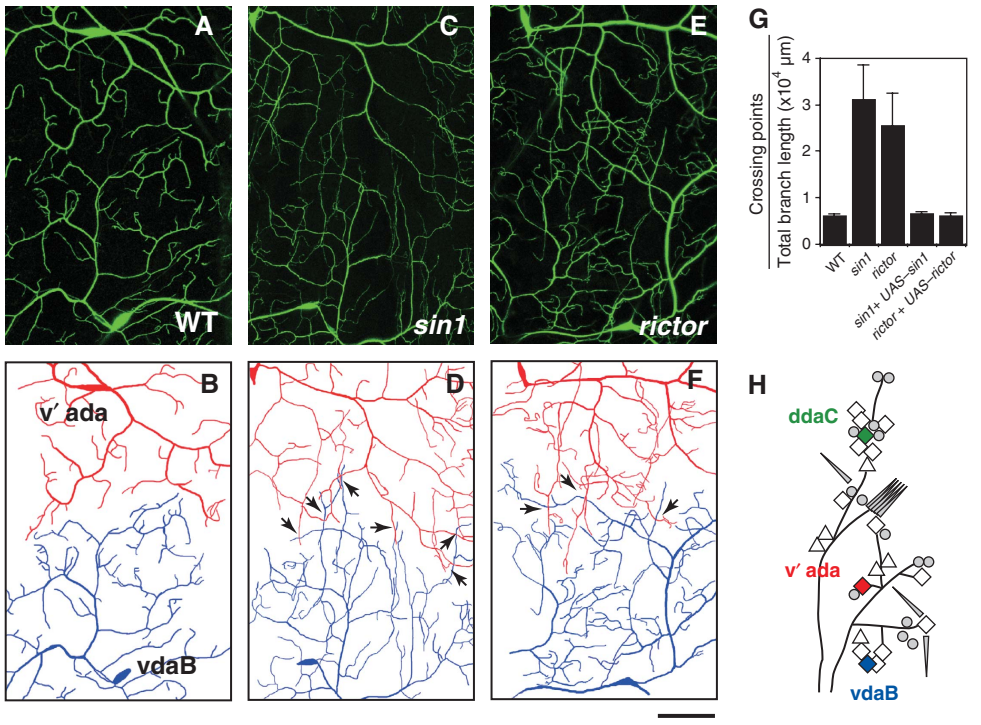

Figure 2 Hetero-neuronal dendritic tiling defect in *sin1* and *rictor* mutants. (**A**–**F**) Live images and their traces of adjacent v'ada and vdaB dendrites. In wild-type (WT) larvae (A), the dendrites of the adjacent class IV neurons, v'ada and vdaB, do not overlap; however, class IV dendrites overlap extensively in *sin1* (C) and *rictor* (E) mutants, as evident from the tracing of dendrites derived from v'ada (red) and vdaB (blue) neurons in WT (B), *sin1* (D), and *rictor* (F) larvae. Arrows indicate the crossing points of dendritic branches between the neighbouring neurons. Scale bar = 50 μm. Genotypes: (A) *yw; +/+; ppk*-EGFP/*ppk*-EGFP, (C) *yw; sin1^{PBac}/sin1^{PBac}; ppk*-EGFP/*ppk*-EGFP, and (E) *yw, rictor$^{\Delta 2}$/yw, rictor$^{\Delta 2}$; +/+; ppk*-EGFP/*ppk*-EGFP. (**G**) Quantification of the crossing points in v'ada and vdaB dendrites of the WT and the mutant third instar larvae. We normalized the crossing number to the total branch length of the ventral area of v'ada dendrites and the dorsal area of vdaB dendrites. Error bars indicate mean ± s.d. (WT, $n = 15$, *sin1*, $n = 11$; *rictor*, $n = 9$), $^*P < 0.01$ (Student's *t*-test). Rescue genotypes: *sin1^{PBac}, UAS-sin1-Flag/sin1 ^{PBac}, ppkGal4; ppk*-EGFP/*ppk*-EGFP and *rictor$^{\Delta 2}$/rictor$^{\Delta 2}$; +/ppkGal4; UAS-rictor, ppk*-EGFP/*ppk*-EGFP. (**H**) Schematic representation of an abdominal hemisegment of the *Drosophila* larval peripheral nervous system (PNS). Dendritic arborization (da) neurons are indicated by diamonds; triangles, other multidendritic neurons; circles, extra sensory neurons; and cylinders, chordotonal organs.

HeLa cell extracts and antibodies specific for human TORC2 components and a human Trc homologue NDR1 (Hergovich *et al*, 2006). We found that the mTOR protein isolated with a specific antibody interacted with NDR1 as well as with hSin1, Rictor, and a TORC1-specific component Raptor (Figure 4H). In contrast, the protein complexes isolated with hSin1 or Rictor antibodies contained mTOR and NDR1 but not Raptor, while those isolated with the Raptor antibody contained mTOR but not NDR1, hSin1, nor Rictor (Figure 4H). These results indicate that NDR1 interacts, at least in part, with TORC2 but not with TORC1. As reported previously, both TORC1 and TORC2 were stable in 0.3% CHAPS buffer but were disrupted by 1% Triton X-100 (Figure 4H). Interestingly, although the interaction between mTOR and NDR1 was disrupted by Triton X-100, the interactions between NDR1 and hSin1 or Rictor were stable under these conditions (Figure 4H), suggesting that NDR1 associates with hSin1 and/or Rictor, rather than mTOR.

Previous studies suggest that the avoidance behaviour of class IV dendrites requires dynamic remodelling of the cytoskeletons (Grueber *et al*, 2003; Sugimura *et al*, 2003; Emoto *et al*, 2004; Parrish *et al*, 2007). To examine the possible function of TORC2 and Trc in the cytoskeletal organization, we used an established assay for monitoring actin network reorganization in cultured *Drosophila* S2 cells (Rogers *et al*, 2003). When placed on glass coverslips coated with the lectin concanavalin A, S2 cells reorganize their actin network to build a lamellipodium (Figure 4I, smooth). RNA interference (RNAi) knockdown of Sin1 or Rictor resulted in a significant increase in cells with aberrant organizations of their actin filaments (Figure 4I and J). These cells can be classified into three categories: cells with normal lamellae, cells that spread but showed an abnormal serrated edge, and cells that spread but showing a stellate morphology. Although the stellate morphology was observed in <5% of the control cells (3.0%, $n = 123$) or Raptor RNAi-treated cells (4.2%, $n = 113$), more than 30% of the Sin1 (31.3%, $n = 137$)- or Rictor (41.4%, $n = 150$)-treated RNAi cells exhibited a stellate shape (Jacinto *et al*, 2004; Sarbassov *et al*, 2004; Yang *et al*, 2008). It is interesting that, Trc knockdown cells also showed severe cytoskeletal defects (stellate cells: 31.2%, $n = 164$), which were found to be similar to those observed in Sin1 and Rictor knockdown cells (Figure 4I and J). These observations suggest that TORC2 and Trc may regulate actin cytoskeletal organizations via the same signalling pathway.

TORC2 is essential for the Trc kinase activity

Trc has conserved phosphorylation sites at Ser292 and Thr449 (Figure 5A), and phosphorylation of both of these residues is essential for maximal activation of the NDR family kinases (Millward *et al*, 1999; Mah *et al*, 2001; Tamaskovic *et al*, 2003; Emoto *et al*, 2004, 2006; Hergovich *et al*, 2006).

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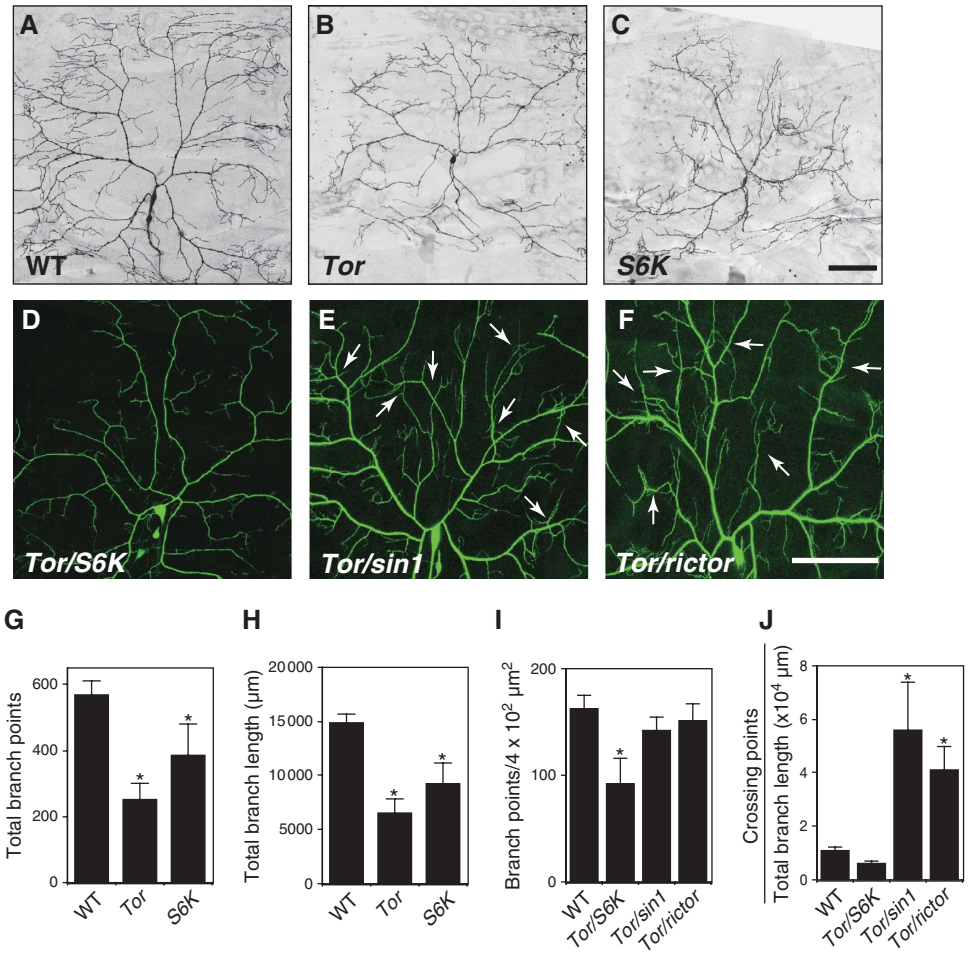

Figure 3 TORC1 and TORC2 regulate dendritic growth/branching and tiling, respectively. (**A–C**) *Tor* and *S6K* MARCM clones are defective in both dendritic arborization and branching. MARCM clones of (A) wild-type (WT), (B) $Tor^{\Delta P}$, and (C) $S6K^{l-1}$ are shown. Bar represents 50 μm. Clone genotypes: (A) *hsFLP, elavGal4, UAS-mCD8-GFP/+; FRT40A*, (B) *hsFLP, elavGal4, UAS-mCD8-GFP/+; FRT40A,* $Tor^{\Delta P}$; (C) *hsFLP, elavGal4, UAS-mCD8-GFP/+; +/+; FRT82B,* $S6K^{l-1}$. (**D–F**) Live images of ddaC dendrites visualized by the *ppk*-EGFP reporter in third instar larvae trans-heterozygous for $Tor^{\Delta P}$ and $S6K^{l-1}$ (D), for $Tor^{\Delta P}$ and $sin1^{PBac}$ (E), and for $Tor^{\Delta P}$ and $rictor^{\Delta 2}$ (F). Arrows in (E) and (F) indicate the crossing points of the dendritic branches. Genotypes: (D) $Tor^{\Delta P}/+$; $S6K^{l-1}$, *ppk*-EGFP/*ppk*-EGFP, (E) $Tor^{\Delta P}/sin1^{PBac}$; *ppk*-EGFP/*ppk*-EGFP, and (F) $rictor^{\Delta 2}/+$; $Tor^{\Delta P}/+$; *ppk*-EGFP/*ppk*-EGFP. (**G**, **H**) Quantification of the total branch points (G) and the branch length (H) of MARCM clones. Error bars indicate the mean ± s.d. (WT, $n=6$; *Tor*, $n=11$; *S6K*, $n=5$), $^*P<0.01$ relative to WT controls (Student's *t*-test). (**I**, **J**) Quantification of the branch points (I) and the crossing points (J) per μm² (4×10^4) of the dendritic branches at the third instar larval stage in ddaC neurons. Error bars indicate the mean ± s.d. ($n=15$), $^*P<0.01$ (Student's *t*-test).

In particular, phosphorylation on the threonine residue in the C-terminal hydrophobic motif (Thr449 in Trc) is tightly correlated with the NDR kinase activity in mammalian cultured cells (Tamaskovic *et al*, 2003; Hergovich *et al*, 2006). To test whether TORC2 might have a function in Trc activation, we generated rabbit polyclonal antibodies directed against phospho-epitopes of Ser292 and Thr449 (Supplementary Figure S3). Using these reagents, we examined phosphorylation states of Trc in WT and mutant embryos and found that phosphorylation on Thr449 was significantly reduced in *sin1*, *rictor*, and *Tor* mutant embryos compared with WT (Figure 5B). This reduction likely reflects a specific requirement of TORC2 for Trc phosphorylation as Thr449 phosphorylation was not obviously reduced in either the *S6K* or *Akt* mutants (Figure 5B). In contrast to Thr449 phosphorylation, phosphorylation on Ser292 was not significantly altered in the *sin1*, *rictor*, or *Tor* mutants (Figure 5B). These results suggest that TORC2 has a potential function in Trc activation *in vivo*.

To further examine whether TORC2 is essential for Trc activity, we next carried out RNAi experiments in cultured *Drosophila* S2 cells. Under basal conditions, Trc phosphorylation on Thr449 was too low to be detected by our phospho-specific antibodies, likely due to the low level of basal Trc phosphorylation on Thr449 (Millward *et al*, 1999; Tamaskovic *et al*, 2003). We therefore examined these RNAi effects under okadaic acid (OA) treatment conditions, which stimulate the basal Trc phosphorylation, and thus render the RNAi inhibitory effects more visible (Figure 5C). Consistently, the Trc kinase activity was elevated by about seven-fold by OA treatment (Figure 5C). Similarly, Trc phosphorylation on Thr449 was significantly increased after OA stimulation, confirming a close correlation between the Trc kinase activity and Thr449 phosphorylation. Knockdown of the TORC2 components Sin1, Rictor, or Tor with double-stranded RNAs (dsRNAs) largely eliminated both OA-induced Trc activation and phosphorylation on Thr449, although the total amount of Trc protein was not significantly affected

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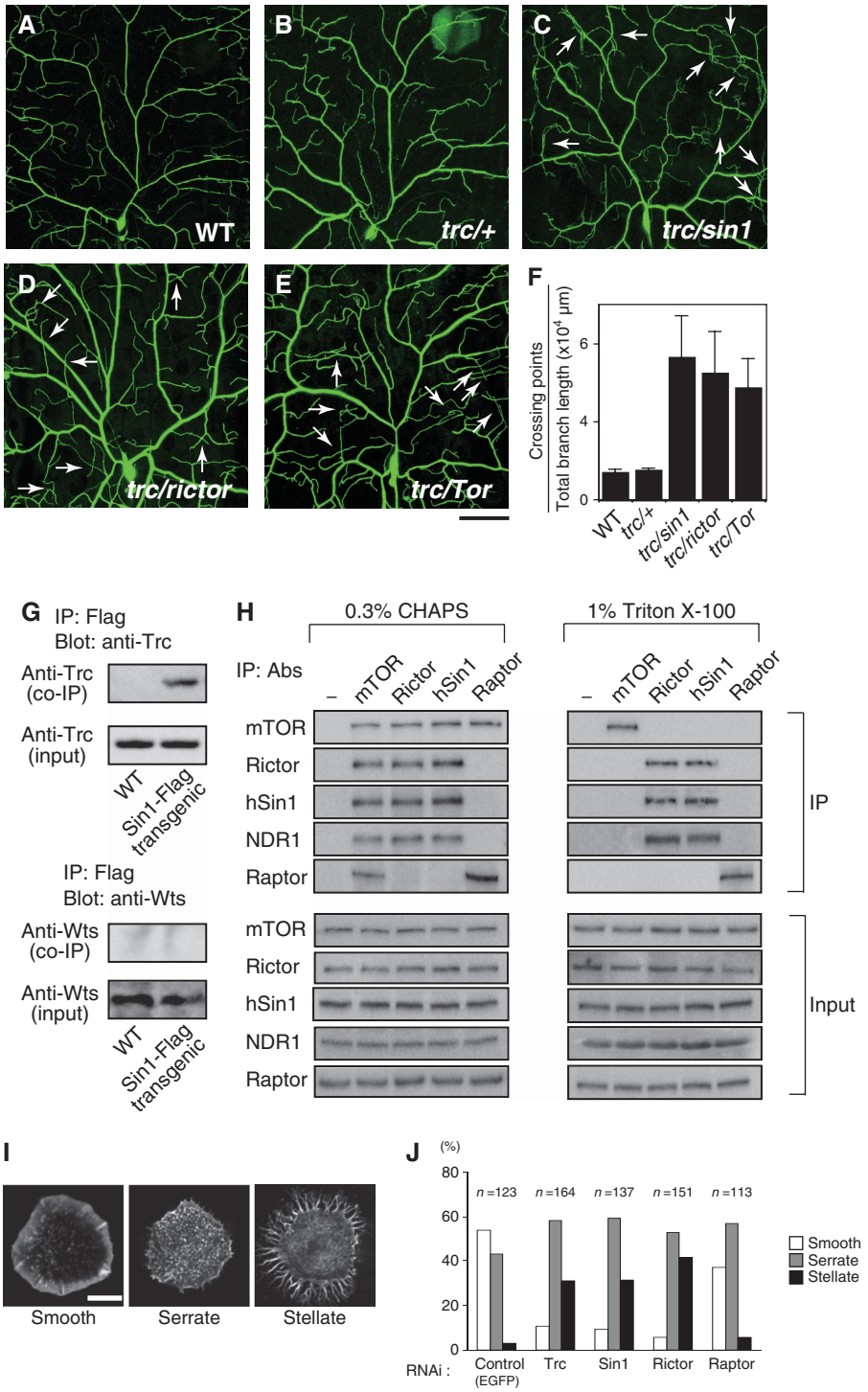

Figure 4 TORC2 genetically and physically interacts with Trc. (**A–F**) Live images of third instar class IV neuron visualized using the *pickpocket*-EGFP reporter in (A) wild-type (WT), (B) *trc/+*, (C) *trc/sin1* trans-heterozygous, (D) *trc/rictor* trans-heterozygous, (E) and *trc/Tor* trans-heterozygous larvae. Anterior is left and dorsal is up. Bar = 50 μm. Genotypes: (A) *yw; +/+; ppk*-EGFP/*ppk*-EGFP, (B) *yw; +/+; trc*^{1}, *ppk*-EGFP/*ppk*-EGFP, (C) *yw; sin1*^{PBac}*/+; trc*^{1}, *ppk*-EGFP/*ppk*-EGFP, (D) *yw, rictor*$^{\Delta 2}$*/+; +/+; trc*^{1}, *ppk*-EGFP/*ppk*-EGFP, and (E) *yw; Tor*$^{\Delta P}$*/+; trc*^{1}, *ppk*-EGFP/*ppk*-EGFP. (F) Quantification of the crossing points in ddaC of WT and trans-heterozygotes. Error bars indicate the mean ± s.d. ($n = 15$) (**G**) Trc can form a complex with Sin1 in *Drosophila* neurons. Trc and Wts were co-immunoprecipitated with neuronally expressed Sin1-Flag from transgenic fly embryos as indicated by western blot analysis using anti-Trc and anti-Wts antibodies, respectively. (**H**) Association of endogenous TORC2 and NDR1 in human HeLa cells. The cells were lysed in buffer containing either 0.3% CHAPS or 1% Triton X-100 as indicated. −, immunoprecipitation control (no primary antibody was used). Co-immunoprecipitation of the TORC components was detected by specific antibodies as described. (**I**) Morphologies of phalloidin-labeled S2 cells on concanavalin (Con) A-coated coverslips were classified into three groups (stellate, serrate, and smooth). Cells were treated with dsRNA against indicated genes for 7 days and then plated on Con A and then stained with rhodamine-phalloidin to visualize filamentous actin. (**J**) Quantification of cell shape on knockdown of the indicated genes. RNAi knockdown of Trc- or TORC2-specific components (Sin1and Rictor) causes a significant increase in the number of stellate cells. Note that the cell morphology was not significantly affected by genetic ablation of Raptor, a TORC1-specific component.

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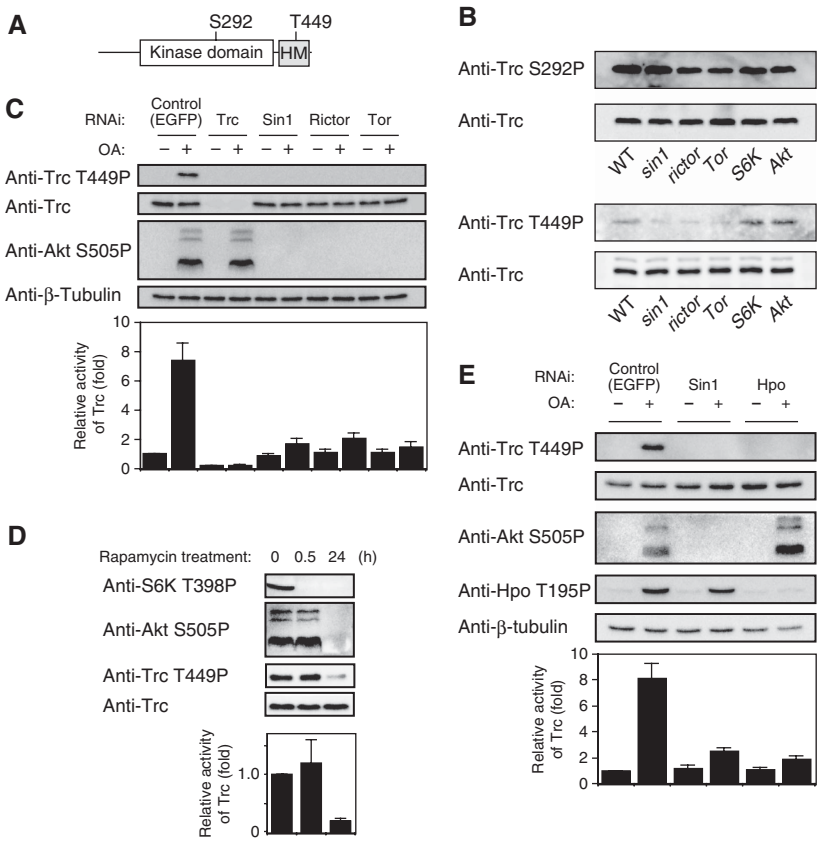

Figure 5 TORC2 is essential for the maximal activation of Trc. (**A**) Schematic representation of the Trc domain structure and phosphorylation sites. HM indicates the hydrophobic motif, which is highly conserved in the NDR kinase family. (**B**) Trc phosphorylation of Thr449 but not Ser292 was reduced in *sin1*, *rictor*, and *Tor* mutant embryos. Embryos (stage 16/17) homozygous for $sin1^{PBac}$, $rictor^{\Delta 2}$, $Tor^{\Delta P}$, $S6K^{l-1}$, and Akt^{1} were selected by their lack of a GFP-expressing balancer chromosome, and then homogenized in sample buffer, boiled, and analyzed by immunoblotting with the indicated antibodies. (**C**) Knockdown of Sin1, Rictor, or Tor inhibits Trc activation in *Drosophila* S2 cells. Different dsRNAs used in the experiments are indicated. 'Control' denotes control dsRNA targeting EGFP. S2 cells were treated with 100 nM okadaic acid (OA+) or with solvent alone (OA−) for 30 min before harvesting where indicated. Cell lysates were analyzed by immunoblotting for the phosphorylation level of Akt (Ser505 for TORC2 activity) and Trc (Thr449). The bottom panel indicates Trc kinase activity relative to the control ('control ' without OA treatment). The kinase activity was determined using the NDR kinase substrate peptides. Error bars indicate the mean ± s.d. ($n = 3$). (**D**) Prolonged treatment of cells with rapamycin inhibits Trc activation. S2 cells were treated with 100 nM rapamycin for the indicated times. Cell lysates were then analyzed by immunoblotting for phosphorylated S6K (Thr398 for TORC1 activity), Akt (Ser505 for TORC2 activity), and Trc (Thr449). The bottom panel indicates Trc kinase activity relative to the control (0 h treatment). (**E**) TORC2 and Hpo regulate Trc activity through different pathways. S2 cells were incubated with dsRNA against the indicated genes for 7 days and then treated with 100 nM okadaic acid (OA+) or with solvent alone (OA−) for 30 min before harvesting. Cell lysates were then analyzed by immunoblotting for the phosphorylated Akt (Ser505 for TORC2 activity), Hpo (Thr195 for Hpo activity), and Trc (Thr449). Bottom panel indicates Trc kinase activity relative to the control ('control' without OA treatment). Error bars indicate the mean ± s.d. ($n = 3$).

(Figure 5C). This is consistent with the results of our analysis of Trc phosphorylation in TORC2 mutant embryos (Figure 5B). Conversely, the knockdown of Trc did not affect Akt phosphorylation on Ser505, a direct target of TORC2 (Figure 5C), indicating that Trc is not required for the TORC2 activity. It therefore appears that TORC2 likely functions upstream of the Trc signalling pathway.

We next attempted to confirm the function of TORC2 in the Trc signalling pathway using a pharmacological approach. Sarbassov *et al* (2006) have shown that although a short rapamycin treatment (0.5–1 h) specifically disrupts TORC1, a prolonged rapamycin treatment (48–72 h) inhibits both TORC1 and TORC2 functions in mammalian cultured cells. We thus treated S2 cells with 100 nM rapamycin for different time periods and examined the effects of this on Akt phosphorylation of Ser505 and S6K phosphorylation on Thr398, well-known phosphorylation sites for TORC2 and TORC1, respectively. Although a 30-min treatment of S2 cells eliminated S6K phosphorylation but not Akt phosphorylation, a 48-h treatment caused a strong inhibition of both S6K phosphorylation and Akt phosphorylation (Figure 5D), indicating that a short rapamycin treatment causes specific disruption of TORC1, whereas a prolonged rapamycin treatment inhibits both TORC1 and TORC2 functions in S2 cells. This is similar to what has been observed in mammalian cultured cells. Under the same rapamycin treatment conditions, neither the Trc activity nor Trc phosphorylation on Thr449 was affected after a 30-min treatment, but both were suppressed by a 48-h treatment (Figure 5D). These results strongly suggest that TORC2, but not TORC1, is required for Trc activation in S2 cells.

Finally, we examined whether TORC2 regulates Trc activity through the Ste20 family kinase Hpo, as Hpo activates Trc by phosphorylating Thr449, thereby controlling the dendritic tiling of class IV neurons (Emoto *et al*, 2006). On the basis of the results of previous reports (Praskova *et al*, 2004), we

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used Hpo autophosphorylation on Thr195 as a marker of Hpo activation. Under OA treatment conditions, the knockdown of Hpo with dsRNAs largely inhibited both Trc activity and Trc phosphorylation on Thr449, consistent with a previous report that Hpo is predominantly responsible for Thr449 phosphorylation (Emoto *et al*, 2006). However, in Hpo knockdown cells, Akt phosphorylation on Ser505 was not significantly reduced (Figure 5E). Similarly, Sin1 knockdown did not affect Hpo activation, although Trc activity and phosphorylation were inhibited (Figure 5E). These results suggest that both TORC2 and Hpo are required for full activation of the Trc kinase, and that TORC2 and Hpo appear to control Trc kinase activity through independent pathways.

A dominant active form of Trc rescues the dendritic tiling defects in sin1 *and* rictor *neurons*

We reasoned that if TORC2 acts upstream of the Trc signalling pathway in class IV neurons to control dendritic tiling, overexpression of Trc might rescue some aspects of *sin1* and *rictor* mutant phenotypes. We first overexpressed WT Trc using a class IV neuron-specific Gal4 driver and found a slight reduction in the tiling phenotypes of *sin1* and *rictor* mutants (Figure 6D and G). As full activation of Trc/NDR kinases requires activator proteins such as MOBs (designated as Mats in *Drosophila*) and Fry (Emoto *et al*, 2004; Hergovich *et al*, 2005; Hirabayashi *et al*, 2008), overexpression of Trc alone might be insufficient to activate the Trc signalling in neurons.

Previous studies indicate that the membrane targeting of human NDR1 leads to its constitutive activation (Hergovich *et al*, 2005). Accordingly, we generated a membrane-anchored version of Trc by fusing a myristylation signal from *Drosophila* Src1 to the N-terminus of Trc (Trc^{Myr}) and expressed it in cultured *Drosophila* S2 cells. Although WT Trc was predominantly localized in the cytosol, the Trc^{Myr} was largely observed at the plasma membrane (Figure 6A), confirming that the myristylation signal facilitates the membrane targeting of Trc. A similar membrane localization was observed with the phosphorylation point mutant Trc^{Myr} T449A and the kinase-dead mutant Trc^{Myr} T122A (Figure 6A). Thus, neither phosphorylation on Thr449 nor kinase activity is necessary for the membrane localization of Trc^{Myr}. Remarkably, Trc^{Myr} became phosphorylated on Thr449 even in the absence of OA treatment (Figure 6B), consistent with a previous report that the myristylated NDR kinases act as constitutively active forms in cultured cells (Hergovich *et al*, 2005). We further found that a single copy of trc^{Myr} rescues the tiling defects in *trc* mutants (Supplementary Figure S4), indicating that Trc^{Myr} retains WT activity.

We next introduced a single copy of the transgene into a *sin1* or *rictor* null background. Strikingly, the expression of Trc^{Myr} in class IV neurons substantially rescued both iso-neuronal and hetero-neuronal tiling defects in *sin1* and *rictor* dendrites (Figure 6E, H and I, Supplementary Figure S5). The total branch length of class IV neurons was not significantly affected by Trc^{Myr}, indicating that the rescue of tiling phenotype is not secondary to the growth defects of dendritic branches (Figure 6J). Furthermore, the ability of Trc^{Myr} to rescue the tiling defects of *sin1* and *rictor* mutants was dependent on the phosphorylation on Thr449, as the Trc^{Myr} T449A transgene in which Thr449 was replaced with alanine was unable to rescue these mutants (Figure 6I). These data are consistent with the model in which TORC2 regulates dendritic tiling at least in part by signalling through the Trc signalling pathway.

Discussion

TORC2 regulates dendritic tiling in da neurons

In this study, we have shown that TORC2, composed of Tor, Sin1, and Rictor, is essential for the dendrite tiling of *Drosophila* sensory neurons. Dendrites of class IV neurons rely on homotypic repulsion to ensure that dendrites do not cross over into other boundary, and thus resulting in a complete and non-redundant coverage of the body wall. However, *sin1* mutant class IV dendrites showed significant tiling phenotypes, presumably due to defects in both iso-neuronal and hetero-neuronal repulsion (Figures 1B and 2C, D). Similarly, mutations in *rictor*, which encodes a component of TORC2 but not TORC1, caused similar defects in dendritic tiling (Figures 1C and 2E, F). In addition, *sin1* strongly interacts with *rictor* in the regulation of dendritic tiling (Figure 1D–F), suggesting that Sin1 and Rictor act together as TORC2 in the dendritic tiling control. The tiling defects can be substantially ameliorated by the expression of *sin1* or *rictor* in mutant neurons (Figure 1L), indicating that TORC2 largely functions cell-autonomously to regulate dendritic tiling.

In cultured hippocampal neurons, the inhibition of mTOR by RNAi knockdown or rapamycin treatment leads to reductions in the number of dendrite branches and in the complexity of dendritic arbors, thus indicating that mTOR has an important function in regulating dendrite growth (Jaworski *et al*, 2005). These dendritic defects can be mimicked by the RNAi knockdown of S6K, suggesting that TORC1 may regulate dendrite growth through translational control. Consistent with these findings, we found in our current experiments that TORC1 has a similar important function in regulating dendrite growth in *Drosophila*, that is, *Tor* and *S6K* null mutants were found to be severely defective in dendritic growth and branching in da neurons (Figure 3A–C). In addition, our genetic studies indicated that *Tor* genetically interacts with *S6K* in dendritic growth and branching, but shows a strong interaction with *sin1* and *rictor* in dendritic tiling (Figure 3D–F). Thus, TOR likely acts through two distinct complexes to regulate different aspects of dendrite development in class IV da neurons: TORC1 for dendritic growth/branching and TORC2 for dendritic tiling. Given the evolutionarily conserved role of TORC1 in the regulation of dendrite growth/branching, it is feasible therefore that the role of TORC2 in tiling control is also conserved. Indeed, mammalian homologues of Sin1 and Rictor are highly expressed in specific neurons in the brain (Makino *et al*, 2006; Shiota *et al*, 2006). Although it has not been established whether a tiling mechanism contributes to dendritic field specification in the central nervous system outside the retina, some neurons are known to exhibit contact-mediated growth inhibition of neurites (Sestan *et al*, 1999). It will thus be of interest to examine the potential roles of TORC2 in the regulation of dendritic tiling in the vertebrate nervous system. In addition, accumulating evidence now suggests that mTOR is involved in several neuronal diseases, including neurodegeneration (Ravikumar *et al*, 2004; Khurana *et al*, 2006), neurofibromatosis (Johannessen

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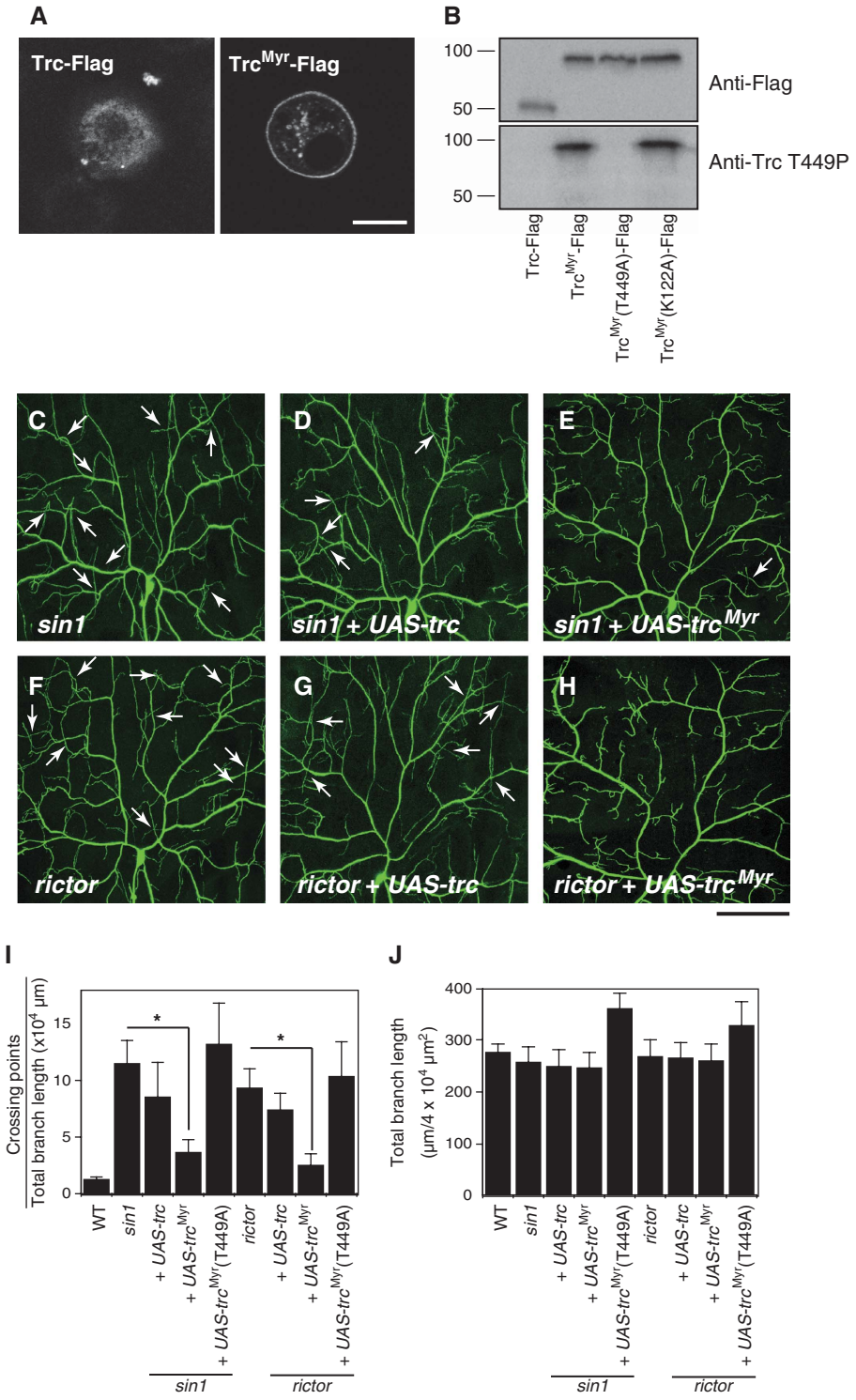

Figure 6 Neuronal expression of a constitutive active form of Trc (Trc^{Myr}) rescues the dendritic tiling defects of the *sin1* and *rictor* mutants. (**A**) S2 cells transfected with wild-type Trc (Trc-Flag), a membrane-targeted Trc (Trc^{Myr}-Flag), or a membrane-targeted Trc with a mutation at the Thr449 site (Trc^{Myr}(T449A)-Flag) were stained with anti-Flag antibodies. Images were taken under a confocal microscope. Scale bar = 10 μm. (**B**) Lysates of S2 cells expressing a Flag-tagged wild-type (WT) (Trc-Flag), a membrane-targeted Trc (Trc^{Myr}-Flag), a membrane-targeted Trc with a mutation at the Thr449 site (Trc^{Myr}(T449A)-Flag), or a membrane-targeted Trc with a kinase-dead mutation at the Lys122 site (Trc^{Myr} (K122A)-Flag) were analyzed by blotting using anti-Flag (top panel) and anti-Thr449P (bottom panel) antibodies. (**C**–**H**) In *sin1* and *rictor* mutant larvae carrying a single copy of *UAS-trc*^{Myr} under the control of *ppk-Gal4* driver, tiling defects in ddaC dendrites were largely rescued. Scale bar = 50 μm. Genotypes: (C) *yw; sin1*^{PBac}*/sin1*^{PBac}*; ppk*-EGFP/*ppk*-EGFP, (D) *yw; UAS-trc, sin1*^{PBac}*/sin1*^{PBac}*; ppk-Gal4, ppk*-EGFP/*ppk*-EGFP, (E) *yw; sin1*^{PBac}*, UAS-trc*^{Myr}*/sin1*^{PBac}*; ppk-Gal4, ppk*-EGFP/*ppk*-EGFP, (F) *yw, rictor*$^{\Delta 2}$*/yw, rictor*$^{\Delta 2}$*; +/+; ppk*-EGFP/*ppk*-EGFP, (G) *yw, rictor*$^{\Delta 2}$*/yw, rictor*$^{\Delta 2}$*; UAS-trc/+; ppk-Gal4, ppk*-EGFP/*ppk*-EGFP, and (H) *yw, rictor*$^{\Delta 2}$*/yw, rictor*$^{\Delta 2}$*; UAS-trc*^{Myr}*/+; ppk-Gal4, ppk*-EGFP/*ppk*-EGFP. (**I**, **J**) Quantification of the dendritic crossing points (I) and the total branch length (J) in the rescue experiments. −, *sin1* or *rictor* mutants carrying no transgene. Error bars indicate the mean ± s.d. ($n = 15$), $^*P < 0.01$ (Student's *t*-test).

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et al, 2005), and schizophrenia (Kalkman, 2006). Although the molecular events underlying the onset of mTOR-related diseases remain poorly understood, much of the current knowledge about these pathologies is associated with TORC1 (Jaworski and Sheng, 2006). In the light of our current findings that TORC1 and TORC2 have distinct functions in dendrite development, it is possible that aberrant signalling through TORC2 may account for some aspects of neuronal disorders. It will thus be intriguing to examine the relationship between TORC2 and mTOR-associated neuronal diseases in future studies.

How does TORC2 regulate dendritic tiling in da neurons? One possibility is that TORC2 may modulate the avoidance behaviour of dendritic branches by regulating cytoskeletal rearrangement. In class IV neurons, an unknown repulsion signal induces the avoidance behaviour of dendrites, which presumably requires dynamic remodelling of the cytoskeleton (Grueber *et al*, 2003; Sugimura *et al*, 2003; Emoto *et al*, 2004; Parrish *et al*, 2007). It can be noted that TORC2 has been implicated in reorganization of the actin cytoskeleton in yeast and mammalian cells (Schmidt *et al*, 1996; Jacinto *et al*, 2004; Sarbassov *et al*, 2004; Yang *et al*, 2008). Similarly, we found that RNAi knockdown of the TORC2 genes *sin1* or *rictor* in S2 cells results in the aberrant organizations of actin fibres (Figure 4I). Hence, TORC2 can regulate cytoskeletal organization in *Drosophila* cultured cells as well. Given that Trc is one of the downstream targets of the TORC2 signalling pathway and that NDR kinases has critical functions in cell morphogenesis by modulating the actin cytoskeleton (Hergovich *et al*, 2006), TORC2 might control the actin cytoskeleton, at least in part, by signalling through Trc in neurons. In support of this model, a Trc knockdown in S2 cells caused defects in actin organization similar to what we observed after TORC2 knockdown (Figure 4I).

Previous reports have suggested that TORC2 phosphorylates PKCα to regulate the cytoskeleton in cultured mammalian cells (Schmidt *et al*, 1997; Sarbassov *et al*, 2004). It is unlikely, however, that Trc acts downstream of PKCα in regulation of the actin cytoskeleton as Trc phosphorylation on Thr449 is unaffected by the RNAi ablation of PKC or Akt (Supplementary Figure S6). Thus, TORC2 might use multiple downstream targets to regulate different aspects of actin organization. Further studies will be required to clarify the functional relationship between PKC signalling and Trc signalling with respect to actin organization.

TORC2 functions upstream of the Trc signalling pathway

Although Trc signalling has been implicated in the control of dendritic tiling in sensory neurons (Emoto *et al*, 2004; Gallegos and Bargmann 2004), little is known about the regulation of Trc activation in neurons. In this study, we provide genetic and biochemical data indicating that Trc functionally interacts with TORC2, but not with TORC1, in class IV neurons and cultured cells (Figure 4). This functional interaction is likely related to the specific association between Trc and the TORC2-specific components Sin1 and/or Rictor (Figure 4G and H). In addition, mutations in TORC2 genes cause a significant reduction in Trc phosphorylation on Thr449 *in vivo* (Figure 5B). Given that the phosphorylation of this residue is critical for Trc activation, it seems likely that TORC2 regulates the Trc activity *in vivo*. Reinforcing this notion, Trc phosphorylation and kinase activity were found to be largely suppressed by RNAi ablation of TORC2 components (Sin1, Rictor, or Tor) and by a pharmacological disruption of the TORC2 complex assembly (Figure 5C and D). These data thus indicate that TORC2 probably functions upstream of the Trc signalling. We thus propose a model in which TORC2 regulates dendritic tiling by signalling through the Trc signalling pathway. This contention is based on the following evidence. First, *sin1* and *rictor* mutants show both iso-neuronal and hetero-neuronal dendritic tiling defects similar to those observed in *trc* mutants (Figures 1 and 2). Second, TORC2 genes including *sin1*, *rictor*, and *Tor* genetically interact with *trc* in the regulation of dendritic tiling (Figure 4A–F). Third, a constitutively active form of Trc can substantially rescue the dendritic tiling defects in *sin1* and *rictor* mutants (Figure 6).

In addition to the dendritic tiling defects, the total number of dendrite branches in *sin1* and *rictor* mutants was reduced to ∼80% of WT (Figure 1E). This branching defect in TORC2 mutants is inconsistent with that of *trc* mutants, as *trc* mutations cause overbranching in class IV dendrites (Emoto *et al*, 2004). Although it remains unknown why TORC2 and *trc* mutants show opposite branching phenotypes, this might be due to the reduction in the Akt activity in TORC2 mutants. Previous studies have shown that Akt activity is required for dendrite growth/branching in cultured neurons (Jaworski *et al*, 2005). Our RNAi experiments in S2 cells indicate that TORC2 is essential for both Trc and Akt activities (Supplementary Figure S6). Therefore, TORC2 mutants might show the combined branching phenotypes of *trc* and *Akt* mutations, resulting in a slight reduction in the branch points.

Our present data indicate that TORC2 is essential for Trc phosphorylation on the Thr449 residue that is critical for the maximal activation of Trc (Figure 5B and C). Recent studies have established that members of the Hpo/MST family of kinases directly phosphorylate the Trc/NDR kinases on this conserved threonine residue in the C-terminal hydrophobic motif (Mah *et al*, 2001; Stegert *et al*, 2005; Emoto *et al*, 2006; Seiler *et al*, 2006). In addition, we found that Hpo and TORC2 are required independently for Trc phosphorylation (Figure 5E). It is thus likely that TORC2 promotes the Hpo-dependent Trc phosphorylation on Thr449 in an indirect manner. The molecular mechanism by which TORC2 regulates Trc phosphorylation is currently unclear. On the basis of the results from recent studies of NDR1, one possible scenario is that TORC2 may have a function in the membrane recruitment of Trc for its activation. Hergovich *et al* (2005) show that the full activation of human NDR kinases requires the recruitment of NDR kinases to the plasma membrane, which presumably induces their proximity to the upstream kinases Hpo/MST kinases and subsequent phosphorylation of the threonine residue in the hydrophobic motif (Thr449 in Trc). MOBs are proposed to have a function in this recruitment process, although no obvious membrane-binding domain is present in these proteins (Hergovich *et al*, 2005). Interestingly, Sin1 contains a pleckstrin homology (PH)-like domain at its C-terminus, and this domain has been shown to be essential for the function and the membrane localization of TORC2 in both yeast and mammalian cells (Schroder *et al*, 2007; Berchtold and Walther, 2009).

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Similarly, we found that *Drosophila* Sin1 overexpressed in S2 cells is localized at the plasma membranes via the C-terminal PH-like domain (data not shown). As Trc associates with TORC2, it is possible that Trc might be recruited to the plasma membrane partially through the activity of TORC2. It is also possible that TORC2 may facilitate the membrane targeting of Trc by phosphorylating MOBs, which are suggested to be important for the membrane recruitment of Trc (Wei *et al*, 2007; Praskova *et al*, 2008). In either scenario, TORC2 is expected to enhance the membrane recruitment of Trc. Given our current finding that a membrane-targeted form of Trc (Trc^{Myr}) can partially rescue dendritic tiling defects in *sin1* and *rictor* mutants (Figure 6), we propose that TORC2 may regulate dendritic tiling by promoting membrane targeting of Trc. It is likely that the precise regulation of Trc activation within dendrites is crucial to its functions in dendritic tiling as the avoidance behaviour of class IV dendrites is induced only when the branches come within a short distance of each other (Grueber *et al*, 2003; Sugimura *et al*, 2003; Emoto *et al*, 2004). Presumably, therefore, TORC2 cooperates with Hpo to regulate the precise recruitment and activation of Trc signalling in specific spatial domains in dendrites.

In summary, we show in our current analyses that there is a novel neuronal function of TORC2 in the control of dendritic tiling of *Drosophila* sensory neurons. We also show that TORC2 regulates dendritic tiling through the Trc signalling pathway. Given the widespread function of mTOR in neuronal development and plasticity, as well as potential implications for neurological diseases, it will be important to determine whether TORC2–Trc signalling is a general mechanism underlying dendritic field specification and neural circuit formation in the nervous system.

Materials and methods

Fly stocks

The following lines were used in this study: $sin1^{PBac}$ (PBac e03756) (Hietakangas and Cohen, 2007), $rictor^{\Delta 2}$ (Hietakangas and Cohen, 2007), *UAS-rictor* (Hietakangas and Cohen, 2007), $Tor^{\Delta P}$ (Knox *et al*, 2007), $S6K^{l-1}$ (Knox *et al*, 2007), Akt^{1} (Stocker *et al*, 2002), trc^{1} (Emoto *et al*, 2004), and Df(2R)BSC11 (a deletion line uncovering *sin1* gene). To visualize class IV dendrites, we used *yw; +/+; ppk*-EGFP/*ppk*-EGFP, *yw;* $sin1^{PBac}/sin1^{PBac}$; *ppk*-EGFP/*ppk*-EGFP, *yw,* $rictor^{\Delta 2}$; *+/+; ppk*-EGFP/*ppk*-EGFP, and *yw; +/+;* $S6K^{l-1}$, *ppk*-EGFP/$S6K^{l-1}$, *ppk*-EGFP. For *pUAS-sin1-Flag* transgenic flies, the encoding region of Sin1 cDNA was amplified by PCR and subcloned into the pUAST vector using *Not*I and *Xba* sites. The trc^{Myr} constructs were prepared by fusing DNA encoding the first 90 amino acids of *Drosophila* Src1 to the first codon of *trc*. Trc mutants were generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and confirmed by sequencing. The PBc insertion lines that we screened in this study were obtained from the Bloomington, Exelixis, and Kyoto Stock Centers. For the genetic screening of mutants defective in dendrite development, we introduced all PBc mutant alleles into the *ppk*-EGFP reporter line and examined the class IV dendrite morphology at the third instar larval stage.

MARCM analysis

MARCM analyses were carried out as described previously, with some modifications (Emoto *et al*, 2004). Briefly, to generate mosaic clones, *yw*; $sin1^{PBac}$, *FRT42D/CyO, yw,* $rictor^{\Delta 2}$, *FRT19A/FM7, w*; $Tor^{\Delta P}$, *FRT40A/CyO, w*; $S6K^{l-1}$, *FRT82B/TM3* were mated with *w, elav-Gal4, UAS-mCD8GFP, hsFLP; FRT42D, tub-Gal4/CyO, w, FRT19A, tub-Gal80, hsFLP/FM7; FRT42D, Gal4[109(2)80], UAS-mCD8GFP/CyO,* or *w, elav-Gal4, UAS-mCD8GFP, hsFLP; FRT82B, tub-Gal80/TM6B* flies. Embryos were kept for 2 h and allowed to grow for 3–5 h at 25°C before being subjected to the following heat-shock regime: 38°C for 45 min, room temperature recovery for 30 min, and finally 38°C for 45 min. The eggs were kept at 25°C and larvae were examined for mutant clones and then dissected, fixed, and stained with anti-mCD8 antibody (Caltag). Dendritic length and branch numbers were quantified using ImageJ (NIH, Bethesda, MD) with a NeuronJ plug-in.

Immunoprecipitation

For immunoprecipitation, HeLa cells were cultured in a 100-mm-diameter dish and then lysed in 200 μl of CHAPS buffer (150 mM NaCl, 50 mM Tris–HCl at pH 7.4, 2 mM EDTA, 1 mM DTT, 20 mM β-glycerophosphate, 0.3% CHAPS, and Complete cocktail (Roche)) or Triton buffer (150 mM NaCl, 50 mM Tris–HCl at pH 7.4, 2 mM EDTA, 1 mM DTT, 20 mM β-glycerophosphate, 1% Triton X-100, and Complete cocktail (Roche)). Extracts were precleaned by Protein G beads (Roche) and then incubated with ~2 μg of primary antibodies for 2 h, followed by Protein G beads for 1 h. Beads were washed five times in lysis buffer for analyses of associated proteins by SDS–PAGE and western blotting. The following antibodies were obtained commercially: mTOR (Cell Signaling), NDR1 (Santa Cruz Biotechnology), Rictor (Cell Signaling, Abcam), and Raptor (Cell Signaling, Bethyl Laboratories). Anti-hSIn1 antibodies were kindly provided by Dr S Ishii (Makino *et al*, 2006).

Phospho-specific antibodies

Phospho-*Drosophila* Akt (Ser505), phospho-*Drosophila* S6K (Thr398), and phospho-MST1 (Thr195 in *Drosophila* Hpo) antibodies were purchased from Cell Signaling. The anti-Trc antibody has been reported previously (Emoto *et al*, 2004). Anti-phospho-Trc antibodies were raised against the synthetic peptides RALAY(pS)TVGT for the Ser292 phosphorylation site and FINY(pT)YKRFE for the Thr449 phosphorylation site. These antibodies were then purified using peptides coupled to Sepharose beads.

RNAi

The primers fused to generate dsRNAs were synthesized mainly as described in previous reports (Sarbassov *et al*, 2005a, b; Yang *et al*, 2008) and are listed in Supplementary Table 1. dsRNAs were produced by *in vitro* transcription using MEGAscript kits (Ambion) according to the manufacturer's instructions. *Drosophila* S2 cell RNAi experiments were performed as described previously (Rogers *et al*, 2003). In brief, cells were plated in 24-well plates, with a starting density of 1×10^5 cells per well. Cells were then treated with 15 μM dsRNA every 3 days for 6 days. At the end of the 7-day treatment, cells were recovered for biochemical analysis, or plated on concanavalin A-treated coverslips and allowed to spread for ~10 h for subsequent phalloidin staining.

Kinase assay

Trc kinase activity was measured as previously described (Tamaskovic *et al*, 2003). Briefly, S2 cells were lysed in CHAPS buffer (150 mM NaCl, 50 mM Tris–HCl at pH 7.4, 1 mM DTT, 20 mM β-glycerophosphate, 0.3% CHAPS, and Complete cocktail (Roche)) and Trc was immunoprecipitated. *In vitro* kinase assays were carried out in kinase buffer (20 mM Tris–HCl at pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 100 μM ATP, 1 μM cAMP-dependent protein kinase inhibitor peptide, 1 mM NDR1 substrate peptide (KKRNRRLSVA), and 20 μCi [γ-^{32}P]ATP) at 25°C for 30 min. The resulting solutions were then spotted onto P81 phosphocellulose paper (Whatman), and then washed five times for 10 min in 1% phosphoric acid and assayed in a liquid scintillation counter.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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