

The phosphoinositide-binding protein TRAF4 modulates tight junction stability and migration of cancer cells

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Abbreviations: TRAF4, tumor necrosis factor (TNF) receptor-associated factor 4; TJ, tight junction; PIP, phosphoinositide; MEC, mammary epithelial cell; TGF- β , transforming growth factor β ; EMT, epithelial-mesenchymal transition; ZF, zinc finger

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Tumor necrosis factor (TNF) receptor-associated factor 4 (TRAF4), a protein localized in TJs in normal epithelial cells, is frequently overexpressed in carcinomas. We recently found that TRAF4 impedes TJ formation/stability and favors cell migration, 2 hallmarks of cancer progression. In addition TRAF4 contributes to the TGF- β -induced epithelial-mesenchymal transition (EMT), metastasis, and p53 destabilization. TRAF4 recruitment to TJs is a prerequisite for its biological function on TJ formation/stability and on cell migration. Interestingly, TRAF4 is targeted to TJs through lipid-binding. The trimeric TRAF domain of TRAF4 binds 3 phosphoinositide (PIP) molecules. These findings shed new light on the role of TRAF4 in cancer progression; they provide a novel link between lipid metabolism and cancer progression and support the notion that TRAF4 could be a relevant target for cancer therapies. TRAF4 belongs to a family of 7 human proteins involved in different biological processes, such as inflammation, immunity and embryonic development. While the lipid-binding ability of the TRAF domain is conserved among the whole TRAF protein family, its functional role remains to be established for the remaining TRAF proteins.

Introduction

In multicellular organisms, epithelia shield the organism from the outside world; they consist of a sheet of cells tightly attached to each other and to an underlying

extracellular matrix, the basement membrane. Different types of intercellular junctions link neighboring cells: desmosomes; adherens; gap; and tight junctions (TJs). These molecular connections promote the mechanical robustness of the tissue as well as allowing intercellular communications. TJs, the uppermost elements of the junctional complex, are specifically involved in paracellular permeability, which is the regulation of diffusion across the intercellular space.^{1,2} Besides this gate function, TJs constitute another barrier within the cell as it separates the apical and baso-lateral membrane compartments. This fence function of TJs allows the maintenance of lipid and protein asymmetry between these 2 compartments.³ For instance, the phosphoinositide species PI(4,5)P2 and PI(3,4,5)P3 are enriched in apical and baso-lateral plasma membranes, respectively.^{4,5} The robustness of epithelia is not associated with stasis; indeed, within the epithelium sheet, epithelial cells divide, move or die, thus intercellular junctions are dynamically remodeled to accommodate these processes.^{6,7} This junctional remodeling is tightly regulated to allow for fine-tuning between the dynamics and the robustness of the epithelium. Remarkably, this balance is altered in cancer; indeed, intercellular junctions are disrupted during the course of tumor progression, indicating that the function of the proteins involved in the regulation of junction dynamics is modified in this pathological process.

Tumor necrosis factor (TNF) receptor-associated factor 4 (TRAF4), a gene originally identified in breast cancer, is

overexpressed in a variety of carcinomas and is associated with poor prognosis.^{8-11,12} TRAF4 belongs to the TRAF family which is composed of 7 members in human.^{13,14} Unlike the other TRAF proteins, which function predominantly in inflammation and immunity, TRAF4 is mainly involved in developmental and morphogenetic processes.¹⁵ Of interest, to date, TRAF4 is the sole TRAF localized to tight junctions in epithelia.¹⁶ While the recruitment of TRAF4 to tight-junction is a dynamic and complex process, we have recently demonstrated that it requires the targeting of the protein at the plasma membrane which is controlled by the binding of the TRAF domain with PIPs. Moreover, TRAF4 is involved in the dynamics of TJs: its expression delays TJ assembly and favors cell migration in mammary epithelial cells (MECs) documenting the important function for this protein during cancer progression.¹⁷

The Cancer Protein TRAF4, a Dynamic Protein from TJ

TRAF4 has an unusual cellular localization: it is present in many distinct cellular compartments including the cytoplasm, the nucleus and the plasma membrane.^{16,18,19} We have previously documented that in normal human mammary tissues, TRAF4 is mainly localized in epithelial cell TJs and TRAF4 labeling on sections typically appears as a honeycomb pattern. Moreover, fluorescence recovery after photobleaching (FRAP) experiments on MECs showed that TRAF4 association with TJs is highly dynamic; TRAF4 shuttles rapidly between TJs and the cytoplasm. These data support the notion that TRAF4 relays signals between TJs and the cytoplasm.¹⁶ Interestingly, in mammary cancer cells, TRAF4 localization is altered: while it is still present in TJs in well differentiated tumors, the protein is relocalized in the cytoplasm and the nucleus in poorly differentiated tumors, indicating that TRAF4 functions differently in cancer cells.^{8,9,16,20}

TRAF4 is a typical TRAF protein composed of 3 distinct modular domains, an N-terminal RING domain, a succession

of 7 zinc fingers (ZF), and a C-terminal TRAF domain.¹⁵ The RING domain of TRAF4 was shown to confer an E3-ligase activity to the protein, the 7 central ZF have an unknown function but, by analogy with other TRAF proteins, are most probably involved in protein-protein interactions, and the TRAF domain governs the trimerization of the protein.^{12,17,21,22} Deletion mutants of the protein showed that the TRAF domain is necessary and sufficient for TRAF4 targeting to the plasma membrane.^{16,18} The N-terminal part of TRAF4 composed of the RING and ZF domains is cytoplasmic and nuclear and often generates cytoplasmic foci of protein accumulation.^{16,18}

TRAF4 is Targeted to Tight Junctions Through its TRAF Domain in a PIP-Binding-Dependent Manner

How TRAF4 is localized in TJs in normal MEC and lost from TJs in cancer MECs remained unclear for many years. We failed to identify TJ resident proteins interacting with TRAF4, which could explain the recruitment of the protein to this specific compartment of the plasma membrane (our unpublished data). The finding that an interaction with membrane lipids, namely phosphoinositides (PIPs), was crucial for the positioning of TJ proteins, such as the Partitioning Defective 3 protein (PAR3),²³ led us to investigate a similar addressing mechanism for TRAF4. Using lipid overlay assays we showed that recombinant TRAF4 binds all PIPs and phosphatidic acid (PA) through its TRAF domain.¹⁷ Liposome flotation assays, mass spectrometry and isothermal titration calorimetry analyses confirmed this binding and showed that, in solution, the TRAF domain of TRAF4 forms a homotrimer which binds up to 3 PIP molecules. The affinity between one lipid-binding site of the TRAF trimer and one PIP molecule was around 5 μ M, which is consistent with the lipid-binding affinity of other TJ-related proteins such as PAR3, Zonula Occludens-1 (ZO-1) and ZO-2.^{23,24} Molecular and structural analyses provided mechanistic insights about the

interaction of the TRAF domain with PIPs. The crystal structure of the TRAF domain of human TRAF4 was solved to 1.85 Å (PDB 3ZJB) and showed the details of the constitutive trimeric structure of the protein (Fig. 1). Generally, the interaction between PIPs and protein domains, such as PH (pleckstrin homology) or FYVE (Fab1, YotB, Vac1p, and EEA1) domains, require the presence of several basic residues. Consistently, basic residues exposed at the surface of the TRAF domain (lysine 313 and lysine 345) were functionally involved in the TRAF-PIP interaction. A 3D model of this interaction shows that 3 PIP molecules can simultaneously bind a trimer of TRAF4 protein. Of interest, each PIP molecule binds at the interface between 2 neighboring protomers making contacts with 3 basic (R297, K313 and K345) and one aromatic (Y338) residues (Fig. 2).

The binding of PIPs to the TRAF domain of TRAF4 supports the idea that this lipid-protein interaction could be the mechanism of membrane recruitment for TRAF4. Accordingly, functional assays showed that TRAF4 is recruited to both synthetic and cellular membranes thanks to its interaction with PIPs. In addition, in living cells, while the wild-type TRAF domain of TRAF4 in isolation was localized all along the plasma membrane, PIP-binding deficient TRAF domains were cytoplasmic. Altogether, these data showed that the TRAF domain of TRAF4 is a bona fide PIP-binding module which addresses the protein to the plasma membrane.

While in isolation the TRAF domain localizes all along the plasma membrane, the full-length protein is predominantly targeted to TJs. In addition, a mutant of TRAF4 unable to bind to PIPs is no longer addressed to the plasma membrane. Therefore, the TRAF domain allows the recruitment of the protein to the membrane in a PIP-dependent manner, which is a prerequisite step in the fine-tuning of the localization of the protein in TJs mediated by the other domains of the protein, the RING and/or the 7 zinc fingers. Therefore, the peculiar localization of TRAF4 in TJs results from the combined action of its different structural domains.

TRAF4 Weakens Tight Junctions and Favors Cell Migration, 2 Crucial Events for Tumor Progression

As TRAF4 is a TJ-resident protein frequently overexpressed in carcinomas, we hypothesized that TRAF4 might contribute to cancer progression by weakening TJs. Cellular studies showed that TRAF4 negatively regulates TJ assembly/stability in MECs. Of interest, the PIP-binding-deficient TRAF4-K345E mutant was not able to impair TJs, highlighting that TRAF4 has to be recruited to TJs to act as a negative regulator. Loss of intercellular junctions is a crucial step in cancer progression; indeed, loss of cell-cell contacts favors cell migration. Cell migration assays revealed that TRAF4 promotes MEC migration. These results support the notion that TRAF4 might participate to cancer progression by destabilizing TJs and favoring cell migration. However, the molecular mechanism leading to TJ destabilization after TRAF4 recruitment remains to be established. Since TRAF4 is an E3-ligase, its action might involve the ubiquitination of yet to be identified target proteins, which could either be activated or sent to the degradation pathway following ubiquitination (Fig. 3).

One candidate signaling pathway on which TRAF4 might act in TJs is the Transforming growth factor- β (TGF- β) pathway. Indeed, TRAF4 was shown to potentiate TGF- β -induced signal transduction in MECs.¹² Notably, TRAF4 promoted TGF- β -induced migration and epithelial-to-mesenchymal transition. Upon TGF- β -mediated activation of the TGF- β receptor complex (TGF- β type I –TGFBR1- and type II –TGFBR2-

receptors), TRAF4 recruitment allows the protein to inhibit the E3 ligase SMURF2 (SMAD specific E3 ubiquitin protein ligase) on one hand, and to recruit the deubiquitinase USP15 (Ubiquitin carboxyl-terminal hydrolase 15) on the other hand. Since SMURF2 and USP15 are involved in the ubiquitination and deubiquitination of TGFBR1, respectively, TRAF4 recruitment globally decreases

ubiquitination and therefore stabilizes the TGFBR1 receptor at the membrane and favors its signaling. Interestingly, TGFBR2 activation was directly linked to TJ disruption; TGFBR2 was shown to phosphorylate the PAR6 protein which allows the recruitment of SMURF1; SMURF1 then targets RhoA to degradation.²⁵ This mechanism is believed to be responsible for TJ destabilization upon

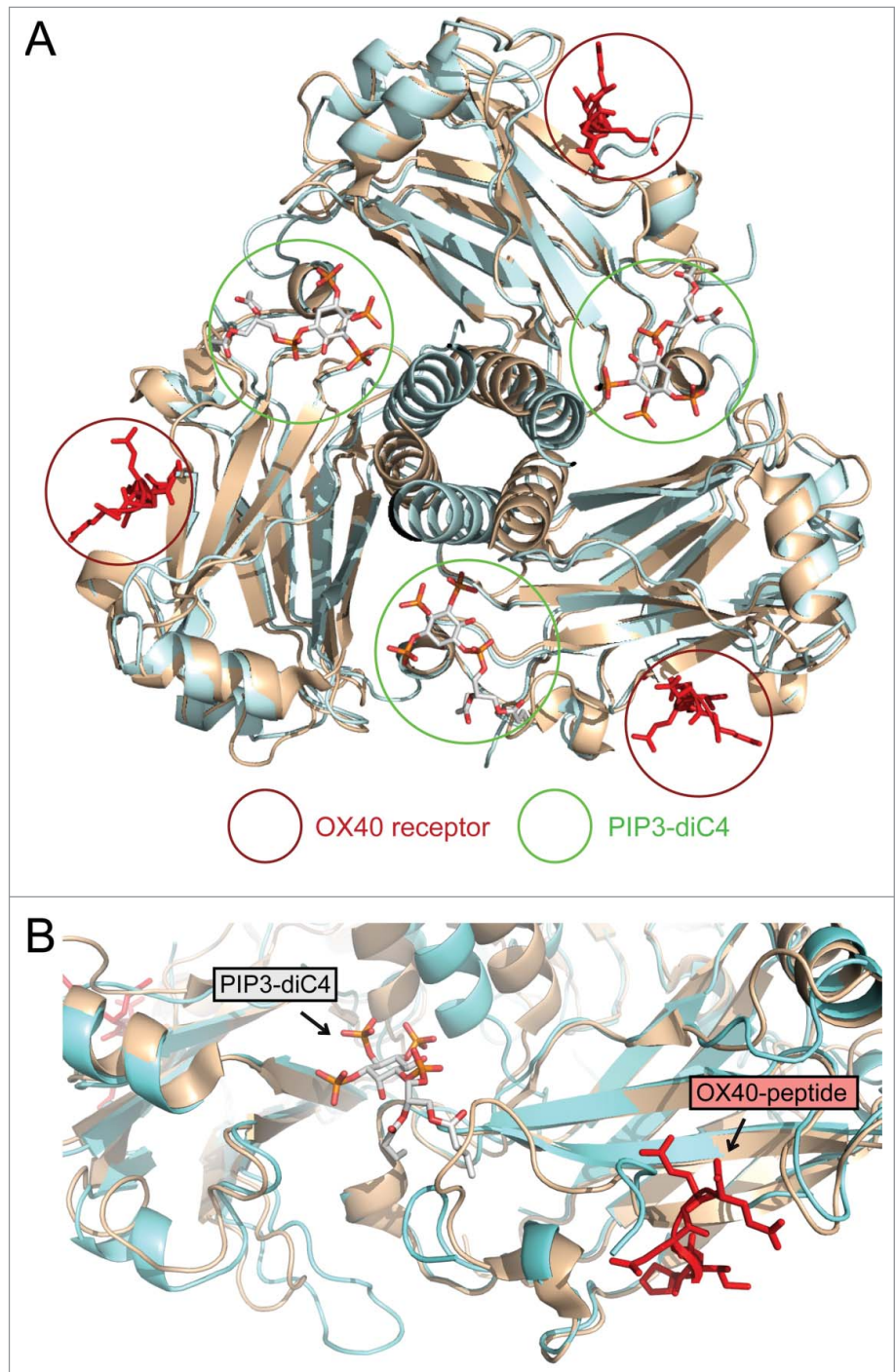


Figure 1. PIP- and receptor-binding sites are not overlapping on the TRAF domain. Structural superposition of PIP3-diC4-bound TRAF of TRAF4¹⁷ and OX40-bound TRAF of TRAF2³⁴ (PDB 1D0A) complexes. Into the page and 3-fold axis vertical are shown in (A) and (B), respectively. The TRAF domain of TRAF4 and TRAF2 are colored in blue and brown, respectively. PIP3-diC4 and the OX40-peptide are depicted in white and red, respectively. The PIP-binding site and the receptor-binding site are not overlapping suggesting that they are not mutually exclusive.

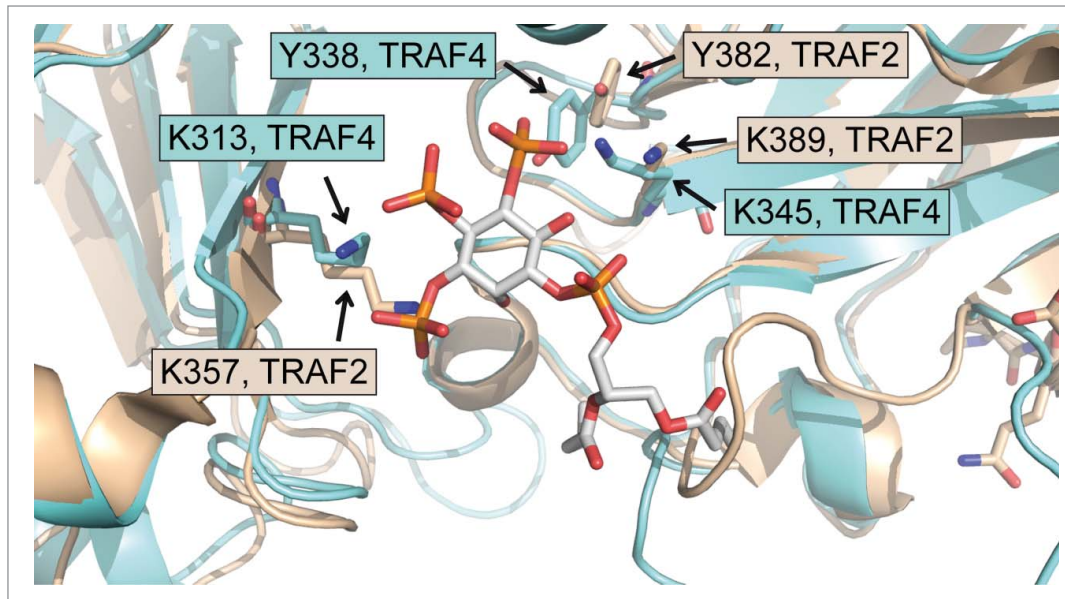


Figure 2. Position and orientation of PIP-interacting residues of TRAF4 are conserved in TRAF2. Superposition of the model of PIP3-dic4-bound TRAF domain of TRAF4¹⁷ and the TRAF domain of TRAF2²¹ (PDB 1CA4) crystal structure. TRAF4, TRAF2 and PIP3-dic4 are depicted in light blue, brown and white, respectively. Phosphorus, nitrogen and oxygen atoms are colored in orange, blue and red, respectively. The relative position and orientation of lysine 313 and 345 of TRAF4, as well as tyrosine 338, are conserved in TRAF2 3D structure. The figure was generated using PyMOL.³⁵

TGF- β pathway activation. Interestingly, TRAF4 is itself the target of ubiquitination by the 2 human SMURF proteins, SMURF1 and SMURF2, which may provide a mechanistic link between TJ destabilization and TRAF4 function.^{26,27} Moreover, the association of TRAF4 to the TGF- β receptor was shown to activate the TGF- β -activated kinase (TAK) 1 through a poly-ubiquitination mechanism. Interestingly, TGF- β receptors are localized in TJs in polarized epithelial cells.²⁵ It is tempting to speculate that TRAF4 association with PIPs directs TRAF4 to the vicinity of TGF- β receptors to regulate the pathway (Fig. 3).

All these data link TRAF4 overexpression in tumors with TJ disruption. Once TJ are destroyed, TRAF4 is more abundant in the cytoplasm and/or in the nucleus^{8,9,16,20}; its role in these compartments remained unclear. A recent study addressed the role of TRAF4 in signaling events occurring in the cytoplasm and in the nucleus. Indeed, TRAF4 was shown to bind and inactivate the deubiquitinase HAUSP (herpesvirus-associated ubiquitin-specific protease).²⁰ HAUSP antagonizes the action of MDM2 on P53. While the E3-ligase MDM2 ubiquitinates p53 to induce its proteasomal degradation,

HAUSP deubiquitinates P53 and stabilizes the protein. Therefore, TRAF4, by inactivating HAUSP favors P53 degradation. These data converge with previous studies linking TRAF4 with P53 as they clearly involve TRAF4 in the regulation of the tumor suppressor gene P53 which plays a major role in most human cancers;^{28,29} moreover, they suggest that TRAF4 is involved in the resistance of tumor cells to cytotoxic agents, by way of a P53-dependent pathway (Fig. 3).

Altogether, these studies show that TRAF4 favors tumor progression by operating on different signaling pathways. The distinct cellular localizations of the protein, from the plasma membrane to the nucleus, have different functional consequences which all promote tumor progression.

Therefore, TRAF4 should be a valuable prognostic marker and a potential therapeutic target in human carcinomas.

PIPs as Novel Modulators of TRAF Proteins

TRAF proteins are widely found in metazoans, from cnidaria to human.³⁰ Interestingly, the TRAF domain is the

most conserved domain within the 7 human TRAF proteins, with an average amino acid identity around 45%. Three-dimensional structure comparison of the TRAF domain from different TRAF proteins also revealed a strong conservation of their tertiary and quaternary structures. These data reinforce the notion that all TRAF domains are highly related in terms of function. Thus, we reasoned that the ability to interact with PIPs might be a common ability of TRAF proteins. Lipid overlay and flotation assays showed that, like TRAF4, the other TRAF paralogs (TRAF1 to TRAF6) also interact with PIPs, thus demonstrating that the TRAF domain is a bona fide PIP-binding domain. Moreover, these results suggested that PIP-interacting residues, identified by our molecular docking model and mutagenesis experiments, might be conserved among TRAFs: except for arginine 297, all PIP-interacting residues (K313, Y338 and K345 in TRAF4) are fully conserved in the other TRAF proteins. Furthermore, the 3D structure superimposition of the PIP3-dic4-bound TRAF of TRAF4 and TRAF of TRAF2 showed that the relative position and orientation of these 3 lipid-interacting residues are comparable (Fig. 2). Similar observations were made

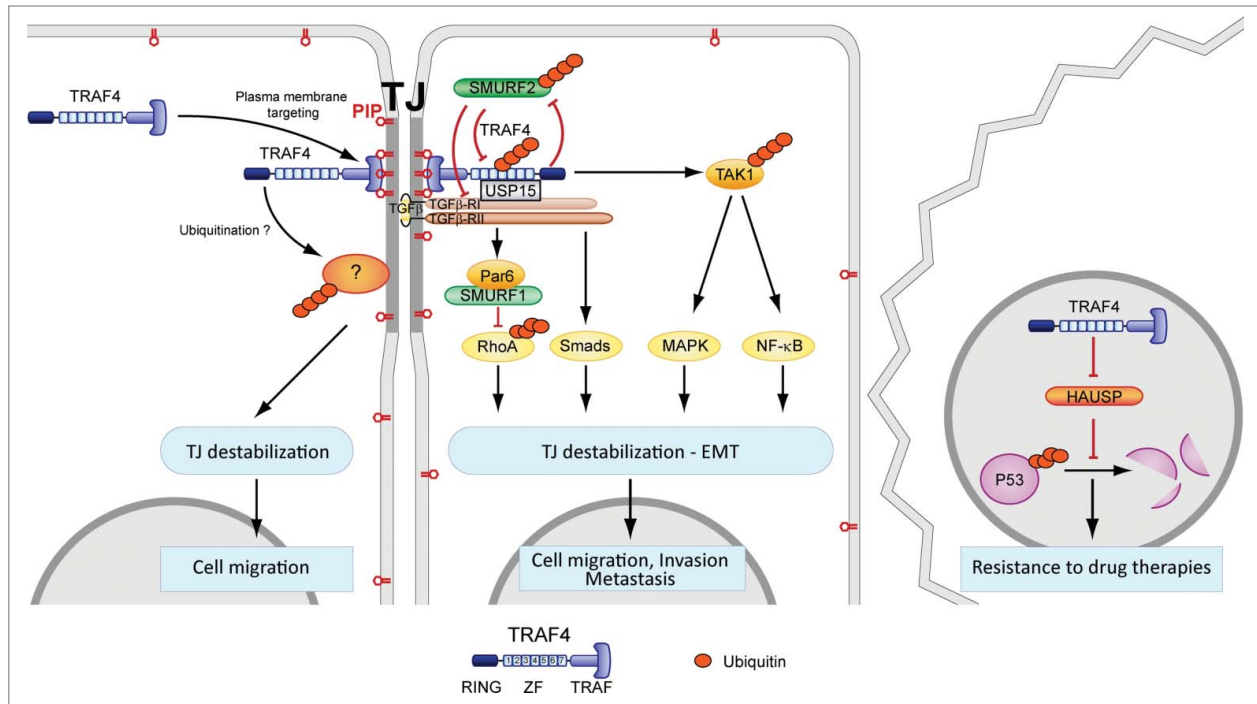


Figure 3. Model of TRAF4 function on TJs. Left: the interaction between PIPs and the TRAF domain of TRAF4 allows the recruitment of the protein onto the plasma membrane in TJs. Through a mechanism possibly involving the E3-ligase activity of the protein, TRAF4 destabilizes TJs and promotes migration. Middle: TRAF4 an adaptor from the TGF- β pathway. Upon TGF- β binding on its receptor, the activation of the pathway leads to the recruitment of TRAF4 on the receptor complex. TRAF4 inhibits SMURF2 and activates USP15, which leads to the stabilization of the receptor complex on the membrane and potentiates its signaling. TRAF4 also allows the activation of TAK1 through a K63-linked ubiquitination. Right: in cells devoid of tight junctions, TRAF4 is localized both in the cytoplasm and in the nucleus. TRAF4 interacts with the deubiquitinase HAUSP, presumably in the nucleus, and inactivates its action on P53. This favors the ubiquitination of P53 and its subsequent degradation, thereby limiting the ability of the cell to respond to a genotoxic stress.

with the 3D structures of TRAF3 and TRAF6 (data not shown). This structural conservation of the PIP-binding site supports the notion that PIP-binding by the TRAF domain is a common ability of TRAF proteins. Consistently, this PIP-binding ability is also conserved through evolution since dTRAF1, the TRAF protein of the fly which shares the highest homology with human TRAF4, also binds PIPs and PA.¹⁷ This suggests that the ability to bind lipids is an ancestral property of the TRAF domain. Interestingly, dTRAF1 was shown to be apically localized in cells of the early drosophila embryo.³¹ This localization of TRAF4 is required for the proper apical positioning of the adherens junctions protein Armadillo (drosophila β -catenin ortholog) in constricting cells during gastrulation.

The seventh member of the TRAF family lacks the C-terminal TRAF domain but possesses a C-terminal domain composed of 7 WD40 repeats.³² Similarly to a TRAF

domain, the WD40 repeats of TRAF7 are involved in protein-protein interactions.¹⁴ The functional similarity between these 2 domains suggested that TRAF7 might bind to PIPs through its WD40 repeats. Lipid-binding analyses showed that, similarly to the TRAF domain, the WD40-repeats of TRAF7 interact with PIPs (our unpublished data). Consistently, it has already been described that the WD40-repeats of WIPI-1 (WD-repeat protein Interacting with Phosphoinositides-1) also bind to PIPs. These results indicate that the PIP-binding ability is common to all members of the TRAF family and is achieved either by the TRAF domain (TRAF1–6) or WD40-repeats (TRAF7).

TRAF domains assemble as a constitutive mushroom-shaped trimer which binds to the cytoplasmic tail of activated TNF and interleukin-1/Toll-like receptors to mediate a wide range of biological processes.³³ Structural determinations of the receptor-bound TRAF domains of

TRAF2 and TRAF6 revealed that the interaction interface corresponds to a shallow surface depression on the side of one protomer without contact to the adjacent protomer (Fig. 1). Interestingly, in our model the PIP is bound at the interface between 2 adjacent protomers. The superposition of PIP3-dic4-bound TRAF of TRAF4 and OX40-bound TRAF of TRAF2 complexes showed that the lipid binding site and the receptor binding site do not overlap suggesting that they are not mutually exclusive (Fig. 1). This is of great interest as the association of TRAF proteins with their cognate receptors is of low affinity (40–60 μ M range) which is unusual for specific receptor recruitment. Since upon ligand stimulation a trimeric receptor-TRAF complex is formed, it has been suggested that both affinity and specificity are amplified through an avidity effect.²¹ Our work sheds new light on the molecular mechanism of TRAF proteins membrane recruitment and suggests that

PIPs might participate to receptor recruitment by stabilizing TRAF proteins at the plasma membrane. Thus, PIPs would constitute a lipid platform allowing spatial containment around the receptor of the TRAF signaling molecules. This suggests that lipid signaling, by modulating the interaction of TRAF proteins with their receptor at the membrane, might be an additional level of regulation of the different TRAF-mediated signaling pathways.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

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