Rescue of tropomyosin deficiency in *Drosophila* and human cancer cells by synaptopodin reveals a role of tropomyosin α in RhoA stabilization

Jenny S Wong^{1,6}, Elizabeth Iorns^{2,6},
Michelle N Rheault^{3,6}, Toby M Ward²,
Priyanka Rashmi⁴, Ursula Weber⁵,
Marc E Lippman², Christian Faul²,
Marek Mlodzik⁵ and Peter Mundel^{4,*}

¹Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA, ²Department of Medicine, University of Miami Miller School of Medicine, Miami, FL, USA, ³Department of Pediatrics, University of Minnesota, Minneapolis, MN, USA, ⁴Harvard Medical School and Department of Medicine, Massachusetts General Hospital, Boston, MA, USA and ⁵Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY, USA

Tropomyosins are widespread actin-binding proteins that influence numerous cellular functions including actin dynamics, cell migration, tumour suppression, and *Drosophila* oocyte development. Synaptopodin is another actin-binding protein with a more restricted expression pattern in highly dynamic cell compartments such as kidney podocyte foot processes, where it promotes RhoA signalling by blocking the Smurf1-mediated ubiquitination of RhoA. Here, we show that synaptopodin has a shorter half-life but shares functional properties with the highly stable tropomyosin. Transgenic expression of synaptopodin restores *oskar* mRNA localization in *Drosophila* oocytes mutant for *TmII*, thereby rescuing germline differentiation and fertility. Synaptopodin restores stress fibres in tropomyosin-deficient human MDA-MB 231 breast cancer cells and TPM α -depleted fibroblasts. Gene silencing of TPM α but not TPM β causes loss of stress fibres by promoting Smurf1-mediated ubiquitination and proteasomal degradation of RhoA. Functionally, overexpression of synaptopodin or RhoA(K6,7R) significantly reduces MDA-MB 231 cell migration. Our findings elucidate RhoA stabilization by structurally unrelated actin-binding proteins as a conserved mechanism for regulation of stress fibre dynamics and cell motility in a cell type-specific fashion.

The EMBO Journal (2012) 31, 1028–1040. doi:10.1038/emboj.2011.464; Published online 13 December 2011

Subject Categories: cell & tissue architecture

Keywords: actin filaments; cell migration; *oskar* mRNA; RhoA; Smurf1

*Corresponding author. Division of Nephrology, Harvard Medical School, Massachusetts General Hospital, 149 13th Street, Room 8.203, Charlestown, MA 02129, USA. Tel.: +1 617 643 8710;

Fax: +1 617 726 5669; E-mail: mundel.peter@mgh.harvard.edu
⁶These authors contributed equally to this work

Received: 20 December 2010; accepted: 23 November 2011; published online: 13 December 2011

Introduction

Tropomyosins form a family of actin-binding proteins that influence numerous intracellular functions including actin cytoskeleton dynamics, cell migration, and tumour suppression (Cooper, 2002; Hitchcock-DeGregori *et al*, 2007; O'Neill *et al*, 2008).

Tropomyosin (Tm) has a well-defined function in *Drosophila*. During oogenesis, the assembly of the posterior pole plasm requires the polarized microtubule-mediated transport of *oskar* (*osk*) mRNA to the posterior pole (Clark *et al*, 1994; Shulman *et al*, 2000; Tomancak *et al*, 2000; Doerflinger *et al*, 2006; Zimyanin *et al*, 2008). *Osk* mediates the nucleation of the pole plasm, which is required for germ cell and abdomen development (Ephrussi *et al*, 1991; Kim-Ha *et al*, 1991). Failure to localize and enrich the pole plasm results in offspring lacking germ cells, referred to as grandchildlessness. Besides mutations in the factors that are required to assemble the pole plasm (e.g., *Osk*), *TmII* loss of function also causes grandchildlessness (Erdelyi *et al*, 1995). In the developing oocytes of *TmII* mutant females, the germline determinants do not get localized to the posterior pole plasm because *osk* mRNA is not properly transported to and localized to the posterior pole, subsequently giving rise to very few germ cells (Erdelyi *et al*, 1995). More recent data suggest that *TmII* is required for *osk* mRNA trafficking and affects the speed and reversal of the directional bias of particle movement (Singer, 2008; Zimyanin *et al*, 2008). Of note, similar to *TmII* deficiency, mutations in the *Drosophila moesin* gene that encodes for another actin-binding protein also result in loss of *osk* mRNA and protein from the posterior subcortical region and failure of oocyte development (Jankovics *et al*, 2002). The importance of the actin cytoskeleton for proper targeting and function of Oskar during oocyte development is underscored by the observation that long filamentous actin projections at the oocyte posterior pole are induced by and intermingled with Oskar protein. These data suggest that Oskar maintains its localization to the posterior pole through dual functions in regulating endocytosis and F-actin dynamics (Vanzo *et al*, 2007).

The downregulation of high molecular weight Tm isoforms is a common feature observed in many transformed cell lines (Hendricks and Weintraub, 1981; Cooper *et al*, 1985) and in human tumours (Bhattacharya *et al*, 1990; Wang *et al*, 1996; Hughes *et al*, 2003; Pawlak *et al*, 2004). The loss of Tm contributes to the neoplastic phenotype of altered cell shape, loss of stress fibres, and anchorage-independent growth (Boyd *et al*, 1995). The transformed phenotype in *v-ki-ras* transformed NIH3T3 cells and human MDA-MB 231 breast cancer cells can be rescued by reintroduction of Tm1, an isoform of TPM β (Prasad *et al*, 1993; Bharadwaj *et al*, 2005), implying that the loss of Tm is a crucial event in oncogenic transformation. Tm2, an isoform of TPM α , is necessary for

transforming growth factor- β -induced stress fibre formation in NMuMG mouse mammary epithelial cells and SiHa human cervical carcinoma cells (Bakin *et al*, 2004). Thus, in non-muscle cells Tm acts as a tumour suppressor and is necessary to maintain actin filament integrity, but the underlying molecular mechanisms remain elusive (Gunning *et al*, 2005; Hitchcock-DeGregori *et al*, 2007; O'Neill *et al*, 2008).

Rho GTPases can switch between an inactive GDP-bound and an active GTP-bound form (Jaffe and Hall, 2005). The E3 ubiquitin ligase Smurf1 can bind to nucleotide-free RhoA and promote RhoA ubiquitination, leading to its proteasomal degradation (Wang *et al*, 2003; Ozdamar *et al*, 2005). Synaptopodin is a proline-rich scaffolding protein that is strongly expressed in highly dynamic cell compartments such as dendritic spines in the brain and podocyte foot processes (FPs) in the kidney (Mundel *et al*, 1997a; Faul *et al*, 2007). Similarly to Tm, synaptopodin binds to actin and α -actinin and regulates the actin-bundling activity of α -actinin (Asanuma *et al*, 2005). The clinical importance of synaptopodin is underscored by the observation that it is a direct target of the anti-proteinuric effect of the calcineurin inhibitor cyclosporine A (Faul *et al*, 2008). Synaptopodin is not a target of proteasomal degradation but instead is degraded by cathepsin L-mediated cleavage (Faul *et al*, 2008). Synaptopodin induces stress fibres in podocytes by blocking the Smurf1-mediated ubiquitination of RhoA, thereby preventing the targeting of RhoA for proteasomal degradation (Asanuma *et al*, 2006; Faul *et al*, 2007). As it stands, the general relevance of this mechanism for the regulation of RhoA signalling beyond highly specialized podocytes is unclear.

Tm has long been known to regulate actin dynamics, but the precise mechanisms remained elusive (Gunning *et al*, 2005; Hitchcock-DeGregori *et al*, 2007; O'Neill *et al*, 2008). Here, we describe the important observation that synaptopodin is sufficient to rescue *TmII* deficiency in *Drosophila* by regulating *osk* mRNA localization and function, which suggests that these two structurally distinct proteins share functional properties. We confirmed this by showing that the mammalian orthologue *TPM α* regulates stress fibre formation through inhibition of Smurf1-mediated ubiquitination of RhoA in fibroblasts and human cancer cells. Our findings unveil the stabilization of RhoA protein abundance as a mechanism by which *TPM α* but not *TPM β* can modulate stress fibre dynamics and cell motility.

Results

Synaptopodin rescues *Drosophila* *TmII* loss of function

During *Drosophila* development, *TmII* is required for germ cell formation because mutations in *TmII* (*TmII^{eg9}*) virtually abolish *osk* mRNA localization to the posterior pole of the oocyte (Erdelyi *et al*, 1995). Both Tm and synaptopodin induce actin filaments. To test whether these two structurally distinct actin-binding proteins are interchangeable *in vivo*, we attempted to rescue the grandchildless phenotype of *Drosophila* *TmII* mutants (Erdelyi *et al*, 1995) by introducing synaptopodin via the Gal4/UAS system (Brand and Perrimon, 1993). Synaptopodin was expressed in the developing oocyte via the maternal *nanos*-Gal4 driver (*nosGal4*, *UAS-synpo*) and crossed into the *TmII^{eg9}* mutant background (Supplementary Figure S1). The *nosGal4*, *UAS-synpo*, *TmII^{eg9}* genotype was analysed for *osk* mRNA localization by *in-situ* hybridization

and quantification of F2 progeny (grandchildren; see Supplementary Figure S1 for details). In both assays, the expression of synaptopodin induced a rescue to wild-type conditions. *Osk* mRNA localized tightly to the posterior egg pole in rescue flies in a pattern indistinguishable from wild-type flies (Figure 1A). The rescue of *osk* mRNA targeting was functionally relevant, as it led to the production of normal numbers of germ cells deduced by the number of F2 progeny/grandchildren (Figure 1B). Of note, the eggs laid by *nos-synpo*, *TmII^{eg9}* developed and hatched normally. We thus conclude that vertebrate synaptopodin can fully substitute for the function of *TmII* in this *in-vivo* assay in *Drosophila*.

Overexpression of synaptopodin induces stress fibres and reduces motility in cancer cells

To test whether synaptopodin and Tm share overlapping functions at the cellular level, we analysed MDA-MB 231 cells that lack stress fibres (Bharadwaj *et al*, 2005) and express a Tm isoform not found in NIH3T3 fibroblasts (Supplementary Figure S2A). The transformed phenotype of MDA-MB 231 cells including the loss of stress fibres can be rescued by introduction of *TmI* (Bharadwaj *et al*, 2005). To determine whether the ectopic expression of synaptopodin would have a similar effect, we infected MDA-MB 231 cells, which do not express endogenous synaptopodin, with synaptopodin or empty vector (Figure 2A). We then visualized the actin cytoskeleton by phalloidin staining (Figure 2B). Similar to non-infected MDA-MB 231 cells, only a few cells infected with the empty control vector contained stress fibres. In contrast, synaptopodin overexpression induced stress fibres (Figure 2B). Quantitative analysis showed a significant increase in the percentage of stress fibre containing cells after infection with synaptopodin (Figure 2C). Functionally, the restoration of stress fibres was associated with the reduction of cell migration as determined by a wound-healing assay (Figure 2D and E).

Synaptopodin restores stress fibres in *TPM α* -depleted fibroblasts

Similar to synaptopodin in podocytes (Mundel *et al*, 1997a), Tm is found along actin filaments in fibroblasts (Gunning *et al*, 2005; Hitchcock-DeGregori *et al*, 2007; O'Neill *et al*, 2008; Figure 3A). Given that synaptopodin can functionally replace *TmII* deficiency in *Drosophila* and human cancer cells, we hypothesized that it may also rescue *TPM α* depletion in fibroblasts. To directly test this hypothesis, we used a gene silencing approach to reduce the expression of *TPM α* isoforms in NIH3T3 cells, which do not express endogenous synaptopodin (Figure 3B). Gene silencing with a Tm shRNA that recognizes a sequence in *TPM α* transcript variants 2, 3, 4, 6, and 7, but not with a control shRNA caused the loss of stress fibres (Figure 3A), reduction of focal contacts (Supplementary Figure S2B), and downregulation of Tm protein abundance (Figure 3B). Of note, the lentiviral overexpression of synaptopodin (Figure 3B) was sufficient to restore stress fibres in *TPM α* -depleted cells (Figure 3C, lower panels). Quantitative analysis revealed that synaptopodin significantly increased the percentage of stress fibre containing *TPM α* knockdown fibroblasts (Figure 3D). In order to determine if isoform specificity is important in the loss of stress fibres, we silenced *TPM β* expression using three independent *TPM β* -specific shRNAs (Supplementary Figure

Figure 1 Synaptopodin rescues the grandchildless phenotype of *Drosophila* *TmII* mutants. (A) *In-situ* localization of *osk* mRNA in stage 10 egg chambers of indicated genotypes. Transgenic expression of synaptopodin restores proper *osk* mRNA localization to the posterior pole in oocytes of *TmII^{eg9}* mutants. The average normal *osk* mRNA localization in wild type was $82 \pm 10\%$ ($n = 325$), *nos-synpo* $80 \pm 7\%$ ($n = 478$), *nos-synpo, TmII^{eg9}* 59 ± 14 ($n = 393$), and *TmII^{eg9}* $35 \pm 6\%$ ($n = 302$). Data were obtained from three independent experiments. (B) F2 egg count confirms the rescue of *TmII^{eg9}* grandchildless phenotype by synaptopodin. Two separate experiments were counted twice and averaged. *TmII^{eg9}* mutant, *nos-synpo* transgenic, and *nos-synpo/TmII^{eg9}* transgenic flies were analysed. The average egg count was $110 \pm 16\%$ ($n = 1253$) for wild-type (WT) flies and $90 \pm 16\%$ ($n = 1085$) for synaptopodin transgenic flies versus $10 \pm 6\%$ ($n = 146$) for the *TmII^{eg9}* genotype. The *nosGal4, UAS-synpo, TmII^{eg9}* flies displayed WT levels: $94 \pm 15\%$ ($n = 1008$). * $P < 0.0001$, Student's *t*-test.

S3). Because the Tm311 antibody detects many Tm isoforms (Tm6, 1, 2, 3, α , β , γ muscle Tm) (Nicholson-Flynn *et al*, 1996), we performed a semi-quantitative RT-PCR to monitor RNA knockdown. The TPM β shRNAs specifically depleted TPM β isoforms including Tm1 but not TPM α isoforms (Supplementary Figure S3A). By western blot analysis, we found a reduction of TPM β (upper band) but not TPM α (lower band) protein abundance (Supplementary Figure S3B). In contrast to the depletion of TPM α (Figure 3A), the depletion of TPM β did not cause the loss of stress fibres (Supplementary Figure S3C and D). Collectively, these results support the conclusion that TPM α and synaptopodin but not TPM β have overlapping cellular functions in spite of their structural differences.

Lactacystin restores stress fibres in TPM α -depleted fibroblasts

NIH3T3 cells, MDA-MB 231 cancer cells, and podocytes, serving as a positive control (Asanuma *et al*, 2006), express the E3 ubiquitin protein ligase Smurf1 (Supplementary Figure S2C), which can induce the proteasomal degradation of RhoA (Wang *et al*, 2003; Asanuma *et al*, 2006; Sahai *et al*, 2007). In fibroblasts, gene silencing of TPM α reduced RhoA steady-state protein levels (Figure 4A). This raised the possibility that TPM α depletion, by analogy to synaptopodin depletion (Asanuma *et al*, 2006), may result in Smurf1-induced degradation of RhoA. In keeping with this hypothesis, the protea-

some inhibitor lactacystin increased RhoA protein abundance (Figure 4A) and the percentage of stress fibre containing TPM α knockdown fibroblasts (Figure 4B and C). The upregulation of stress fibres by lactacystin was independent of Tm, because Tm protein abundance remained unchanged (Figure 4A). To confirm these results with an independent approach, we infected TPM α knockdown fibroblasts with wild-type RhoA or RhoA(K6,7R) that is resistant to Smurf1-mediated ubiquitination and proteasomal degradation (Wang *et al*, 2003; Ozdamar *et al*, 2005). Infection with FLAG-RhoA(K6,7R) but not with wild-type FLAG-RhoA or empty vector restored stress fibres in TPM α knockdown fibroblasts (Figure 4D). Overexpression of FLAG-RhoA was confirmed by western blot analysis (Figure 4E). Quantitative analysis showed a significant increase in the percentage of stress fibre containing Tm knockdown cells infected with Smurf1-resistant FLAG-RhoA(K6,7R) but not with wild-type FLAG-RhoA (Figure 4F). Collectively, these data establish a competitive relationship between Smurf1-mediated ubiquitination of RhoA and Tm activity.

Rho kinase inhibition abrogates synaptopodin- or lactacystin-induced stress fibres in TPM α -depleted fibroblasts

RhoA and its downstream effector Rho kinase are principal mediators of stress fibre formation through their effects on actin and myosin (Etienne-Manneville and Hall, 2002).

Figure 2 Synaptopodin rescues Tm deficiency in human cancer cells. (A) Western blot analysis for synaptopodin after infection of MDA-MB 231 cells with synaptopodin (synpo) or empty vector. Wild-type MDA-MB 231 cells do not express synaptopodin. Protein extracts from isolated mouse kidney glomeruli (Glom) serve as positive control. GAPDH serves as loading control. (B) Overexpression of synaptopodin, but not the empty vector control, induces stress fibres in MDA-MB 231 cells as shown by phalloidin labelling. Scale bar: 50 μ m. (C) Synaptopodin significantly increases the percentage of stress fibre containing MDA-MB 231 cells: wild-type cells: 24.36 $\pm$ 5.13% stress fibre containing cells; empty vector: 19.01 $\pm$ 3.08%; synaptopodin: 60.06 $\pm$ 4.32%. $P < 0.005$, Student's *t*-test. (D) Synaptopodin but not the empty vector control reduces migration of MDA-MB 231 cells. Scale bar: 125 μ m. (E) Quantitative analysis shows a significant delay of wound closure at 12 h for MDA-MB 231 cells infected with synaptopodin: wild type: 80.39 $\pm$ 8.07%; empty vector: 60.55 $\pm$ 3.95%; synaptopodin 35.39 $\pm$ 4.73%. $P < 0.005$, Student's *t*-test.

Synaptopodin induces stress fibres in podocytes by increasing RhoA abundance and activation (Asanuma *et al*, 2006). Now, we find that ectopic synaptopodin expression also induces stress fibres in TPM α -depleted fibroblasts (Figure 3). To demonstrate that this effect is dependent on an increase of RhoA, we used the Rho kinase inhibitor Y-27632 to block synaptopodin-induced stress fibres in TPM α -depleted fibroblasts (Figure 5A and B). Similarly, Y-27632 blocked lactacystin-induced stress fibres (Figure 5A and C). These results confirm that synaptopodin- or lactacystin-induced stress fibre formation depends on RhoA and Rho kinase signalling.

Tm regulates stress fibre formation and cell migration in MDA-MB 231 breast cancer cells through inhibition of RhoA ubiquitination

Given that lactacystin restores stress fibres in TPM α -depleted fibroblast, we asked if lactacystin also affected RhoA protein abundance and stress fibre formation in MDA-MB 231 cells. Similarly to podocytes (Asanuma *et al*, 2006) and TPM α knockdown fibroblasts (Figure 4A), lactacystin increased RhoA steady-state protein levels in MDA-MB 231 cells (Figure 6A). As in TPM α knockdown cells (Figure 4B and C), lactacystin induced stress fibres in MDA-MB 231 cancer cells (Figure 6B and C). The induction of stress fibres was

independent of Tm because it did not change Tm protein abundance (Figure 6A). To determine whether the ectopic expression of Smurf1-resistant RhoA(K6,7R) (Wang *et al*, 2003; Ozdamar *et al*, 2005) would have a similar effect as the overexpression of synaptopodin or lactacystin treatment, we infected MDA-MB 231 cells with empty vector, FLAG-RhoA or Smurf1-resistant FLAG-RhoA(K6,7R) (Figure 6D). Similarly to synaptopodin (Figure 2B) or lactacystin (Figure 6B), Smurf1-resistant RhoA(K6,7R), but not wild-type RhoA induced stress fibres (Figure 6E and F). As in fibroblasts (Figure 5), the Rho kinase inhibitor Y-27632 suppressed synaptopodin- or lactacystin-induced stress fibres (Supplementary Figure S4A–C), thereby confirming that RhoA is a downstream mediator of synaptopodin- or lactacystin-induced stress fibre formation in MDA-MB 231 cells. Functionally, RhoA(K6,7R) reduced the migration of MDA-MB 231 cells (Figure 6G and H). Thus, the lack of Tm in MDA-MB 231 cells leads to the loss of stress fibres and increased cell migration through proteasomal degradation of RhoA.

Tm2, an isoform encoded by the TPM α gene, blocks the Smurf1-mediated ubiquitination of RhoA

Synaptopodin can bind to RhoA, thereby blocking the binding of Smurf1 to RhoA (Asanuma *et al*, 2006). Therefore, we

Figure 3 Synaptopodin restores stress fibres in TPM α -depleted fibroblasts. (A) Tm is localized along stress fibres in NIH3T3 wild-type and control shRNA fibroblasts. Gene silencing of TPM α (TPM α shRNA) causes the loss of stress fibres. Scale bar: 25 μ m. (B) Tm protein levels are decreased in TPM α shRNA cells. Synaptopodin is detected in NIH3T3 cells after infection with synaptopodin (Synpo) but not in wild-type (wt) cells or in cells infected with the empty vector control. Con: non-silencing control shRNA. (C) Synaptopodin restores stress fibres in TPM α knockdown cells. Synaptopodin staining is found along stress fibres. The secondary antibody alone also stains non-specifically the nuclei of all cells. Scale bar: 25 μ m. (D) Quantitative analysis shows a significant decrease in the percentage of stress fibre containing TPM α knockdown cells, which can be rescued by ectopic expression of synaptopodin (Synpo) but not by the empty vector control: wild-type cells: 72.74 $\pm$ 1.11 % stress fibre containing cells; control shRNA + empty vector: 70.03 $\pm$ 6.31 %, TPM α shRNA + empty vector: 33.41 $\pm$ 5.61 %; TPM α shRNA + synaptopodin: 68.28 $\pm$ 4.78 %; TPM α shRNA + empty vector versus TPM α shRNA + synaptopodin; $P < 0.005$, Student's *t*-test. Wild type: non-infected wild-type cells; control: non-silencing control shRNA.

Figure 4 TPM α induces stress fibres by blocking the proteasomal degradation of RhoA. (A) Western blot analysis shows decreased baseline (0 h) RhoA steady-state protein levels in TPM α knockdown fibroblasts (TPM α) compared with non-infected wild-type cells or cells expressing a non-silencing control shRNA (control). Lactacystin (16 h) causes the upregulation of RhoA steady-state protein levels in TPM α knockdown cells but does not alter Tm protein abundance. GAPDH serves as loading control. (B) Phalloidin labelling shows that lactacystin restores stress fibres in TPM α knockdown cells. Scale bar: 25 μ m. (C) Lactacystin does not change the percentage of stress fibre containing wild-type and control shRNA infected fibroblasts but significantly increases the percentage of stress fibre containing TPM α knockdown cells: 0 h lactacystin: 30.96 $\pm$ 4.33 % stress fibre containing cells versus 16 h lactacystin: 63.86 $\pm$ 5.15 %; $P < 0.05$; Student's *t*-test. (D) Smurf1-resistant FLAG-RhoA(K6,7R), but not wild-type FLAG-RhoA (WT) or empty vector induces stress fibres in TPM α knockdown fibroblasts. Scale bar: 25 μ m. (E) Western blot analysis of FLAG-RhoA levels after infection with empty vector, wild-type FLAG-RhoA or FLAG-RhoA(K6,7R). The first lane shows co-infection with the non-silencing control shRNA (con) and the empty pLentix control vector. The second lane shows co-infection with the TPM α shRNA and the empty pLentix control vector. The third lane shows co-infection with the TPM α shRNA and FLAG-tagged wild-type RhoA. The last lane show co-infection with the Tm shRNA and FLAG-tagged Smurf1-resistant RhoA(K6,7R). GAPDH serves as loading control. Con: non-silencing shRNA. (F) Quantitative analysis shows a significant increase in the percentage of stress fibre containing TPM α knockdown cells infected with Smurf1-resistant RhoA(K6,7R): control shRNA + empty vector: 68.63 $\pm$ 8.18 % stress fibre containing cells; TPM α shRNA + empty vector: 35.73 $\pm$ 6.05 %; Tm shRNA + FLAG-RhoA(wt): 31.15 $\pm$ 5.93 %; TPM α shRNA + FLAG-RhoA(K6,7R): 70.30 $\pm$ 1.59 %; TPM α shRNA + empty vector versus TPM α shRNA + FLAG-RhoA(K6,7R): $P < 0.0005$, Student's *t*-test.

tested whether Tm can also bind to RhoA. We found that both GFP-tagged Tm1 (encoded by the *TPM α* gene) and GFP-tagged Tm2 (encoded by the *TPM β* gene) but not the GFP control (GFP-sui) could bind to dominant-active FLAG-

RhoA(G14V) and dominant-negative FLAG-RhoA(T19N) in co-transfected HEK293 cells (Figure 7A). GFP-Tm1 and GFP-Tm2 did not bind to the FLAG control (FLAG-Cdc42) (Figure 7A), confirming the specificity of the interaction.

Figure 5 Rho kinase inhibition suppresses synaptopodin- or lactacystin-induced stress fibres in TPM α knockdown fibroblasts. (A) Synaptopodin (synpo)-induced stress fibre formation in TPM α -depleted fibroblasts is blocked by the Rho kinase inhibitor Y-27632 but not the vehicle control. Similarly, Y-27632, but not the vehicle control, abrogates lactacystin-induced stress fibres. Scale bar: 25 μ m. (B) Quantitative analysis shows a significant loss of synaptopodin-induced stress fibre formation in TPM α -depleted fibroblasts after Y-27632 treatment: Vehicle control: $60.30 \pm 0.99\%$ stress fibre containing cells; Y-27632: $0.0 \pm 0.0\%$; $P < 0.0005$, Student's *t*-test. (C) Quantitative analysis shows a significant loss of lactacystin-induced stress fibre formation in TPM α -depleted fibroblasts after Y-27632 treatment. Vehicle control: $63.84 \pm 9.25\%$ stress fibre containing cells; Y-27632: $0.0 \pm 0.0\%$; $P < 0.0005$, Student's *t*-test.

Synaptopodin can displace Smurf1 from GDP-bound RhoA, thereby suppressing the Smurf1-mediated ubiquitination of GDP-RhoA (Asanuma *et al*, 2006). To test whether Tm2 can compete with Smurf1 for RhoA binding, we conducted *in vitro* reconstitution assays according to our published protocols (Faul *et al*, 2005; Asanuma *et al*, 2006; Yanagida-Asanuma *et al*, 2007). In the absence of Tm2, purified Smurf1 bound to GST-RhoA(T19N), but not to the GST control. However, with the addition of increasing amounts of purified FLAG-Tm2 protein, the binding of FLAG-Smurf1 to GST-RhoA(T19N) progressively decreased, with a concomitant increase in Tm2 binding to GST-RhoA (Figure 7B). To test whether the observed inhibition of Smurf1 binding to RhoA by Tm2 is functionally relevant, we performed co-transfection studies in HEK293 cells using FLAG-RhoA(T19N), Myc-Smurf1 (wild-type or catalytically inactive, CA) and HA-ubiquitin in the presence of GFP-Tm1, GFP-Tm2, or GFP alone (Figure 7C). In cells expressing the GFP control, RhoA was strongly ubiquitinated by Smurf1, as expected from previous studies (Wang *et al*, 2003; Asanuma *et al*, 2006). In keeping with previous studies (Asanuma *et al*, 2006), the ubiquitination of RhoA was markedly reduced in cells co-transfected with synaptopodin. Similarly, the ubiquitination of RhoA was markedly reduced in cells co-

pressing Tm2 but not in cells coexpressing Tm1 or α -actinin-4, another actin-binding protein serving as a negative control (Asanuma *et al*, 2006; Figure 7C). Reduced ubiquitination of RhoA was also found in cells expressing catalytically inactive Smurf1 (CA) (Figure 7C). Although we did not conduct a two step IP experiment to explore the effects of Tm on RhoA ubiquitination, collectively our data suggest that at least one isoform of TPM α , Tm2, but not the TPM β isoform Tm1, can block the Smurf1-mediated ubiquitination and proteasomal degradation of RhoA.

The half-life of synaptopodin is markedly shorter than that of Tm

In podocytes, Tm is localized to the cell body (Drenckhahn *et al*, 1990), whereas synaptopodin is found in FPs (Mundel *et al*, 1997a; Faul *et al*, 2007). FPs are highly dynamics structures, so rapid changes in FP morphology require dynamic control of their actin cytoskeleton, a function thought to be subserved by synaptopodin (Faul *et al*, 2007). Synaptopodin is readily degraded by TRPC5/Ca²⁺-induced (Tian *et al*, 2010) and calcineurin/cathepsin L-mediated proteolysis (Faul *et al*, 2008). On the other hand, Tm in cardiac myocytes is highly stable (Martin, 1981), and therefore an unlikely candidate for dynamic, rapid actin cytoskeleton remodelling. To compare the

Figure 6 Synaptopodin induces stress fibres and reduces cell migration in human MDA-MB 231 breast cancer cells. (A) Lactacystin causes an upregulation of RhoA steady-state protein levels in MDA-MB 231 breast cancer cells without affecting Tm protein abundance. GAPDH serves as loading control. (B) Lactacystin induces stress fibres in MDA-MB 231 breast cancer cells. Scale bar: 25 μ m. (C) Quantitative analysis shows a significant increase in the percentage of stress fibre containing MDA-MB 231 after lactacystin treatment: 0 h lactacystin: $16.09 \pm 5.00\%$ stress fibre containing cells versus 8 h lactacystin: $43 \pm 2.20\%$; $P < 0.05$, Student's *t*-test. (D) Western blot analysis shows FLAG-RhoA protein abundance after infection of MDA-MB 231 cells with empty vector, wild-type FLAG-RhoA or Smurf1-resistant FLAG-RhoA(K6,7R). GAPDH serves as loading control. Non-infected wild-type cells serve as baseline control. (E) Overexpression of FLAG-RhoA(K6,7R) but not wild-type FLAG-RhoA or the empty vector control induces stress fibres in MDA-MB 231 cells. Scale bar: 25 μ m. (F) FLAG-RhoA(K6,7R) significantly increases the percentage of stress fibre containing MDA-MB 231 cells: wild-type cells: $20.99 \pm 9.38\%$ stress fibre containing cells; empty vector: $10.29 \pm 2.94\%$; wild-type RhoA: $16.47 \pm 5.20\%$; RhoA(K6,7R): $45.87 \pm 7.21\%$; $P < 0.005$, Student's *t*-test. (G) FLAG-RhoA(K6,7R) but not wild-type FLAG-RhoA reduces MDA-MB 231 cell migration. Scale bar: 125 μ m. (H) Quantitative analysis shows a significant delay of wound closure at 12 h for MDA-MB231 cells infected with RhoA(K6,7R): wild type: $78.17 \pm 11.13\%$; empty vector: $76.37 \pm 2.63\%$; wild-type RhoA: $64.65 \pm 9.06\%$; RhoA(K6,7R): 36.91 ± 4.60 ; $P < 0.0005$, Student's *t*-test.

half-life of synaptopodin with that of Tm, we overexpressed FLAG-synaptopodin or FLAG-Tm2 in HEK293 cells. The cells were treated with cycloheximide to block *de-novo* protein synthesis and the levels of both proteins were followed over

time. Synaptopodin was rapidly degraded (Supplementary Figure S3D) with a calculated half-life of 14.6 ± 4.5 h. In contrast, Tm2 remained stable over the entire course of the experiment (24 h), in keeping with its previously reported

long half-life of about 5.5 days in myocytes (Martin, 1981). To validate the results in the context of endogenous proteins, we examined the turnover of synaptopodin and Tm in podocytes. The estimated half-life of endogenous synaptopodin in podocytes was 17.1 ± 2.4 h (Figure 7D), similarly to exogenous synaptopodin in HEK293 cells. As in HEK293 cells, endogenous Tm in podocytes remained stable over the course of the experiment (Figure 7D).

Discussion

The results of this study revealed that synaptopodin can rescue Tm deficiency by functional mimicry *in vitro* in cancer cells and fibroblasts and *in vivo* in *Drosophila*. The stabilization of RhoA abundance offers a molecular mechanism by which Tm can regulate stress fibre dynamics. Our results show further that Tm can induce stress fibres by blocking the Smurf1-mediated ubiquitination of RhoA, thus protecting RhoA against proteasomal degradation. Our findings support the notion that, in addition to the regulation by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide-dissociation inhibitors (GDIs) (Jaffe and Hall, 2005), the proteasomal degradation of RhoA is a conserved mechanism for the control of RhoA signalling, stress fibre dynamics, and cell motility (Figure 7E). The level of RhoA protein can be regulated by the ubiquitin-proteasome system through multiple mechanisms. Besides Smurf1, the Cullin3-BACURD complex can also regulate RhoA degradation (Chen *et al*, 2009). This study showed that Smurf1-resistant RhoA(K6,7R) is sufficient to rescue stress fibres in TPM α depleted fibroblast and MDA-MB 231 cancer cells. Thus, Tm probably does not affect Cullin3-BACURD-mediated degradation of RhoA. Future studies are needed to confirm or refute this idea.

A further interesting outcome of this study is the identification of RhoA as Tm-binding protein. In muscle cells, Tm interacts with troponin T, caldesmon, and tropomodulin as part of the contractile apparatus (Perry, 2001). In non-muscle cells, various Tm isoforms bind to calponin (Takahashi *et al*, 1986), pEL98 (Takenaga *et al*, 1994), and glutamic dehydrogenase (Akutsu and Miyazaki, 2002) although the physiological significance of these interactions is largely unknown. By linking Tm directly to RhoA signalling, we establish a new paradigm in which structurally unrelated actin-binding proteins such as Tm and synaptopodin not only serve as passive structural components of actin filaments, but also can actively regulate stress fibre dynamics (Figure 7E).

Our results demonstrate that Tm and synaptopodin employ similar molecular mechanisms by which they regulate actin dynamics, cell migration, and oocyte differentiation. The functional similarities between both proteins are underscored by the observation that synaptopodin is not only sufficient to rescue RhoA signalling in TPM α -depleted fibroblasts and human cancer cells *in vitro*, but also *osk* mRNA localization and fertility in *TmII^{es9}* mutant flies *in vivo*. Synaptopodin not only blocks the proteasomal degradation of RhoA (Asanuma *et al*, 2006), but also elongates α -actinin-induced actin filaments (Asanuma *et al*, 2005), another function that is shared by Tm (Hitchcock-DeGregori *et al*, 2007). Synaptopodin and Tm2 can prevent ubiquitination of RhoA and thereby cause its stabilization, thus allowing the formation of actin stress fibres necessary for proper regulation of cell migration and possibly *osk* mRNA localization. Mammalian Tm2 is not the same as TmII in *Drosophila*. Therefore, future studies will be necessary to establish more precisely the molecular link between TmII in *Drosophila* and Tm2 in mammals and to determine whether synaptopodin rescues TPM α deficiency by virtue of restoring RhoA signalling, through the restoration of Oskar-induced long action projections (Vanzo *et al*, 2007) or via regulation of *osk* mRNA trafficking or anchoring (Singer, 2008; Zimyanin *et al*, 2008).

The outcome of the half-life studies suggests that synaptopodin is better poised to mediate rapid temporal changes in actin dynamics, while Tm seems better suited for maintaining a more static actin cytoskeleton. This notion is further supported by the observation that synaptopodin is a target of rapid TRPC5/Ca²⁺ and calcineurin-dependent degradation (Faul *et al*, 2008; Tian *et al*, 2010). Therefore, our results suggest another mechanism for spatial and temporal regulation of the actin cytoskeleton in highly dynamics polarized cells such as podocytes or neurons.

To start exploring the question of isoform specificity, we established that in contrast to TPM α depletion, gene silencing of TPM β is not sufficient to cause a loss of stress fibres in fibroblasts. Together with our finding that Tm1, which is encoded by the *TPM β* gene, is not sufficient to block the ubiquitination of RhoA, these findings merit further investigation in future studies to determine the underlying molecular basis of isoform specificity. For example, Tm1 or a subset of other Tm isoforms may be proven to control stress fibre dynamics via other mechanisms such as regulation of RhoA GEFs or GAPs (Jaffe and Hall, 2005).

In summary, this work provides novel insights into Tm signalling. Our results suggest that the regulation of stress

Figure 7 Tm2 blocks the ubiquitination of RhoA by Smurf1. (A) GFP-Tm1 and GFP-Tm2 interact with dominant-active (G14V) and more strongly with dominant-negative (T19N) RhoA but not with the negative control GFP-sui (GFP control). GFP-Tm1 and GFP-Tm2 do not interact with FLAG-Cdc42 (FLAG control) also serving as negative control. (B) Immobilized GST-RhoA(T19N) but not GST alone directly binds to purified FLAG-Smurf1. In the presence of increasing amounts of FLAG-Tm2, the binding of RhoA to Smurf1 is gradually lost, and concomitantly increased binding of Tm2 to RhoA is detected. In the presence of 1 μ g of FLAG-Tm2, only Tm2 binds to RhoA. (C) FLAG-RhoA is ubiquitinated by wild-type (WT) but not by catalytically inactive (CA) Myc-Smurf1 in transfected HEK293 cells expressing GFP as revealed by anti-HA immunoblotting. GFP-Tm2 and GFP-Synaptopodin (Synpo) but not GFP-Tm1 or GFP- α -actinin-4 (Act-4) markedly reduces the ubiquitination of RhoA. No RhoA ubiquitination is detected in the absence of HA-ubiquitin. (D) Synaptopodin and Tm differ in their protein half-life. Treatment of podocytes with cycloheximide results in the rapid degradation of endogenous synaptopodin with a calculated half-life of 17.1 ± 2.4 h. In contrast, Tm protein abundance remains stable over the entire 24-h observation period. (E) An extended model for the regulation of RhoA signalling. In the canonical model of the Rho GTPase cycle, the activity of Rho GTPases is regulated by a switch between an inactive GDP-bound and an active GTP-bound form. The cycle is tightly regulated mainly by guanine exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine dissociation inhibitors (GDIs). Tm and synaptopodin promote stress fibre formation through the inhibition of Smurf1-mediated ubiquitination of RhoA. Thus, repression of Smurf1-induced proteasomal degradation of RhoA by structurally unrelated actin-binding proteins is a conserved mechanism for the regulation of RhoA signalling, in addition to the GDP/GTP switch.

fibre dynamics by Smurf1-mediated degradation of RhoA is not only essential in highly specialized cell such as podocytes (Asanuma *et al*, 2006), but also represents a conserved mechanism for the regulation of stress fibre dynamics and cell motility by the structurally distinct but functionally similar actin-binding proteins Tm2 and synaptopodin.

Materials and methods

Plasmid constructs

Mouse Tm2 cDNA was obtained from ATCC (GenBank ID BC026720). Rat Tm1 cDNA was provided by Peter W Gunning, GST-RhoA(T19N) by Tadaomi Takenawa, Smurf1-resistant RhoA(K6,7R) by Jeffrey L Wana, wild-type and CA Smurf1 by Takeshi Imamura. cDNAs were subcloned by PCR amplification into pEGFP-C1 (Clontech), pFLAG-6c or pFLAG-5a (Sigma). All constructs were verified by DNA sequencing. FLAG-synaptopodin long and FLAG- α -actinin-4 constructs have been described previously (Asanuma *et al*, 2005, 2006). Synaptopodin was amplified by PCR from GFP-synaptopodin long (Asanuma *et al*, 2005) and cloned into the LXSN vector (Clontech). Synaptopodin, wild-type FLAG-RhoA, and FLAG-RhoA(K6,7R) were also cloned into the pLentix (Clontech) vector. The GIPZ TPM α shRNA and GIPZ non-silencing control shRNAs were acquired from Open Biosystems. The GIPZ TPM α shRNA (Open Biosystems, Catalog Number RMM4431-98754654, Clone ID V2LMM_25293shRNA) is targeted to the following sequence: CACTACATATGTAATTGGT. When blasted, the shRNA matches TPM1 transcript variants 2, 3, 4, 6, and 7. The GIPZ TPM β shRNAs (Open Biosystems) target the following sequences: AAATACGAAGAAGAGATCA (#1), CGGACAAGTATTCACCAA (#2), and CCGAGCAGAGTTTGCTGAA (#3). The Open Biosystems TPM α shRNA targets a sequence in exon 9D of TPM α that is predicted to knockdown isoforms Tm6, Tm2, Tm3, Tm5a, and Tm5b. This is not predicted to knockdown any TPM β isoforms, including Tm1.

RNA isolation and RT-PCR

NIH3T3 cells were lysed from a 10-cm dish and homogenized by passing through a QIAshredder (Qiagen). RNA was isolated using the RNeasy Mini Kit according to the manufacturer's protocol. cDNA was synthesized from 200 to 350 ng total RNA using oligo(dT)₂₀ and SuperScript III RT (Invitrogen) according to the manufacturer's protocol. In all, 1 μ l cDNA was amplified by PCR (30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s) using Choice-Taq DNA Polymerase (Denville Scientific). The primer sets used were GAPDH (5'-CCAATGTGTCGCTCGTGATCT-3'; 5'-GTGAAGTCCGAGAGACACC-3'), TPM β (5'-TGGCTGAGAGTAAATGTGGGACC-3'; 5'-CAGGCTGCTGTGCAACGTG-3'), and TPM α (5'-AGGAGGCTGA AACTCGGGCTGA-3'; 5'-TGGCCGAGCTAAGGAGGGT-3'). TPM α amplifies TPM α transcript variants 2, 3, 4, 6, and 7.

Cell culture and transient transfection

NIH3T3 cell, which express Tm1, Tm2, Tm3, Tm6, Tm5a, Tm5b, Tm5NM1, and Tm5NM2 (Percival *et al*, 2000; Schvezov *et al*, 2005), was cultured in DMEM (Gibco) with 10% newborn calf serum (Invitrogen or Sigma) and 100 units/ml of penicillin-streptomycin (Invitrogen). Podocytes and HEK293 cells were cultured as described previously (Mundel *et al*, 1997a; Asanuma *et al*, 2005, 2006). MDA-MB 231 cells were obtained from ATCC and cultured as recommended. Transient transfection of HEK293 cells was performed as described before (Asanuma *et al*, 2005). For proteasome inhibition, NIH3T3 was treated for 16 h with 5–10 μ M lactacystin (Santa Cruz) and MDA-MB 231 for 8 h with 2.5 μ M lactacystin (Asanuma *et al*, 2006). For the Rho kinase inhibitor studies, MDA-MB 231 cells were treated with 10 μ M Rho kinase inhibitor (Y-27632, Sigma) or the vehicle control (DMSO) for 1 h prior to fixation and stress fibre quantification. In experiments where NIH3T3 cells were treated with both lactacystin and Y-27632, fibroblasts were first treated with 5 μ M lactacystin for 15 h before 5 μ M Y-27632 was added to the media for another hour. GFP fusion proteins were analysed by direct fluorescence microscopy in living cells or after fixation and double labelling confocal microscopy (Asanuma *et al*, 2005, 2006). Stress fibres were counted manually in each individual cell in independent images. A stress fibre was defined as a phalloidin-positive structure that was represented by a

line spanning across the length of the cell. In all, 200 TPM α -depleted NIH3T3 cells and 100 MDA-MB 231 cells from three independent experiments were analysed for the presence of stress fibres. In addition, for each TPM β shRNA 100 cells were counted. A *P*-value <0.05 with the Student's *t*-test was considered statistically significant.

Lentivirus production

The GIPZ plasmids (TPM α , TPM β shRNA, or control shRNA) and the pLentix plasmids (FLAG-RhoA, FLAG-RhoA(K6,7R), and synaptopodin), along with the helper plasmids psPAX2 and pMD2.G were transfected into HEK293T cells at 70% confluence using Lipofectamine 2000 (Invitrogen) or Fugene 6 (Roche). LXSN vector (LXSN vector only and LXSN-synaptopodin)-based retroviruses were prepared by transient transfection of the retroviral vectors in Phoenix cells, which stably express the packaging plasmids. Media was replaced 16–18 h after transfection. In all, 36 and 60 h after transfection, virus-containing supernatants were harvested and centrifuged at 3000 r.p.m. for 5 min. Viral particles were then passed through a 0.45- μ m filter (Corning) and supernatants were used for infection of target cells in the presence of 4 μ g/ml polybrene (Sigma). NIH3T3 cells were infected for 24 h and then selected. Non-infected cells were removed by selection in either 2 μ g/ml puromycin (Sigma) for GIPZ vectors or 0.7 μ g/ml G418 (Gibco) for LXSN and pLentix constructs, respectively. Non-infected MDA-MB 231 cells were selected with 3 mg/ml G418.

Drosophila histology and in-situ hybridization

Nos-Gal4 is Gal4::VP16-nos.UTR. Females of wild type, *TmII^{eg9}* mutant, nos-Gal4, UAS-synaptopodin/+ and nos-Gal4, UAS-synaptopodin/+; *TmII^{eg9}* genotypes were selected from the intercross of nos-Gal4/w FM6; *TmII^{eg9}*/TM6b and UAS-synaptopodin; *Tm2^{eg9}*/TM6 Ubx. Expression of synaptopodin in the germline during oogenesis was confirmed by immunofluorescence microscopy. *In-situ* hybridization was performed on all four genotypes according to Tautz and Pfeifle (1989) with modifications for RNA probes and ovaries. A digoxigenin-labelled antisense *osk* mRNA probe was generated with T7 RNA polymerase (Roche). Hybridization was performed at 55°C. Ovaries of 10–15 females per genotype were separated into ovarioles and fixed in 4% paraformaldehyde (PFA) in PBS containing 0.1% Tween-20/DMSO/heptane in a 20:2:60 mixture at room temperature for 20 min with agitation. The heptane phase was removed and replaced by 50 parts 4% PFA in PBS containing 0.1% Tween-20 followed by further incubation for 5 min with agitation. Ovaries were rehydrated in methanol/4% PFA in PBS containing 0.1% Tween-20 in a 1:1 mixture for 5 min and processed as described by others (Tautz and Pfeifle, 1989). Immunolabelling of *Drosophila* ovaries was done according to protocols described by the Cooley laboratory (<http://info.med.yale.edu/cooley/Protocol%20Ovaries.html>).

Immunocytochemistry

Cultured cells were plated on coverslips and fixed with 2% PFA and 4% sucrose in PBS for 10 min followed by permeabilization with 0.3% Triton X-100 in PBS for 5 min at room temperature. After three 5 min washes with PBS, non-specific binding sites were blocked with 2% FCS, 2% BSA, and 0.2% fish gelatin in PBS for at least 30 min. Primary antibodies (prediluted in blocking solution) were applied for 60 min at room temperature. Antigen-antibody complexes were visualized using Alexa Fluor 488 or 647 conjugated secondary antibodies at 1:500 to 1:1000 (Molecular Probes). Stress fibres were visualized by staining with rhodamine-labelled phalloidin (Molecular Probes) at 1:750 for 30 min along with the secondary antibodies. Cells were washed with PBS, rinsed with ddH₂O, and mounted in 15% Mowiol and 50% glycerol in PBS. Monoclonal anti-Tm (Tm311, Sigma) and anti-vinculin (Sigma) were used at 1:400. Confocal images were captured on a Leica TC5 SP5 or a Zeiss LSM 510 upright confocal microscope. ImageJ and Photoshop softwares were used for image analysis.

Western blotting and immunoprecipitation

SDS-PAGE, western blotting, and co-immunoprecipitation of FLAG- and GFP-tagged fusion proteins from transfected HEK293 cells were performed as described previously (Asanuma *et al*, 2005, 2006). It was previously found by others that an N-terminally tagged Tm1 is unable to induce stress fibres in transformed cells (Bharadwaj *et al*, 2004). Therefore, we cloned the tropomyosin constructs used in the

present study into pFLAG-CMV-5 vectors, thereby placing the tags on the C-terminus and avoiding interfere with the critical function of the N-terminal domain (Bharadwaj *et al*, 2004). This is in keeping with other studies that have also successfully used C-terminal FLAG-Tm constructs for localization and functional studies (Michele *et al*, 1999; Hillberg *et al*, 2006). Protein extraction from isolated mouse glomeruli has been described before (Mundel *et al*, 1997b; Asanuma *et al*, 2005). Antibodies were used as follow: Synaptopodin NT (Mundel *et al*, 1997a) at 1:1000, FLAG (Sigma) at 1:10 000, GFP (BD Transduction Laboratories) at 1:1000, GAPDH (Calbiochem) at 1:3000, RhoA (Cell Signaling) at 1:1000, and Tm (Tm311, Sigma) at 1:1000. The Tm311 antibody recognizes a common epitope in the long Tm isoforms (Nicholson-Flynn *et al*, 1996). HRP-conjugated secondary antibodies (Promega) were used at 1:10 000.

GST binding assays

To study the competitive binding of Tm2 and Smurf1 to RhoA, GST pull-down studies with purified recombinant proteins were carried out as described previously for synaptopodin (Asanuma *et al*, 2006). Recombinant proteins were bound to GST or GST-RhoA(T19N) in Triton buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100) and washed five times in Triton buffer. We immobilized 1 µg purified GST-RhoA(T19N) on GSH beads and incubated it with 0.5 µg of purified FLAG-tagged catalytically inactive Smurf1(CA) (Wang *et al*, 2003) and increasing amounts (0, 0.25, 0.5, or 1 µg) of FLAG-Tm2.

Wound-healing assays

Cell migration of wild-type MDA-MB 231 cells and MDA-MB 231 cells infected with empty pLentix vector, pLentix-FLAG-RhoA(wt), pLentix-FLAG-RhoA(K6,7R) or pLentix-synaptopodin was assessed using a wound-healing assay. The cells were plated in 24-well plates and allowed to proliferate to form a confluent monolayer. A 200-µl pipette tip was used to scratch a single wound through the middle of the cell monolayer. Cells were fixed with methanol and stained with crystal violet at 0 and 12 h post wounding. Stained cells were imaged by use of a light microscope equipped with an Olympus digital camera. The percent wound closure at the 12-h time point was compared with determine differences in the rate of cell migration. In each experiment, we analysed a total $n = 6$.

Protein half-life assays

To determine the turnover of exogenous synaptopodin and Tm2, we transfected HEK293 cells with FLAG-synaptopodin or FLAG-Tm2. Forty-eight hours later, the cells were treated with 10 µg/ml cycloheximide (Sigma-Aldrich) for indicated times (0, 1, 2, 4, 8,

12, and 24 h). Protein extracts were prepared in CHAPS buffer (20 mM Tris pH 7.5, 500 mM NaCl, 0.5% CHAPS) and synaptopodin and tropomyosin levels were quantified using ImageJ software. GAPDH levels were used as an internal loading control and the protein levels at the beginning of the experiment (0 h) as 100%. Using the best-fit curve, half-life of each protein was determined and reported as average $\pm$ standard deviation of four independent experiments. To analyse the half-life of endogenous synaptopodin and Tm in podocytes, we differentiated podocytes for 10 days as described before (Mundel *et al*, 1997b). The podocytes were then treated with 10 µg/ml cycloheximide for indicated time points (0, 4, 8, 12, 16, and 24 h). Protein extracts were prepared in CHAPS buffer and the half-life of synaptopodin and tropomyosin was determined from three independent experiments as described above. Tropomyosin was detected with Tm311 antibody as described above.

Supplementary data

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

Acknowledgements

We thank Miklos Erdelyi, Szeged, Hungary for providing *oskar* plasmid and *TmII* mutant flies; the Drosophila stock center, Bloomington, IN, USA for fly strains; and Sophy Okello, New York, NY, USA for transgenic injections. We thank Anna Greka, Boston, MA, USA for help with confocal microscopy. We also thank Peter Gunning Westmead, Australia for Tm1 cDNA; Tadaomi Takenawa, Tokyo, Japan for GST-RhoA(T19N) cDNA; Jeffrey Wrana, Toronto, Canada for RhoA(K6,7R) cDNA; Keiji Tanaka, Tokyo, Japan for HA-ubiquitin cDNA; and Takeshi Imamura (The JFCR Cancer Institute, Tokyo, Japan) for wild-type Smurf1 and catalytically inactive Smurf1(C710A) cDNAs. MNR was supported by a Child Health Research Center grant HD052890. This work was supported by NIH grants DK57683 and DK062472 to PM and EY14597 to MM. This manuscript has been used by JSW to fulfil the requirements for obtaining a PhD degree from the Graduate School at the Mount Sinai School of Medicine, New York, NY, USA.

Conflict of interest

The authors declare that they have no conflict of interest.

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