Tight junction protein ZO-2 modulates the nuclear accumulation of transcription factor TEAD

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ABSTRACT The presence of tight junction protein zonula occludens 2 (ZO-2) at the nucleus inhibits the transcription of genes regulated by TEAD transcription factor. Here, we analyzed whether the movement of ZO-2 into the nucleus modulates the nuclear concentration of TEAD. In sparse cultures of ZO-2 knockdown Madin–Darby canine kidney cells, nuclear TEAD was diminished, as in parental cells transfected with a ZO-2 construct without nuclear localization signals, indicating that ZO-2 facilitates the entry of TEAD into the nucleus. Inhibition of nPKCδ in parental cells triggers the interaction between ZO-2 and TEAD at the cytoplasm and facilitates TEAD/ZO-2 complex nuclear importation. Using proximity ligation, immunoprecipitation, and pull-down assays, TEAD/ZO-2 interaction was confirmed. Nuclear TEAD is phosphorylated, and its exit in parental cells is enhanced by activation of a ZO-2 nuclear export signal by nPKCε, while the nuclear accumulation of ZO-2 triggered by the mutation of ZO-2 nuclear export signals induces no change in TEAD nuclear concentration. In summary, our results indicate that the movements of ZO-2 in and out of the nucleus modulate the intracellular traffic of TEAD through a process regulated by nPKCδ and ε and provide a novel role of ZO-2 as a nuclear translocator of TEAD.

INTRODUCTION

Zonula occludens 2 (ZO-2) is a tight junction (TJ) protein member of the MAGUK (membrane-associated guanylate kinase homologue) protein family that translocates to the nucleus in response to low cell density (Islas et al., 2002) and stress induced by chemicals (CdCl2) and heat shock (Traweger et al., 2003). In sparse cultures, newly synthesized ZO-2 temporarily localizes to the nucleus before reaching the TJs at the cell borders (Chamorro et al., 2009). In contrast, in confluent monolayers, ZO-2 goes directly to the cell borders (Quirós et al., 2013). Movement of ZO-2 from the cytoplasm to the plasma membrane involves activation of the calcium-sensing receptor and the novel protein kinase Cε (nPKCε) with no lysine-ε-4 (WNK4) downstream signaling cascade, which leads to ZO-2 serine phosphorylation, its release from 14-3-3 protein in the cytoplasm, and incorporation to TJs (Amaya et al., 2019). ZO-2 moves in and out of the nucleus with nuclear localization and exportation signals (NLS and NES) (Lopez-Bayghen et al., 2006). In canine ZO-2 (cZO-2), one monopartite and two bipartite (bp) NLS are present at the U2 segment located at the amino portion of the molecule between PDZ1 and PDZ2 domains (Quirós et al., 2013). In addition, 16 serine–arginine (SR) repeats are found at this segment, whose phosphorylation...
by the kinase SRPK induces protein entrance to the nucleus and its localization at nuclear speckles (Quiros et al., 2013). cZO-2 has four NES, two located at the PDZ2 domain and the other two at the guanylate kinase (Guk) module (Gonzalez-Mariscal et al., 2006).

ZO-2 has multiple protein-binding domains and motifs that allow it to function as a scaffold that concentrates a wide variety of proteins at both the TJ and the nucleus (for reviews, see Gonzalez-Mariscal et al., 2017, 2019). At the plasma membrane, ZO-2 and ZO-1 act as a platform for the polymerization of claudins into TJ strands (Umeda et al., 2006). In contrast, at the nucleus, even though ZO-2 has no DNA-binding sites, it inhibits the transcription of several genes regulated by AP-1 (activator protein 1) (Betanzos et al., 2004), TEAD (transcriptional enhancer-associated domain) (Domínguez-Calderon et al., 2016), and TCF (T cell factor)/LEF (lymphoid enhancer family) (Tapia et al., 2009; Wetzel et al., 2017) transcription factors. Here we have studied if ZO-2 plays a role as a nuclear translocator of TEAD transcription factor.

Mammals express four TEAD genes, and the particular function of each one remains controversial. Thus, Tead1 null mice are embryonic lethal due to defective cardiac development (Chen et al., 1994); Tead2 knockout (KO) mice either show no phenotype (Sawada et al., 2008) or have defects in neural tube closure (Kaneko et al., 2007); Tead4 null mice are lethal due to embryo implantation failure (Yagi et al., 2007), and a KO of both Tead1 and Tead2 is embryonic lethal due to defects in neural tube closure (Sawada et al., 2008). In humans, an inactivation mutation of Tead1 is associated with degeneration of the choroid and retina or Sveinsson’s chorioretinal atrophy (Fossdal et al., 2004).

Regulation of TEAD involves YAP (Yes-associated protein) and its parologue TAZ (transcriptional activator with PDZ-domain binding). Thus, when YAP/TAZ are phosphorylated by the kinases of the Hippo pathway LATS1/2 (large tumor suppressor kinase 1/2), they remain in the cytoplasm by sequestration to 14-3-3 or are ubiquitinated and degraded. Instead, unphosphorylated YAP/TAZ move into the nucleus and bind to TEAD, driving the transcription of target genes (for review see Zhou et al., 2016).

Hippo-independent coactivators can also regulate TEAD. Such is the case of vestigial-like (VGLL) protein that competes with YAP/TAZ for TEAD binding (Pobbatì et al., 2012; Jiao et al., 2014); of TCF4 (transcription factor 4), whose interaction with TEAD facilitates the expression of Wnt target genes (Jiao et al., 2017); of the p160 family of steroid receptor coactivators that potentiates TEAD transcriptional activity (Belandia and Parker, 2000); and of AP-1 that occupies the same chromatin sites as TEAD and is necessary for the activation of TEAD target genes (Zanconato et al., 2015; Liu et al., 2016).

Although TEAD function can also be regulated by posttranslational modifications, including palmitoylation (Chan et al., 2016) and phosphorylation by PKA (Gupta et al., 2000) and PKC (Jiang et al., 2001), it is noteworthy that TEAD subcellular localization regulates transcriptional programs that determine trophoderm versus inner cell mass (ICM) lineage differentiation in embryos. Thus, in mouse embryonic stem cells (mESCs), TEAD4 localized exclusively in the cytoplasm, while in mouse trophoblast stem cells (mTSCs) TEAD4 was enriched in the nucleus, regulating a trophoderm-specific transcriptional program (Home et al., 2012). Hyperosmotic stress also induces TEAD cytoplasmic localization through a mechanism where p38 binds to TEAD and subsequently translocates the transcription factor to the cytoplasm (Lin et al., 2017).

Previously we demonstrated that ZO-2 silencing in renal epithelial Madin-Darby canine kidney (MDCK) cells induced the nuclear accumulation of YAP and increased the activity of an artificial promoter driven by TEAD-binding sites. In contrast, the overexpression of ZO-2 abolished this activity (Domínguez-Calderón et al., 2016). Moreover, we showed that the promoter for the connective tissue growth factor (CTGF), regulated by TEAD-binding sites (Zhao et al., 2008), is more active in ZO-2 KD cells and that these changes correlated with increased CTGF mRNA (Domínguez-Calderón et al., 2016). These effects, in turn, were obliterated upon ZO-2 transfection (Domínguez-Calderón et al., 2016). These observations prompted us to explore whether the expression and subcellular localization of ZO-2 modulated the nuclear accumulation of TEAD.

Here, in epithelial cells we have found that the entry and departure of ZO-2 from the nucleus modulate TEAD intracellular traffic and that TEAD and ZO-2 form a complex that is negatively regulated by nPKCδ, which facilitates the nuclear importation of TEAD.

RESULTS

ZO-2 facilitates the entry of TEAD into the nucleus of epithelial cells

We first explored by immunofluorescence the subcellular localization of TEAD in parental and ZO-2 KD MDCK cells. For this purpose, we employed sparse and confluent cultures as we had previously shown that in the former, ZO-2 accumulates at the nucleus. In contrast, in confluent monolayers, the amount of nuclear ZO-2 is minimal (Islas et al., 2002). Figure 1A shows that the absence of ZO-2 induces a decreased expression of TEAD in the nuclei of sparse cultures. In contrast, in confluent cultures, an increased expression of nuclear TEAD is observed compared with parental cultures. A Western blot then revealed that in total cellular extracts, the amount of TEAD was diminished in sparse cultures in a ZO-2–dependent manner because the decrease in TEAD was reversed upon transfection with a human full-length ZO-2 construct with an altered short hairpin RNA (shRNA)-binding site (hZO-2). Instead, in confluent cultures, the cellular content of TEAD increased with the lack of ZO-2 and was diminished upon ZO-2 transfection (Figure 1B).

Next, we analyzed by Western blot the amount of TEAD present in nuclear and cytoplasmic fractions. Figure 1C reveals that nuclear TEAD was diminished in ZO-2 KD cells in sparse cultures and that this effect was reversed by hZO-2 transfection. However, the decrease of nuclear TEAD in ZO-2 KD cells was not accompanied by an increase in cytoplasmic TEAD. Instead, in confluent cultures, the lack of ZO-2 augments the cytoplasmic content of TEAD, and the transfection of hZO-2 in ZO-2 KD cells abolishes the presence of nuclear TEAD. These results suggest that ZO-2 regulates the entry of TEAD into the nucleus.

To further test this point, we next analyzed the expression of TEAD in the nucleus of sparse parental cells transfected with Flag-hZO-2 wild type (WT) or a construct lacking the NLS of the molecule (Flag-hZO-2 ΔNLS) present at the U2 segment located between the PDZ1 and PDZ2 domains. Figure 2 shows that the nuclear staining for TEAD is reduced in cells transfected with a ZO-2 that, as previously reported, cannot localize at the nucleus due to the lack of NLS (Jaramillo et al., 2004; Oka et al., 2010; Amaya et al., 2019). However, because the reduction in nuclear TEAD is small in cells transfected with Flag-hZO-2 ΔNLS, our results suggest that the nuclear importation of ZO-2 only favors the movement of TEAD into the nucleus but that other mechanism might also be involved, including signaling by the bP-NLS present in TEAD (Magico and Bell, 2011).

Nuclear TEAD is phosphorylated

The Western blot in Figure 1C also revealed that the band of TEAD in nuclear extracts of ZO-2 KD cells had a lower mobility when compared with that of parental or ZO-2 KD cells transfected with Flag-hZO-2 ΔNLS, suggesting that ZO-2 might modulate the nuclear phosphorylation state of TEAD. Indeed, we show that nuclear TEAD is phosphorylated by the kinase SRPK, and the overexpression of ZO-2 abolished this activity (Domínguez-Calderón et al., 2016). Moreover, we showed that the promoter for the connective tissue growth factor (CTGF), regulated by TEAD-binding sites (Zhao et al., 2008), is more active in ZO-2 KD cells and that these changes correlated with increased CTGF mRNA (Domínguez-Calderón et al., 2016). These effects, in turn, were obliterated upon ZO-2 transfection (Domínguez-Calderón et al., 2016). These observations prompted us to explore whether the expression and subcellular localization of ZO-2 modulated the nuclear accumulation of TEAD.
In silico analysis with GPS 3.0 revealed that TEAD has 58 putative phosphorylation sites. To determine whether the higher molecular weight of nuclear TEAD was due to phosphorylation, we next made a Western blot of nuclear extracts treated or not with alkaline phosphatase and observed that the molecular weight of the nuclear band of TEAD was diminished from 51 to 50 kDa (Figure 1D, left panel), suggesting that nuclear TEAD is more strongly phosphorylated than cytoplasmic TEAD. To confirm this observation, we next made a mobility shift detection assay of TEAD. To this end, we ran nuclear extracts of MDCK cells treated or not with alkaline phosphatase on phosphate-affinity SDS–PAGE with a dinuclear manganese complex of acrylamide-pendant Phos-tag, which binds to and retards the migration of phosphorylated proteins. The right panel of Figure 1D shows that the higher-molecular-weight band of TEAD is not present in the nuclear samples treated with alkaline phosphatase, thus confirming that this band corresponds to phosphorylated TEAD.

Inhibition of nPKCδ promotes the nuclear importation of TEAD and ZO-2

Next, we performed an in silico analysis with GPS 3.0, finding that TEAD has 23 and six putative PKC and PKA phosphorylation sites, respectively. Hence, we explored whether the inhibition of different PKC isozymes and PKA altered the nuclear/cytoplasmic distribution of TEAD in cultures of parental and ZO-2 KD cells. Figure 3A reveals that treatment with 27 nM Ro 31-8220, which inhibits cPKCα, γ, and β1 and nPKCε (Young et al., 2005), significantly increases the concentration of TEAD in the nuclei of ZO-2 KD cells but not of parental cells, thus suggesting that in the absence of ZO-2, cPKCα, γ, β1 or nPKCε induces TEAD nuclear exportation. Instead, treatment with
Inhibition of nPKC δ augments the cytoplasmic content of TEAD, thus suggesting that phosphorylation mediated by nPKC δ blocks the nuclear exportation of TEAD in ZO-2 KD cells. To test whether ZO-2 and TEAD interact and whether this process is inhibited by nPKC δ, we transfected MDCK cells with Flag-hZO-2, treated them or not with 3 μM rottlerin, which inhibits nPKC δ, and made a proximity ligation assay (PLA) with a mouse antibody against Flag and a rabbit antibody against TEAD (Figure 5A). At time 0 (6th hour after transfection), when as previously demonstrated 74–78% of transfected cells have nuclear ZO-2 (Chamorro et al., 2009; Quiros et al., 2013), positive red fluorescent spots are detected at the nucleus of Flag-hZO-2 transfected cells, identified by their green staining (Figure 5, B, a and a′, and C). The number of red spots was above the background present when the antibody against TEAD was omitted (Figure 5, B, e and e′, and C). The abundance of positive red spots at the nucleus of Flag-hZO-2 transfected cells increases when nPKC δ is inhibited with röttlerin (Figure 5, B, b and b′, and C). However, because röttlerin has also been found to inhibit Ca²⁺/calmodulin kinase II (CaM-kinase II), MAP kinase–activated protein 5 (PRK/AK/M5), and MAP kinase–activated protein 2 (MAPKAP-K2) (Gschwendt et al., 1994; Davies et al., 2000), we next proceeded to test the interaction between TEAD and ZO-2 when instead of using röttlerin, nPKC δ was overexpressed by transfection. Figure 5 shows that the number of red spots decreases when nPKC δ was transfected together with Flag-hZO-2 (Figure 5, B, c and c′, and C) and achieves the highest value when transfected nPKC δ is inhibited with röttlerin. In this case, we also observe the appearance of abundant red spots in the cytoplasm (Figure 5, B, d and d′, and C), which further confirms that inhibition allows the interaction of TEAD and ZO-2 in the cytoplasm.
Instead, 24 h posttransfection, when previous observations demonstrated that the percentage of cells with nuclear ZO-2 has diminished to 17% (Chamorro et al., 2009; Quiros et al., 2013), the red spots at the nuclei are barely detectable in all the experimental conditions (Figure 5, B, f, f′ – j, j′, and C) and resemble the background signal obtained when no anti-TEAD antibody was added (Figure 5, B, j and j′, and C).

To further confirm that the interaction of TEAD and ZO-2 is mediated by nPKCδ, we analyzed the presence of TEAD in a ZO-2 immunoprecipitate, finding that inhibition of nPKCδ with rottlerin augments the amount of TEAD associated with ZO-2 in MDCK cells (Figure 6A). In addition, in intestinal epithelial cells IEC-18, we confirmed the presence of TEAD in a ZO-2 immunoprecipitate (Figure 6B). We also showed with a pull-down assay the interaction between TEAD and ZO-2 in the human kidney cell line HEK293T. For this purpose, the cells were transfected with the amino (coding PDZ domains 1, 2, and 3), or AP (coding the acidic and proline-rich regions) constructs of cZO-2, introduced in the pcDNA4/HisMax vector. Then, the corresponding proteins were purified from extracts of HEK293T cell using Ni affinity columns, run in SDS–PAGE, and stained with Coomassie blue. Figure 6C reveals the presence of bands of 62 and 45 kDa that respectively correspond to amino and AP segments of cZO-2. The identity of the amino pulled-down segment was confirmed in a blot with the anti–ZO-2 antibody that recognizes this section of the protein, as indicated by the manufacturer (Figure 6D, top panel). The blot with the antibody against TEAD revealed the presence of this transcription factor in the pull down of the amino and AP segments of ZO-2, thus suggesting that these segments of ZO-2 associate to TEAD (Figure 6D, bottom panel). We also transfected HEK293T cells with the 3PSG (coding PDZ-3, SH3, and GuK domains) segment of ZO-2. However, the identity of this ZO-2 segment could not be confirmed because it generated a very faint band upon staining with Coomassie blue (unpublished data) and cannot be recognized by any commercial anti-ZO-2 antibody.
FIGURE 4: In ZO-2 KD cells inhibition of nPKCδ increases the activity of a promoter regulated by TEAD-binding sites. Reporter gene assays done in parental and ZO-2 KD cells transiently transfected with hCTGF-Luc at a concentration of 100 ng/well (32 mm²). (A) hCTGF promoter activity is higher in ZO-2 KD than in parental cells. hCTGF promoter activity values were normalized to values observed in parental cells. Statistical analysis done with Student’s t test, *p < 0.05. (B) Inhibition of nPKCδ increases the activity of hCTGF promoter in ZO-2 KD cells but not in parental cells. hCTGF promoter activity values were normalized to values observed in ZO-2 KD cells without the nPKCδ inhibitor. Statistical analysis done with Student’s t test, *p < 0.05. In all cases data were normalized to protein content and transfection efficiency with pGL3-Control. Each bar shows the mean ± SEM from triplicates derived from two independent experiments.

DISCUSSION

In epithelial MDCK cells, the subcellular distribution of ZO-2 relies heavily on the degree of cell–cell contact. Thus, in sparse cultures, ZO-2 is found at the TJ region in the plasma membrane (Chamorro et al., 2009). Therefore, we next tested whether blocking the translocation of nuclear ZO-2 to the cytoplasm as the culture becomes confluent altered the nuclear content of TEAD. For this purpose, we transfected sparse parental cells with HA-cZO-2 or a construct where the four NES of cZO-2 are mutated (HA-cZO-2 MutNES) and analyzed 48 h later the expression of TEAD in the nucleus of the transfected cells. Figure 8 shows that the amount of TEAD at the nucleus is not altered when ZO-2 is kept at the nucleus due to the lack of functional NES.

Altogether, our results indicate that the movements of ZO-2 in and out of the nucleus modulate the intracellular traffic of TEAD in a process regulated by PKA and nPKCδ and ε (Figure 9).

Activation by nPKCδ of NES in ZO-2 induces the nuclear exportation of ZO-2 and TEAD

Previously, we demonstrated the activation of ZO-2 NES-1 through the phosphorylation of Ser-369 by PKCa (Chamorro et al., 2009). Hence, we next analyzed in islets of cells what happened with TEAD after treatment with the nPKCδ permeable activating peptide εv1-7. In the islets of MDCK cells treated with peptide εv1-7, ZO-2 is no longer present at the nucleus (Figure 7A, b and e) in comparison to control cultures (Figure 7A, a and d), as had been previously reported (Quiros et al., 2013). Similarly, the expression of TEAD at the nucleus is barely detectable after treatment with the nPKCδ permeable activating peptide εv1-7 (Figure 7A, h and k) in comparison to the control condition (Figure 7A, a and d). Treatment with εv1-2, a nPKCδ permeable inhibitor peptide, concentrated ZO-2 at the nuclei (Figure 7A, c and f) and increased the amount of nuclear TEAD in comparison to control cells (Figure 7A, i and j). The same results were obtained when this experiment was done in intestinal epithelial cells IEC-18 (Figure 7B). Therefore, we conclude that nuclear exportation of ZO-2 triggers the translocation of nuclear TEAD to the cytoplasm, while the nuclear accumulation of ZO-2 facilitates the nuclear concentration of TEAD.

Altogether, these results indicate that ZO-2 and TEAD associate and that the inhibition of nPKCδ allows ZO-2 and TEAD to interact in the cytoplasm and enter together into the nucleus (Figure 6E).
Nevertheless, the blockade of the nuclear entry of TEAD and ZO-2 by nPKCδ could be explained by the fact that at the carboxyl basic region of the bpNLS-2 of ZO-2, three serine residues present there (S257, S259, and S261) are putative nPKCδ phosphorylation sites. Previously we demonstrated that their phosphomimetic mutation obstructs the nuclear accumulation of ZO-2, likely due to the neutralization of the positive charges present at the carboxyl-terminal segment of bpNLS-2 (Quiros et al., 2013).

Our results also indicate that in MDCK cells, the exportation of TEAD from the nucleus is triggered by the activation of nPKCε. This process can happen in a ZO-2–independent manner as observed in ZO-2 KD cells and in a ZO-2–dependent way in parental cells, which involves the activation of ZO-2 NES by nPKCε. The latter observation further confirms that the interaction with ZO-2 facilitates the movement of TEAD. Nevertheless, it should be mentioned that TEAD also contains a bipartite NLS and a NES, both conserved in numerous species whose functionality has been tested in Drosophila (Magico and Bell, 2011). Therefore, the interaction of ZO-2 with TEAD might only reinforce the movement of the transcription factor.

The role of ZO-2 as a nuclear translocator of proteins was previously observed. Thus, ZO-2 was found to enhance the nuclear localization of YAP-2 through a process that required the presence of the NLS of ZO-2 (Oka et al., 2010). YAP is a transcriptional activator of promoters regulated by the TEAD transcription factor (Zhao et al., 2008). It binds to the first PDZ domain of ZO-2 through its carboxy-terminal PDZ-binding motif (Oka et al., 2010). Likewise, the movement of ARVCF into the nucleus requires the interaction of the PDZ-binding motif of this protein with the amino PDZ-containing region of ZO-2 and the presence of the NLS of ZO-2 (Kausalya et al., 2004). ARVCF protein at the nucleus binds to splicing factors and contributes to the regulation of alternating splicing (Rappe et al., 2014).

In summary, our observations indicate that ZO-2 acts as a platform that facilitates the nuclear importation and exportation of TEAD.

**MATERIAL AND METHODS**

Request a protocol through Bio-protocol.

**Cell culture**

Parental (control) and ZO-2 KD MDCK II cells were kindly provided by Alan Fanning (University of North Carolina, Chapel Hill, NC) and cultured as previously described (Van Itallie et al., 2009). These cells stably express a mixture of three different shRNAs against ZO-2 in the pSuper vector. Parental cells instead express only the empty vector. Sparse cultures were plated at a density of 2.5 × 10^4 cells/cm^2, whereas confluent cultures were seeded at a density of 5 × 10^5 cells/cm^2.
IEC-18 epithelial cells derived from the rat small intestine and obtained from the American Type Culture Collection (Cat. CRL-1589; ATCC, Manassas, VA) were grown in DMEM F-12 (Cat. 12500-062; Thermo Fisher Scientific, Waltham, MA) supplemented with 5% fetal bovine serum (FBS) and penicillin–streptomycin 10,000 (U/μg/ml) (Cat. A-01; In Vitro, Mexico).

HEK293T epithelial cells derived from the human embryonic kidney (Cat. CRL-3216; ATCC, Manassas, VA) were grown in a high-glucose DMEM (Cat. 11965-118; Thermo Fisher Scientific, Waltham, MA) supplemented with 5% FBS and penicillin–streptomycin 10,000 (U/μg/ml) (Cat. A-01; In Vitro, Mexico).

Transfections

Transfections were done following the manufacturer’s instructions using Lipofectamine 2000 (Cat. 11668-019; Life Technologies, Carlsbad, CA). Because the Lipofectamine manufacturer indicates that 6 h is the optimal time required for the transfected construct to be expressed as protein, time 0 in Figure 5 corresponds to 6 h after transfection.

ZO-2 KD cells were transfected with a human full-length ZO-2 construct with altered shRNA-binding sites (pTRE-hZO-2), generously provided by Alan Fanning (University of North Carolina, Chapel Hill, NC).

To generate hZO-2 ΔU2 in a construct with CMV10 promoter and a tag of 3 × Flag, we liberated with KpnI and XbaI restriction enzymes the hZO-2 ΔNLS construct inserted into a vector containing CMV2 promoter and a tag of 2 × Flag (generously provided by Marius Sudol, National University of Singapore, Singapore). hZO-2 ΔNLS lacks base pairs 313–873 of hZO-2 that code for the U2 segment (amino acids 105–291). Full-length hZO-2 contained within a vector with CMV10 promoter and a 3 × Flag (Flag-hZO-2) (generously provided by Otmar Huber, Jena University, Germany) was cut with KpnI and XbaI restriction enzymes to eliminate full-length hZO-2 from the vector. The hZO-2 ΔNLS construct was then inserted into this empty vector containing CMV10 promoter and a 3 × Flag. Parental cells were transfected with either Flag-hZO-2 or hZO-2 ΔNLS within a vector containing CMV10 promoter and a 3 × Flag. Parental cells were transfected with either Flag-hZO-2 or hZO-2 ΔNLS within a vector containing CMV10 promoter and three Flag tags (Flag-hZO-2).

In some experiments, parental and ZO-2 KD MDCK cells were transfected with mouse nPKCδ (plasmid 16386; Addgene, Water- town, MA) or Flag-hZO-2 plus nPKCδ. HEK293T cells were transfected with amino (398–962 nucleotides [nt]), 3PSG (1595–3019 nt), or AP (3029–3923 nt) segments of cZO-2, in the pcDNA4/HisMax vector that had been previously reported (Betanzos et al., 2004).

Immunofluorescence

Immunofluorescence experiments on MDCK cells were done as previously described (Quiros et al., 2013) with the only modification that cells were fixed with 10% (vol/vol) formaldehyde in
As first antibodies, we employed a rabbit monoclonal against TEAD (Cat. 12292; dilution 1:200; Cell Signaling, Danvers, MA); mouse monoclonal antibodies against Flag (Cat. F3165; dilution 1:1500; Sigma Aldrich, Darmstadt, Germany); and rabbit polyclonal against ZO-2 (Cat. 71-1400; dilution 1:200; Invitrogen, Carlsbad, CA). As secondary antibodies, we employed donkey antibodies against rabbit immunoglobulin G (IgG) coupled to Alexa Fluor 488 (Cat. A21206; dilution 1:1000; Invitrogen, Carlsbad, CA) or Alexa Fluor 594 (Cat. A21207; dilution 1:1000; Invitrogen, Carlsbad, CA) and against mouse IgG coupled to Alexa Fluor 488 (Cat. A11011; Life Technologies, Eugene, OR).

Quantification of TEAD immunofluorescence intensity at the nucleus was done using ImageJ. Nuclei images stained with DAPI (4′,6-diamidino-2-phenylindole) were used to define the region to be subsequently quantitated for TEAD immunofluorescence.

**Western blot**

Western blot was done following a standard procedure previously described (Quiros et al., 2013), but using a different RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and a commercial sample buffer (Cat. NP0008; Invitrogen, Carlsbad, CA). The following primary antibodies were employed: a rabbit monoclonal against TEAD (Cat. 12292; dilution 1:200; Cell Signaling, Danvers, MA); mouse monoclonals anti-GAPDH (Cat. Sc-32233; dilution 1:15,000; Santa Cruz Biotechnology, Santa Cruz, CA) and lamin B1 (Cat. 33-2000; dilution 1:2000; Invitrogen, Carlsbad, CA); and rabbit polyclonal against ZO-2 (Cat. 71-1400; dilution 1:2000; Invitrogen, Carlsbad, CA). As secondary antibodies, we employed goat polyclonals anti-rabbit IgG coupled to peroxidase (Cat. A9169; dilution 1:20,000; Sigma Aldrich, St. Louis, MO) or anti-mouse IgG coupled to peroxidase (Cat. 62-6420; dilution 1:10,000; Invitrogen, Carlsbad, CA). The procedure was followed by Immobilon chemiluminescence detection (Cat. WVKLS 0500; Merck KGaA, Darmstadt, Germany).

For the mobility shift detection assay of phosphorylated TEAD, phosphate affinity SDS–PAGE was done with the acrylamide-pendant Phos-tag ligand (Cat. AAL-107; Wako Pure
Chemical Industries, Richmond, VA) following the manufacturer’s instructions. In Phos-tag SDS–PAGE, molecular weight estimations using molecular weight markers are not possible. Because the manufacturer recommends using a dephosphorylated sample of the target protein as a marker, in Figure 1D, right panel, we have placed the indication of 50 kDa in the lower band of TEAD that is still present after alkaline phosphatase treatment.

Alkaline phosphatase treatment

Nuclear pellets obtained from confluent MDCK monolayers were resuspended in NEB buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, and 1 mM dithiothreitol, pH 7.9; Cat. B7003S; New England Biolabs, Ipswich, MA). The nuclei were then sonicated and incubated for 60 min at 37°C with 20 U of calf intestine alkaline phosphatase (Cat. M0290S; New England Biolabs, Ipswich, MA). Samples were then processed for Western blot.

Proximity ligation assay

The Duolink in situ proximity ligation assay (PLA) (Cat. DUO92101; Sigma Aldrich, Darmstadt, Germany) was done according to the manufacturer’s instructions. Using a rabbit antibody against ZO-2 (Cat. 12292; dilution 1:200; Cell Signaling, Danvers, MA) and a mouse antibody anti-Flag (Cat. F3165; dilution 1:1500; Sigma Aldrich, Darmstadt, Germany) was done according to the manufacturer’s instructions. Using a rabbit antibody against TEAD (Cat. 71-1400; Invitrogen, Carlsbad, CA) per 300 μg of protein in the lysate of parental MDCK cells and following a protocol previously reported by others (Shaiken and Opekun, 2014). Briefly, cells were lysed in isotonic buffer A (40 mM HEPES, pH 7.4, 120 mM KC1, 2 mM EGTA (ethylene glycol-bis(2- aminoethylether)-N,N,N',N'-tetraacetic acid), 0.4% glycerol, 10 mM β-glycerophosphate, and 0.2% NP-40) while rotating for 30 min at 4°C. Nuclei were pelleted by centrifugation at 1000 × g for 5 min. The supernatant was centrifuged further at 10,000 × g for 10 min to obtain the cytosolic fraction. The pellet of nuclei was sequentially and gently washed with 0.1% NP-40 and non–detergent containing buffer A and centrifuged at 1000 × g for 5 min. The supernatant was discarded, and the nuclear pellet was resuspended in RIPA buffer.

Immunoprecipitation

Immunoprecipitation of ZO-2 was done using 1 μl of ZO-2 antibody (Cat. 71-1400; Invitrogen, Carlsbad, CA) per 300 μg of protein in the lysate of parental MDCK cells and following a protocol previously described (Raya-Sandino et al., 2017). The radioimmunoprecipitation assay buffer employed contained 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40 (vol/vol), and the protease inhibitor cocktail Complete (Cat. 11697498001; Roche Diagnostics, Mannheim, Germany). The blot for TEAD in the ZO-2 immunoprecipitate was done according to the manufacturer’s instructions. Using a rabbit antibody against TEAD (Cat. STAR209; BioRad, Hercules, CA) per 300 μg of protein in the lysate of parental MDCK cells and following a protocol previously reported by others (Shaiken and Opekun, 2014). Briefly, cells were lysed in isotonic buffer A (40 mM HEPES, pH 7.4, 120 mM KC1, 2 mM EGTA (ethylene glycol-bis(2- aminoethylether)-N,N,N',N'-tetraacetic acid), 0.4% glycerol, 10 mM β-glycerophosphate, and 0.2% NP-40) while rotating for 30 min at 4°C. Nuclei were pelleted by centrifugation at 1000 × g for 5 min. The supernatant was centrifuged further at 10,000 × g for 10 min to obtain the cytosolic fraction. The pellet of nuclei was sequentially and gently washed with 0.1% NP-40 and non–detergent containing buffer A and centrifuged at 1000 × g for 5 min. The supernatant was discarded, and the nuclear pellet was resuspended in RIPA buffer.

Proximity ligation assay

The Duolink in situ proximity ligation assay (PLA) (Cat. DUO92101; Sigma Aldrich, Darmstadt, Germany) was done according to the manufacturer’s instructions. Using a rabbit antibody against TEAD (Cat. 12292; dilution 1:200; Cell Signaling, Danvers, MA) and a mouse antibody anti-Flag (Cat. F3165; dilution 1:1500; Sigma Aldrich, Darmstadt, Germany). After the Duolink reaction was completed, the preparations were washed three times with Duolink B-buffer. The cells were stained with a donkey anti-mouse antibody coupled to Alexa Fluor 488 to detect cells transfected with Flag-hZO-2.

Quantitative analysis of PLAs was done using BlobFinder (Allalou and Wahlby, 2009) developed by the Centre for Image Analysis at Uppsala University.

Immunoprecipitation

Immunoprecipitation of ZO-2 was done using 1 μl of ZO-2 antibody (Cat. 71-1400; Invitrogen, Carlsbad, CA) per 300 μg of protein in the lysate of parental MDCK cells and following a protocol previously described (Raya-Sandino et al., 2017). The radioimmunoprecipitation assay buffer employed contained 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40 (vol/vol), and the protease inhibitor cocktail Complete (Cat. 11697498001; Roche Diagnostics, Mannheim, Germany). The blot for TEAD in the ZO-2 immunoprecipitate was done using the Tidy blot reagent (Cat. STAR209; BioRad, Hercules, CA) following the manufacturer’s instructions.

Protein purification

HEK293T cells were transfected with previously reported constructs containing the amino (398–962 nt), 3PSG (1595–3019 nt),
or AP (3029–3923 nt) segments of ZO-2 in the pcDNA4/HisMax vector (Betanzos et al., 2004). After 24 h the cells were lysed and the extracts were subjected to affinity chromatography with Complete His-Tag Purification Columns (Cat. COHISC-RO; Sigma Aldrich, St. Louis, MO), following the manufacturer’s instructions. The purified fractions were run in a SDS–PAGE, stained with Coomassie blue, and blotted with antibodies against ZO-2 and TEAD.

**Receptor gene assays**

Parental and ZO-2 KD MDCK cells were plated in 96-well plates at a density of 2.5 x 10^4 cells/cm². After 24 h, cells were transfected using Lipofectamine 3000 (Cat. L3000015; Thermo Fisher, Waltham, MA) with 100 ng of a construct of the human CTGF promoter region that contains three putative TEAD-binding sites cloned into the basic luciferase reporter vector pGL3-6xOSE-Luc (Zhao et al., 2008) generously provided by Kun-Liang Guan from the Moors Cancer Center, University of California at San Diego, or with 75 ng of the pGL3-Control (Cat. E1741; Promega, Madison, WI) plasmid with constitutive activity. After 6 h, the transfection medium was removed and replaced with a fresh culture medium or medium with 2 μM nPKCe permeable inhibitor peptide εv1-2. After 20 h of treatment, the cells were harvested and suspended in reporter lysis buffer (Cat. E3971; Promega, Madison, WI). Protein extraction was done by a heat shock lysis cycle of 5 min at 70°C followed by 1 min at 37°C and 3 min in agitation. Finally, luciferase activity was determined using the luciferase assay system (Cat. E1500; Promega, Madison, WI) and the Infi nite M200 Pro series (Tecan, Männedorf, Switzerland) equipment. Luciferase activity values were normalized to protein content and expressed relative to those recorded in parental cells or in parental and ZO-2 KD cells without the nPKCe inhibitor. In all cases, transfection efficiency was normalized using pGL3-control as a reference.

**Drugs**

The nPKCe permeable inhibitor peptide εv1-2 and the nPKCe permeable activating peptide εv1-7 were kindly provided by Daria Mochly-Rosen (Stanford University, Stanford, CA) and dissolved in water as a stock at a concentration of 1 mM and used at a final concentration of 2 μM. Röttler, an nPKCe inhibitor (Cat. S57370; Calbiochem, Darmstadt, Germany), was prepared as a 12 μM stock in dimethyl sulfoxide (DMSO) and used at a final concentration of 3 μM.

**ACKNOWLEDGMENTS**

This work was supported by grants to L.G.-M. from the Miguel Aleman Valdéz Foundation 2018, SEP-Cinvestav FIDSC2018/33, and National Council of Science and Technology of Mexico (Conacyt) FORDECYT-PRONACES-140644/2020. H.G.-G. and L.G.-G. were recipients of doctoral fellowships from the Conacyt FORDECYT-PRONACES-140644/2020. H.G.-G. and National Council of Science and Technology of Mexico (Conacyt) MEXT the Mexiquense Council of Science and Technology (282075 and 340209), and H.G.-G. was also the recipient of a scholarship from the Mexiquense Council of Science and Technology (2018ABD0009-11).

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