Functional complex between YAP2 and ZO-2 is PDZ domain dependent, regulates YAP2 nuclear localization and signaling

Tsutomu OKA*, Eline REMUE[†‡], Kris MEERSCHAERT[†‡], Berlinda VANLOO[†‡], Ciska BOUCHERIE[†‡], David GFELLER[§], Gary D. BADER[§], Sachdev S. SIDHU[§], Joël VANDEKERCKHOVE [†‡], Jan GETTEMANS[†‡] and Marius SUDOL[*#]

*Weis Center for Research, Danville, PA 17822, USA
†Department of Medical Protein Research, VIB, B-9000 Ghent, Belgium and ‡ Department of Biochemistry, Ghent University, Faculty of Medicine and Health Sciences, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium, §Terrence Donnelly Center for Cellular and Biomolecular Research, University of Toronto, 160 College Street, Toronto, Ontario M5S 3E1, Canada; #Mount Sinai School of Medicine, Department of Medicine, New York, NY 10029, USA

Address correspondence to: Marius Sudol, Ph.D. 100 North Academy Avenue, Danville, PA 17822-2608. Fax: 570-271-6701; E-mail: msudol1@geisinger.edu

The HIPPO pathway regulates the size of organs by controlling two opposing processes: proliferation and apoptosis. YAP2, one of the three isoforms of YAP, Yes kinase-associated protein, is a WW domain-containing transcriptional co-activator that acts as the effector of the HIPPO pathway in mammalian cells. In addition to WW domains, YAP2 has a PDZ-binding motif at its carboxy-terminus. We previously documented that this motif was necessary for YAP2 localization in the nucleus and for promoting cell detachment and apoptosis. Here, we report that a tight junction protein, ZO-2 uses its first PDZ domain to form a complex with YAP2. The endogenous ZO-2 and YAP2 proteins co-localize in the nucleus. We also found that ZO-2 facilitates nuclear localization and pro-apoptotic function of YAP2, and that this activity of ZO-2 is PDZ-domain dependent. This is the first report on PDZ-based nuclear translocation mechanism. Moreover, since the HIPPO pathway acts as a tumor suppressor pathway, the YAP2-ZO-2 complex represents a target of cancer therapy.

Short title: Function of the first PDZ domain of ZO-2 and YAP

Key words: YAP gene, Zona occludens proteins, Hippo pathway, nuclear translocation,

The abbreviations used: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HEK293, human embryonic kidney cell line 293; WW domain, Tryptophan-Tryptophan domain; YAP, Yes kinase-associated protein; Yki, Yorkie; ZO, Zonula Occludens

Introduction

YAP, Yes kinase-associated protein is one of the effectors of the HIPPO (Hpo) pathway that controls the size of organs by coordinating two opposing processes, proliferation and cell death [1]. Originally, the YAP gene was isolated by functional cloning, as Yes- and Src-kinase binding

protein [2]. Subsequently, YAP was shown to act as a transcriptional co-activator downstream of various signaling complexes [3-6]. However, the most compelling evidence for YAP function came from genetic analyses in Drosophila, which revealed an oncogene-like activity of YAP and its fly ortholog, Yorkie (Yki) [7].

There are three major isoforms of YAP, named YAP1, YAP2 and YAP2-Long (-L), which are generated by differential splicing [4, 8]. YAP1 contains one Tryptophan-Tryptophan domain (WW domain), YAP2 contains two WW domains, and YAP2-L has a 16 amino acid long insert within its transcription activation domain [4]. WW domains are small protein modules known to mediate protein complexes by interaction with linear, proline-rich peptide motifs in cognate proteins [9, 10]. Apart from WW domains, YAP also contains a transcriptional activation domain and a carboxy (C)-terminally located PDZ domain-binding motif. YAP has the ability to translocate from the cytoplasm to the nucleus, a process controlled by phosphorylation and also by WW and PDZ domain-mediated complexes with several cytoplasmic proteins [11-14].

Previously, we reported that when human embryonic kidney cells (HEK293) were grown in media containing 1% serum, YAP2, but not YAP1, was able to stabilize the pro-apoptotic protein, p73, and thus promote cell apoptosis [13]. We also confirmed that the cytoplasmic localization of YAP2 was dependent on YAP2 phosphorylation at Serine 127 by Lats1 kinase. We further showed that the PDZ-binding motif was required for promoting apoptosis in cells grown in low serum [14]. In addition, our data documented that the PDZ-binding motif was necessary for YAP2 localization in the nucleus, as YAP2 mutant with deleted PDZ-binding motif was found only in the cytoplasm. In sum, our recent data pointed to the existence of an unknown PDZ domain-containing protein (or proteins), which could act as a shuttle, facilitating YAP2 translocation from the cytoplasm to the nucleus. In this report we show that Zona Occludens-2 (ZO-2), which associates with tight junctions, is one of the candidate proteins that control the localization of YAP2.

Among the prominent components of the tight junction-associated proteins are the ZO proteins: ZO-1, -2 and -3. They belong to the MAGUK (membrane-associated guanylate kinase) protein family. Like other MAGUKs, the ZO-proteins contain three, amino-terminally located PDZ domains, one SH3 domain, a guanylate kinase (GUK) homology domain, and a proline rich C-terminal region. All three proteins show extensive sequence similarity to each other, especially within their equivalent PDZ domains [15]. Both ZO-2 and -3 can heterodimerize with ZO-1 through their second PDZ domain, but not with each other [16-19]. At tight junctions, ZO-1, -2, and -3 bind to claudins, junction adhesion protein known as JAM (junction adhesion molecule), and occludin [20-22]. They also bind actin filaments [19, 23, 24] and other signalling proteins, serving as putative adaptors in the assembly of signaling complexes [25]. However, the exact function of ZO proteins in the formation and maintenance of tight junctions is still unknown.

The subcellular distribution of ZO-proteins seems sensitive to the degree of cell-cell contact [26, 27]. Generally, ZO-1 and -2 concentrate in the nuclei of sparsely populated epithelial cells, whereas in confluent monolayers, they tend to accumulate at tight junctions. This suggests that ZO proteins could transmit information about the state of cell-cell contact to the nucleus, and such signals could maintain balance between proliferation and differentiation. Both ZO-1 and ZO-2 proteins contain nuclear localization and export signals (NLS and NES), but the function of these signals was verified only for ZO-2 [28, 29]. Nuclear ZO-2 regulates gene expression through its association with various transcription factors including Jun, Fos, and C/EBP [30]. ZO-1 has also been shown to regulate gene expression through the transcription factor

ZONAB/DbpA. ZONAB stimulates cell proliferation in epithelial cells, and this process is negatively regulated by ZO-1, which sequesters the transcription factor at tight junctions [31, 32].

The recent reports showing the involvement of YAP2 in the regulation of cell adhesion [13] and in cell density-dependent nuclear localization of YAP [33] prompted us to study the function of the potential complex between YAP2 and ZO-2 proteins. We show here that: (i) the complex between endogenous YAP2 and ZO-2 proteins could be visualized by immuno-staining and co-immuno-precipitation; (ii) that the PDZ-binding motif in YAP2 and the 1st PDZ domain in ZO-2 are required for the formation of the complex; (iii) that the over-expression of ZO-2 affected the subcellular localization of YAP2; and (iv) that ZO-2 cooperated with YAP2 to enhance detachment of HEK293 cells but to inhibit YAP2-induced proliferation of MDCK cells. The novelty of our work is in implicating PDZ domain and one of the PDZ domain-containing proteins, ZO-2, in the mechanism of nuclear transport.

Experimental Procedures

Cell culture and transfections

HEK293 and MDCK cells were from ATCC and cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). MCF7 cells were cultured in Eagle's Minimum Essential Medium containing 10% FBS and 10 μg/ml insulin (Sigma). MCF10A cells were cultured in MEGM containing 10% FBS, 50 μg/ml bovine pituitary extract (BPE), 0.5 μg/ml hydrocortisone (Sigma), 10 μg/ml insulin, 20 ng/ml EGF (Sigma), 0.1 μg/ml cholera toxin (CALBIOCHEM). HEK293 cells were transfected using Lipofectamine (Invitrogen) according to manufacturer's instructions, and GenJet In Vitro DNA Transfection Reagent (SignaGen) was used to transfect plasmids into MDCK cells.

Antibodies and immunoprecipitation

Flag-M2 antibody and ZO-2 antibody were from Sigma and Santa Cruz Biotechnology, respectively. Antibodies to ZO-1 and ZO-2 for immunostaining were from Invitrogen. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Abcam and poly-[ADP-ribose]-polymerase (PARP) was from Roche Applied Science, respectively. Polyclonal YAP antibody was generated in rabbits as described previously [8]. Immunoprecipitation was conducted as described before [13, 14]. To purify over-expressed Flag YAP2 WT from HEK293 cell lysates, 3x Flag peptide (Sigma) was used according to manufacture's instructions.

Immunostaining

Cultured cells were grown on glass coverslips, washed with PBS, and fixed in 3% paraformaldehyde. After permeabilization with 0.1 % Triton X-100 in PBS, cells were blocked in 1% BSA in PBS and incubated at 37°C with primary antibody for 1 hour or overnight at 4°C, followed by Alexa Fluor-488 goat anti-rabbit (for YAP) or anti-mouse (for ZO proteins) IgG (Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Stained cells were examined using an Apotome Zeiss Axioplan II epifluorescence microscope. Images were captured using a cooled CCD Axiocam Camera and Axiovision 4.4 software (Zeiss, Götingen, Germany).

Plasmids

Human ZO-2 cDNA in pOTB7 vector was purchased from OPEN BIOSYSTEMS. The amplified PCR product was subcloned into pBluescript vector (STRATAGENE) by utilizing Kpn I and Xba I sites that had been incorporated into the primers. This insert was subcloned into pEGFP-C3 vector (Clontech), p2xFlag-CMV2 vector, and pcDNA4/TO/myc-His vector (Invitrogen) using

Kpn I and Xba I sites. ZO-2 N, a truncated fragment of ZO-2 WT, was subcloned from pBluescript-ZO-2 WT into pEGFP-C3 vector, p2xFlag-CMV2 vector, and pcDNA4/TO/myc-His vector using Kpn I and BamH I/Bgl II sites. ZO-2 PDZm has 4 point mutations in its 1st PDZ domain (K38E, F44H, I46E, V48E). ZO-2 gene containing these mutations was synthesized by Epoch Biolabs and the mutated fragment was exchanged with ZO-2 WT in p2xFlag-CMV2 vector using Kpn I and Bgl II sites. The resulting plasmid was subcloned into pEGFP-C3 vector, and pcDNA4/TO/myc-His vector using Kpn I and Xba I sites. ZO-2 ΔNLS lacks two putative NLS, and the ZO-2 deleted fragment (313-873 bp) was synthesized by Epoch Biolabs prior to subcloning into p2xFlag-CMV2 vector. ZO-2 ΔN lacking from 1 to 351 bp had its 1st PDZ domain completely deleted. Its PCR fragment was subcloned into p2xFlag-CMV2 vector using Kpn I and Xba I sites embedded into the primers. Other ZO-2 ΔNLS and ZO-2 ΔN expression plasmids were constructed as ZO-2 PDZm. YAP2 WT and several Flag ZO-2 cDNAs in p2xFlag-CMV2 vector were subcloned into pBABE-puro vector or pBABE-hygro vector. HA-YAP2 5SA was a gift from Dr. Xiaolong Yang (Queen's University, Ontario, Canada) and its cDNA was subcloned into pBABE-puro vector.

ZO-2 WT and delta NLS in p2xFlag-CMV2 vector were used as templates for PCR to construct GST fused human ZO-2. PCR was performed to amplify the cDNA of human ZO-2 using two primer sets. 5'- agaggatccatgccggtgcgaggagaccgc-3'; 5'- agactcgagcggccgccccctaggctcggg -3' and 5'- agaggatccgaagatgaagcaatatatgg-3'; 5'- agactcgagagccaggatgtctctatacac-3'. The amplified fragments were inserted into pGEX 4T3 vector to generate GST-human ZO-2 1st PDZ WT and GST-human ZO-2 1st PDZ ΔNLS, respectively.

Mouse ZO-2 cDNA was a gift from Dr. S. Tsukita (Osaka University, Suita, Japan). Its single PDZ domain was cloned in pGEX 2TK vector using BamH I and EcoR I sites (Amersham Pharmacia Biotech AB-GE Healthcare) and were named GST 1st PDZ, GST 2nd PDZ and GST 3rd PDZ, respectively. All other plasmids were as described previously [13, 14].

Human ZO-1 cDNA in pCR-Blunt II-TOPO vector was purchased from OPEN BIOSYSTEMS. The amplified PCR product was subcloned into p2xFlag-CMV2 vector like ZO-2.

GST-tagged protein expression and binding assay

The expression of GST-tagged proteins was induced by adding IPTG. The cells were harvested prior to adding Triton buffer (50mM Tris-HCl (pH7.45), 150mM NaCl, 1mM EDTA, 1% Triton). Cell debris were removed by centrifugation, and Glutathione Sepharose 4B (Amersham Biosciences) was added to the supernatant and incubated for 1 hour. The resin was washed with Triton buffer, and purified Flag YAP2 WT from HEK293 cell lysates was added to the resin followed by 2 hour incubation. The resin was washed again with Triton buffer and bound proteins were separated by SDS-PAGE followed by immunoblotting or Coomassie blue staining.

RNAi treatment and cell counting assay

The RNAi oligo for ZO-2 and the control oligo were purchased from Santa Cruz Biotechnology and Eurogentec (Seraing, Belgium). siRNA duplexes targeting canine ZO-2 were as follows: sense sequence- 5'-GCAGCAGUAUUCCGACUAUdTdT-3', antisense sequence- 5'-AUAGUCGGAAUACUGCUGCdTdT-3'. MDCK cells were transfected with siRNA duplexes (50nM) using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. A negative control siRNA duplex obtained from Eurogentec was used under similar conditions. HEK293 cells that express YAP2 WT in an inducible system [13, 14] were plated at about 5% confluency in DMEM, 1% FBS, blasticidin (5 μg/ml). Expression of YAP2 was induced by Tetracycline (1 μg/ml). Transfections of the RNAi oligos were conducted by using Lipofectamine (Invitrogen). 96 hours later, floating cells were removed, and attached cells were trypsinized and counted. For the cell counting assay without RNAi treatment, HEK293 cells were transfected with Flag-tagged plasmids. 24 hours after the transfection, the cells were distributed into new

plates and maintained in DMEM with 1% FBS for 96 hours. After the removal of floating cells, attached cells were counted as described above. MDCK cells, were maintained in DMEM with 0.5% FBS for 96 hours. The expression of proteins was checked by Western blotting.

Establishment of MDCK and MCF10A cells that stably express YAP2 and ZO-2

MDCK cells were transfected with pBABE-puro YAP2 WT, pBABE-puro Flag ZO-2 (WT, ΔNLS) or pBABE-puro control vector. Cells which failed to express these plasmids were removed by adding Puromycin (Sigma) to a final concentration of 2 μg/ml. After the selection, cells were transfected with either pBABE-hygro vector or pBABE-hygro ZO-2s. Selection was performed again by adding both Puromycin (2 μg/ml) and Hygromycin (400 μg/ml, Invitrogen). Single cell isolation was never performed when cells were selected. The same procedure was performed in case of MCF10A cells except for lower concentration of Puromycin (0.5 μg/ml) and Hygromycin (200 μg/ml), and pBABE-puro YAP2 5SA was used instead of pBABE-puro YAP2 WT.

Results

Endogenous ZO-2 co-localizes with endogenous YAP in several cell lines

The C-terminal sequence of YAP (-FLTWL) represents a well-conserved motif that binds to one of the 16 subclasses of PDZ domains [34, 35]. By scanning billions of random peptides with 82 PDZ domains from human and *Caenorhabditis elegans* proteomes, a map of binding specificities was completed [35]. This map was used to predict several PDZ domains and their host proteins as putative partners of YAP. Four PDZ domain-containing proteins displayed a specificity profile that matched the C-terminus of YAP: ZO (Zonula Occludens) family, MPDZ, SLC9A3R2 and INADL (Figure S1). To test these predictions we used commercial polyclonal antibodies against human ZO-2, MPDZ and INADL and documented that YAP could be co-precipitated from HEK293 only with ZO-2 antibody (not shown). Therefore, we focused our efforts on the ZO-YAP complex. The -FLTWL C-terminal sequence of YAP matches the consensus sequence of peptides that bind to the 1st PDZ domain of the ZO family of proteins [36]. The sequence similarity among the 1st PDZ domains of ZO family members ZO-1, ZO-2 and ZO-3, is high (between ZO1-and ZO-2 domains identities are 70%; between ZO-1 and ZO-3 identities are 55%). Therefore, one could predict that each of the three ZO proteins would bind to YAP through their 1st PDZ domain. ZO proteins are integral components of tight junctions [37]. However, several reports described that ZO-2 has a propensity to localize to the nucleus [28, 29, 38], and ZO-2 has putative Nuclear Localization Signal*s* (NLS*s*) (Figure S2). ZO-1 is usually present in tight junctions, but under some conditions, it was also observed in the cell nucleus [26]. ZO-3 is the least well characterized member of the ZO family in terms of the potential ability to shuttle between cytoplasm and the nucleus, therefore we decided not to include it the present study.

To determine if YAP and ZO proteins co-localize in cells, we immunostained MDCK cells for endogenous ZO-1, ZO-2 and YAP proteins (Figure 1A). YAP gave a strong signal in the nucleus, while most of the ZO-1 signal was in the membrane. YAP and ZO-1 barely co-localized in MDCK cells. In contrast, ZO-2 immuno-reactivity was concentrated in the nucleus and its distribution in MDCK cells was almost identical to that of YAP (Figure 1A). These results suggest that in contrast to ZO-1, ZO-2 may form a functional complex with YAP in MDCK cells. In MCF10A cells and MCF7 cells, ZO-2 and YAP co-localized in the nucleus as well (Figure 1B). Knowing that ZO-1 and ZO2 could heterodimerize via their second PDZ domains, we investigated whether YAP2, ZO-1 and ZO-2 could form a tripartite complex. Both YAP2 and ZO-2 were co-precipitated with ZO-1, suggesting that these three proteins could form a tripartite

complex *in vivo* (Figure 1C). Judging from the ratio of signal between the input and the immunoprecipitated fraction, ZO-1 binds to ZO-2 more tightly than to YAP2. To test whether the association of YAP and ZO-2 changes depending on the state of cell confluency, MDCK cells were maintained in sparse or dense conditions; however, consistently, we did not detect significant changes in the YAP2-ZO-2 complex as a function of cell density (Figure 1D). The association of endogenous ZO-2 and YAP in the nucleus and cytosol was confirmed when immunoprecipitation was conducted using nuclear and cytosol fractions of MDCK cells (Figure 1E). It was previously shown that ZO-2 accumulated in the nucleus when MDCK cells were sparsely distributed, whereas when the cells were densely distributed, ZO-2 could be also found in the cyoplsm [27]. To investigate whether localization of ZO-2 is accompanied by that of YAP in MDCK cells, nuclear and cytosol fractions of MDCK cells were isolated and the distribution of endogenous ZO-2 and YAP was monitored. However, we observed a less robust change when compared with the data reported previously [27]. Nevertheless, we re-confirmed that the ratio of ZO-2 in the nucleus versus cytoplasm increased when MDCK cells were sparsely distributed. As observed for ZO-2, a similar change in the subcellular distribution was observed for YAP2 (Figure 1F). The amounts of ZO-2 and YAP in the nucleus slightly decreased, when MDCK cells were densely populated (Figure 1F). Based on these observations, we decided to further investigate the role of the ZO-2/YAP association in mammalian cells.

YAP2 PDZ-binding motif binds the 1st PDZ domain of ZO-2

Because ZO-2 has three PDZ domains and YAP2 has a PDZ-binding motif at its C-terminus, we hypothesized that ZO-2 associates with YAP2 via its PDZ domain(s). From the comprehensive map of PDZ binding specificities [35] we predicted that only the 1st PDZ domain of ZO-2 would bind YAP2. The 1st, 2nd, and 3rd PDZ domains of mouse ZO-2 were individually expressed as GST-fused domains and used to pull down Flag YAP2 from HEK293 cells (Figure 2A). As expected, only the 1st PDZ domain of ZO-2 pulled-down YAP2. Using Isothermal Titration Calorimetry (ITC) we determined that the dissociation constant, Kd, for the 11-mer peptide of YAP (KLDKESFLTWL) and the GST-PDZ1 of ZO-2 is 2.3 μM. A control peptide with the deletion of the last 3 amino acids (-TWL) did not result in thermodynamic changes in the ITC assay (not shown). Therefore we concluded that most likely the truncated peptide did not interact with the PDZ domain. Interestingly, the PDZ1 domain of ZO-1 interacted with the YAP peptide with similar strength (Kd = 3.7 μM) (Figure 2B).

To study the function of the ZO-2/YAP2 complex in detail, several ZO-2 mutants were constructed. Both ZO-1 and ZO-2 were recently shown to form a complex with ARVCF protein, and their PDZ domains were responsible for the association [39]. In that report, 4 point mutations within the binding pocket of the 1st PDZ domain of ZO-1 were shown to be required to abolish the binding. The equivalents of the ZO-1 PDZ domain mutations were introduced into the ZO-2 PDZ1 domain (K38E, F44H, I46E, V48E) (see construct ZO-2 PDZm in Figure 3A, Figure S3). Since we showed that the 1st PDZ domain bound YAP2, a ZO-2 ΔN mutant that lacks the 1st PDZ domain was constructed. We also generated a mutant of ZO-2 in which two putative NLS*s* [21, 29, 38] were eliminated (ZO-2 ΔNLS). A truncated version of ZO-2 that has only the 1st PDZ domain (ZO-2 N) was also constructed. Binding assays were conducted with these mutants. ZO-2 WT could co-precipitate YAP2 effectively (Figure 3B). This binding between ZO-2 and YAP2 was weakened significantly when the 1st PDZ domain of ZO-2 was impaired by the 4 point mutations (Figure 3B). The binding of ZO-2 N to YAP2 was as strong as ZO-2 WT, and this binding signal was stronger than those of ZO-2 PDZm, ΔN or ΔNLS mutants, suggesting again that the 1st PDZ domain of ZO-2 associates with YAP2.

Based on the map of PDZ domain binding specificities (35) we hypothesized that the complex between YAP2 and ZO-2 is mediated mainly, if not exclusively, by the 1st PDZ domain. To test this hypothesis, we used a ΔC mutant of YAP2 lacking five C- terminal residues [14], which constitute the PDZ binding motif (Figure 3C). Flag tagged YAP2 WT, YAP2 ΔC and control vector were transfected into HEK293 cells followed by immunoprecipitation and immunoblotting. YAP2 WT bound strongly to endogenous ZO-2, while binding between YAP2 ΔC and the ZO-2 protein was barely detectable, suggesting that the PDZ binding motif in YAP2 is necessary to form complex with ZO-2 (Figure 3D). In sum, our results indicate that binding between YAP2 and ZO-2 is mediated mainly by the PDZ binding motif of YAP2 and the1st PDZ domain of ZO-2.

YAP2 localization is regulated by ZO-2

We showed that the endogenous YAP2 and ZO-2 proteins co-localize in the nucleus of several cells (Figure 1), and both ZO-2 WT and ZO-2 ΔNLS bound to YAP2, although ZO-2 WT bound to YAP2 more tightly than ZO-2 ΔNLS did (Figure 3B). To test whether ZO-2 WT and ZO-2 ΔNLS have any influence on YAP2 localization in MDCK cells. We established MDCK cells stably expressing ZO-2 WT, ZO-2 ΔNLS or control vector. ZO-2 WT was distributed in both cytosol and nucleus, but stronger signal was detected in the nuclear fraction. On the other hand, ZO-2 ΔNLS was detected only in the cytosol fraction (Figure 4A, upper panel). Interestingly, distribution of endogenous YAP in the nucleus consistently increased when ZO-2 WT was expressed and it significantly decreased when ZO-2 ΔNLS was present (see lane 2, 4, 6 in Figure 4A, middle panel). Similar result was obtained in HEK 293 cells (Figure S4). These data support our assumption that ZO-2 regulates the distribution of YAP in cells. To confirm this assumption further, GFP ZO-2 WT or GFP ZO-2 ΔNLS were individually co-transfected with pDsRed YAP2 into MDCK cells and their localizations were observed (Figure 4B, C). As we had expected, ZO-2 ΔNLS was generally excluded from the nucleus while ZO-2 WT was predominantly nuclear in MDCK cells. GFP ZO-2 WT co-localized with pDsRed YAP2 WT in the nucleus as well as in the cytosol (Figure 4B). It was reassuring to see the same locale for both GFP-tagged ZO-2 and YAP2 as the one we observed for the endogenous proteins (Figure 1). The percentage of cells in which pDsRed YAP2 WT was observed in the nucleus was more than 75% when GFP ZO-2 WT was present (Figure 4C). Whereas, GFP ZO-2 ΔNLS and pDsRed YAP2 WT co-localized predominantly in the cytosol. The percentage of cells in which pDsRed YAP2 WT was observed in the nucleus was significantly decreased to 11.2% when GFP ZO-2 ΔNLS was present (Figure 4C). These data suggest that ZO-2 could regulate the subcellular localization of YAP2.

To make sure that by generating the ZO-2 ΔNLS deletion mutant we did not affect the neighboring PDZ domain, two binding assays were conducted. Since ZO-2 forms homodimers as well as heterodimers with ZO-1 via 2nd PDZ domain [19, 40-42], we verified that both GFP ZO-2 WT and GFP ZO-2 ΔNLS were precipitated by Flag ZO-2 WT (Figure 4D). This data suggest that our ZO-2 ΔNLS construct is still capable of binding to ZO-2. Next, the binding ability to YAP2 was tested to rule out the possibility that ZO-2 ΔNLS fails to bind YAP2 because its 1st PDZ domain is not intact. ZO-2 1st PDZ is responsible for the association with YAP2 (Figure 2A). The domain consists of 85 amino acids, and in the ZO-2 ΔNLS construct the PDZ1 domain lacks 13 amino acids at the C-terminus (see orange brackets in Figure S3). Although notably weaker than WT 1st PDZ domain, GST tagged 1st PDZ ΔNLS was able to bind to YAP2 *in vitro* (Figure 4E and also Figure 3B).

To further confirm that GFP-ZO-2 functions with YAP2 in the nucleus, we used the YAP2-Runx2 transactivation assay of the osteocalcin gene, which was originally reported as the functional "read-out" of YAP [6]. We showed that the increasing amounts of GFP-ZO-2 had an

inhibitory effect on the transactivation function of YAP2-Runx2 for the osteocalcin gene luciferase reporter (Figure S5).

Decrease of endogenous ZO-2 affects YAP2 ability to promote cell detachment

We previously reported that YAP2 promotes cell detachment and apoptosis in HEK293 cells [13], and that this function was impaired when the PDZ-binding motif of YAP2 was deleted [14]. Since we detected the binding between PDZ-binding motif of YAP2 and 1st PDZ domain of ZO-2 (Figure 3), and YAP2 accumulated in the nucleus when ZO-2 was co-transfected (Figure 4), we hypothesized that ZO-2 regulated the function of YAP2 in terms of cell detachment. To investigate this assumption, we down-regulated the level of ZO-2 by RNAi in HEK293 cells and monitored changes in YAP2 induced cell detachment. The expression of YAP2 WT was induced in HEK293 cells followed by transfection of either ZO-2 specific RNAi oligo or control RNAi oligo. The efficiency of this RNAi oligo was confirmed by immunoblotting and immunostaining (Figure 5A, B), and it was able to reduce the amount of ZO-2 by more than 99%.

After 96 hours of culture in DMEM containing 1% serum, the attached cells were counted. When the control RNAi was transfected, the ratio of attached cells with induced YAP2 WT to attached cells without induced YAP2 WT was 0.495 (Figure 5A). However, when ZO-2 RNAi was transfected, the ratio was increased to 0.650 (Figure 5A). The amount of cleaved PARP fragment was decreased when endogenous ZO-2 was down-regulated (Figure 5A bottom panel, lane2 and 4), suggesting that apoptosis was repressed. In addition, the amount of YAP in the nucleus of HEK 293 cells was decreased by 37.2% when ZO-2 was removed (Figure 5C). These results indicate that the ability of YAP2 to localize in the nucleus and promote cell detachment and apoptosis in HEK 293 cells was partially impaired when the level of ZO-2 protein was diminished.

We conducted a comparable assay with MDCK cells. However, unlike HEK293 cells, the suppression of ZO-2 in MDCK was never complete in our hands but only partial, which might be a reason why ZO-2 RNAi treatment had no influence on YAP localization in MDCK cells (Figure S6).

ZO-2 protein expression enhances YAP2 ability to promote cell detachment

When endogenous ZO-2 was removed, the ability of YAP2 to promote cell detachment was decreased (Figure 5A). This result prompted us to test whether the over-expression of ZO-2 WT (as well as of several ZO-2 mutants) has any effect on the function of YAP2. YAP2 was transfected with several ZO-2 constructs into HEK293 cells. Twenty-four hours after transfection cells were trypsinized and plated at the same density (25, 000 cells per plate). After 4 days of culture the number of cells was counted (Figure 6A). The number of cells transfected with vector alone increased 16.4-fold. This number of cells in which YAP2 and the vector were transfected increased to a lesser level than control, namely: 11.6-fold, in agreement with our previous findings [13, 14]. Interestingly, the number of cells in which ZO-2 was co-transfected with YAP2 increased to even lower level: 9.4-fold. ZO-2 N, a mutant localized in the nucleus, showed a slightly stronger inhibitory effect (8.8-fold) than the WT ZO-2. Other mutants (ZO-2 PDZm, ΔN, ΔNLS) had minor effects on YAP2 function to reduce cell proliferation. This might be due to their weak association with YAP2 (Figure 3). In contrast to ZO-2, ZO-1 WT did not enhance YAP2 ability to promote cell detachment. PARP cleavage was clearly detectable, especially when ZO-2 WT or ZO-2 N was over-expressed.

To see the effects of YAP2 and ZO-2 in other cells, MDCK and MCF10A cells stably expressing YAP2 and ZO-2 were established. In contrast to HEK293 cells, YAP2 promotes cell proliferation in MDCK cells. This effect was abrogated when ZO-2 WT was co-expressed (Figure 6B). This result is consistent with previous findings which suggested that ZO-2 in some settings could act

as a tumor suppressor gene [43, 44]. ZO-2 mutants slightly diminished pro-proliferate effects of YAP2, but these results were not statistically significant ($p>0.05$). For unknown reasons, the expression of ZO-2 N was consistently weaker than that of other ZO-2 constructs in these MDCK cells (data not shown). In MCF10A cells, it was shown that YAP2 induced epithelial-to-mesenchymal transition (EMT) [45]. Therefore, we decided to see if ZO-2 had any effect on EMT in MCF10A cells. While using positive and negative controls, we reproduced the EMT in MCF10A cells, but the over-expression of ZO-2 had little or no influence on EMT in MCF10A cells (data not shown).

Our data suggest that ZO-2 cooperates with YAP2 to promote cell detachment and apoptosis in HEK293 cells. However, in MDCK cells, YAP2 promotes cell proliferation while ZO-2 prevents it. Even though YAP2 accumulates in the nucleus in the presence of ZO-2, anti-proliferative effects of ZO-2 are more dominant than pro-proliferate effects of YAP2 in MDCK cells. Identification of signaling partners downstream of YAP2 and ZO-2 complex in both cells will help explain the functional duality of YAP, which can act either as an oncogene or a pro-apoptotic factor.

Discussion

The Hpo signaling pathway was delineated as a string of signaling complexes that involve several tumor suppressors, and at least two effectors: YAP and TAZ, which display oncogene-like activity [e.g. 46, 47]. When not affected by genetic lesions, the components of this pathway control the intrinsic size of organs by regulating a balance between proliferation and cell death. Therefore, the detailed understanding of the molecular mechanisms that govern this pathway should help us better understand normal growth and its abnormal forms, including cancer.

In this report, we elucidated a new aspect of the mammalian Hpo pathway, namely the signaling by YAP2-ZO-2 complex that is mediated by the PDZ domain of ZO-2. We showed that a tight junction protein, ZO-2 uses its first PDZ domain to form a complex with YAP2. We also documented that endogenous ZO-2 and YAP2 co-localize in the nucleus and that ZO-2 regulates nuclear localization and the pro-apoptotic function of YAP2 in HEK293 cells and inhibits YAP2 induced proliferation in MDCK cells. Since the Hpo pathway is activated by cell-to-cell contacts [33], the identification of a member of the ZO family of tight junction proteins, as a partner of YAP signaling, further adds to the characterization the quickly expanding Hpo signaling network [47].

The following aspects of this study deserve further comment: (i) the role of ZO-2 among other ZO family members as the "preferred" partner of YAP2; (ii) cell-density-dependent localization of ZO-2 and YAP2 in cells; (iii) the functional implication of the absence of the PDZ domain-binding region in Drosophila YAP, yki; (iv) targeting YAP complexes with PDZ domain-containing proteins for cancer therapy; and (v) the role of YAP paralog, TAZ (WWTR1), in assembling functional complexes with ZO proteins.

We focused our study on ZO-2 as the partner of YAP2 because unlike ZO-1, ZO-2 co-localized with YAP2 in nuclei of kidney epithelial cells. Also, co-immunoprecipiations showed a robust complex between ZO-2 and YAP2 in several cell lines. In contrast to ZO-1 and ZO-2, ZO-3 has not been reported in the nucleus, where YAP2 functions as a transcriptional co-activator. We anticipate that all three ZO proteins have a propensity to form complexes with YAP2 via their first PDZ domains. Such complexes could function in specific locales, in specific cells, and under various physiological conditions. For example, ZO-2 is generally not expressed in non-epithelial cells, where ZO-1 is expressed and therefore it could "replace" the ZO-2 function.

It has been shown that at low cell density YAP is unphosphorylated and nuclear. However, when cells reach confluent state, YAP is phosphorylated and mainly cytoplasmic [33]. Interestingly, the subcellular distribution of ZO-1 and ZO-2 proteins is similar to YAP. In epithelial cells cultured in sparse conditions, ZO-1 and ZO-2 are nuclear. However, in a confluent state, both ZO-s accumulate in tight junctions [26, 27]. However, we do observe that in some cell lines, including MDCK cells, certain amount of YAP and ZO-2 stay concentrated in the nuclei, even after the cells were kept in a confluent state for extended periods of time. Nuclear phospholipids may be a contributing factor in this observation. Phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2 is known to reside in nuclear speckles of MDCK cells and a recent study has shown that ZO-2 interacts with PtdIns(4,5)P_2 via its second PDZ domain [48]. Moreover, in that study ZO-2 was shown to co-localize with nuclear (PtdIns(4,5)P_2. The possibility of ZO-2 forming a tripartite complex with YAP and PtdIns(4,5)P_2 and the regulatory role of PtdIns(4,5)P_2 on the stability or function of the nuclear YAP-ZO-2 complex remains the subject of further study. Apparently, those cells that harbor a mutation in *Merlin*, the gene that encodes one of the cytoplasmic and possibly also nuclear component [49] of the Hpo pathway, have YAP localized in the nucleus even in high-density cultures [50]. To see if *Merlin* is mutated in our stock of MDCK cells, we amplified its cDNA and performed sequence analysis. The analyzed sequence of Merlin was identical to that of the wt dog Merlin cDNA (Gaffney, C., Oka, T and Sudol, M - unpublished data). It is possible that a mutation in other upstream components of the Hpo pathway could be responsible for enhanced and cell density-independent nuclear localization of YAP in MDCK cells.

In the case of Drosophila YAP, yki, the C-terminal sequence does not contain a known PDZ-binding sequence and there is no obvious internal PDZ ligand motif either [14]. Therefore, the signaling by the mammalian YAP in the Hpo pathway differs in that particular aspect from the signaling by yki in the fly. Moreover, the fly seems to be an exception, as the PDZ-binding motif is well conserved in YAP orthologs throughout the evolution of animal kingdom. This is true even in simple Hydra, whose divergence is estimated to have occurred circa 1 billion years ago (Figure S7) [14]. We suggest that Drosophila YAP, yki, must interact with other partner(s) that acts similarly to mammalian ZO-2 protein, regulating the nuclear transport of yki and sensing cell-to-cell contacts.

The loss of main components of the Hpo pathway leads to uncontrolled cell growth, which confirms that this is a tumor suppressor pathway. YAP, as the effector of the pathway, was proposed to act as both, an oncogene [51] or tumor suppressor [52]. The study of YAP expression in common solid tumors revealed relative changes in the subcellular localization of YAP in cancer cells, compared to the normal controls [53]. Increases in both, cytoplasmic and nuclear localization of YAP protein was generally observed in these tumors. Since YAP is a nuclear effector of the Hpo pathway [7, 13], controlling its translocation to the nucleus by interfering with the PDZ domain-mediated complex(es) through small molecular inhibitors, one should be able to control growth of cancer cells. This approach could be feasible since the -TWL sequence is present in the C-termini of only three human proteins, and the C-terminus of YAP was not found to bind to other PDZ domains. Apart from YAP, and its close paralog TAZ, the –TWL end is also present in a mitochondrial ribosomal protein MRPL43, suggesting that peptides or mimetics of this sequence would be specific as drugs, with possible minor side effects, as such drugs would not significantly affect signaling by other PDZ domain complexes.

Two comments are appropriate here. Because regulatory interactions are mostly cooperative, and regulatory decisions in signaling are generally made by large multi-component complexes and not

by single proteins, or simple pair of proteins [54], we have to consider that there could be other PDZ domain-containing proteins, outside of the ZO family, which could also interact with YAP2 and contribute to the global regulation of YAP2 nuclear transport. Such regulations are detectable by signaling studies using sensitive "read-outs", as shown by our analyses, but could not always be easily delineated by genetic studies because of the inherent redundancy.

In a separate study, we most recently documented that YAP paralog, TAZ (WWTR1) also interacts with ZO-2 via PDZ domain, co-localizes in the nucleus in various cells and affects transcription of a TAZ-regulated gene [55].

Our work implicates PDZ domain and PDZ domain-containing protein ZO-2 in the mechanism of nuclear translocation of YAP protein, one of the main effectors of the Hippo tumor suppressor pathway.

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Figure legends

Figure 1 Endogenous ZO-2 co-localizes with endogenous YAP in MDCK cells

(A) Endogenous ZO-1, ZO-2 and YAP2 in MDCK cells were immunostained and observed by a microscope. Size bar = 20μm.

(B) Endogenous ZO-2 and YAP2 in indicated cells were immunostained and observed by a microscope. Size bar = 20μm.

(C) ZO-1, ZO-2 and YAP2 form a complex. Flag ZO-1 WT, pcDNA4/TO/myc-His ZO-2 WT, and pcDNA4/TO/myc-His YAP2 WT were co-transfected into HEK293 cells. Cell lysates were

immunoprecipitated with Flag antibody, resolved on SDS gels and immunoblotted with indicated antibodies. Molecular masses are indicated in kDa.
(D) Endogenous ZO-2 binds to endogenous YAP in MDCK cells. MDCK cells were maintained in two different conditions. One was very sparsely distributed and the other was very dense. To keep the cells healthy, the medium was exchanged every 24 hours for 5 days after the cells had reached confluent. Same amount of MDCK cell lysates were used for immunoprecipitation. The cell lysates were immunoprecipitated with either control IgG or YAP antibody, resolved on SDS gels and immunoblotted with ZO-2 or YAP antibody. Molecular masses are indicated in kDa.
(E) Endogenous ZO-2 binds to endogenous YAP in both cytosol and nucleus of MDCK cells. Cytosol and nuclear fractions of MDCK cells were separated by using CelLytic NuCLEAR Extraction Kit (Sigma). These fractions were immunoprecipitated with either control IgG or YAP antibody, resolved on SDS gels, and immunoblotted with indicated antibodies. Molecular masses are indicated in kDa.
(F) Both YAP and ZO-2 were slightly increased in the nucleus when MDCK cells were sparsely distributed. MDCK cells were maintained as described in D and cytosol and nuclear fractions were separated as described in E. 50μg of extracts was run on each lane, followed by immunoblotting with indicated antibodies to monitor the distribution of endogenous proteins. Molecular masses are indicated in kDa.

Figure 2 1st PDZ domains of ZO proteins are necessary to bind to YAP2
(A) ZO-2 1st PDZ domain binds to YAP2.
Three PDZ domains of mouse ZO-2 were individually fused to GST and purified with Glutathione Sepharose 4B followed by the incubation with purified Flag YAP2 WT. Bound proteins were separated by SDS-PAGE followed by immunoblotting or Coomassie blue staining. Molecular masses are indicated in kDa.
(B) Kd for GST-PDZ domain 1 of ZO1 and ZO2 with human YAP peptide KLDKESFLTWL. Purified GST fusion proteins were incubated with YAP synthetic peptides to determine Kd of interaction. Microcalorimetric titration measurements were performed in a Microcal Omega isothermal titration calorimeter (Microcal, Northampton, MA, USA). All solutions were degassed under vacuum prior to use. In a typical experiment, 1.33ml of 15 μM PDZ1 ZO-2 in 20mM HEPES, 150mM NaCl, pH 7.5 was titrated by twenty 15μl injections of 150μM YAP peptide. During titration, the injection syringe was rotated at 250rpm. Time between injections was 5 minutes. In a blank experiment, heat evolved from dilution was measured by injecting the peptide solution into the sample cell filled with buffer only. This heat of dilution was subtracted from the peptide binding data for the PDZ1 domain. Data were integrated and fitted to an appropriate binding model using the ORIGIN software supplied by Microcal.

Figure 3 Binding between ZO-2 and YAP2
(A) Schematic structure of ZO-2 protein and its mutants. ZO-2 has three PDZ domains, one SH3 domain and one guanylate kinase-like (GK) domain. It also contains two putative NLS (106a.a. ~122a.a. and 271a.a. ~287a.a.) and two NES (377a.a. ~386a.a. and 744a.a. ~754a.a.). Point mutations (K38E, F44H, I46E, V48E) were introduced into the 1st PDZ domain of ZO-2 and the mutant was named ZO-2 PDZm. ZO-2 ΔNLS lacks both NLS, while ZO-2 N has both NLS but lacks both NES and all the downstream domains.
(B) The 1st PDZ domain of ZO-2 associates with YAP2. Flag tagged ZO-2 WT, PDZm, ΔN, ΔNLS and N were individually transfected with YAP2 WT into HEK293 cells. Cell lysates were immunoprecipitated with either control IgG (left) or YAP antibody, resolved on SDS gels, and immunoblotted with Flag or YAP antibody. Molecular masses are indicated in kDa.
(C) Schematic structure of YAP2 protein and its ΔC mutant. YAP2 has two WW domains in its central region and a PDZ-binding motif at its C-terminal end. The last 5 a. a. (-FLTWL) are

truncated in ΔC mutant. TAD stands for transcription activation domain. S127 is a phosphorylation site for Lats1 and Akt kinases.
(D) The PDZ-binding motif of YAP2 is indispensable for the binding to ZO-2. Flag tagged cDNAs were transfected into HEK293 cells. Cell lysates were immunoprecipitated with Flag antibody, resolved on SDS gels and immunoblotted with ZO-2 or YAP antibody. Molecular masses are indicated in kDa.

Figure 4 Co-localization of YAP2 and ZO-2
(A) YAP2 accumulates in the nucleus in the presence of ZO-2 WT. Cytosol and nuclear fractions of MDCK cells stably expressing Flag ZO-2 WT, Flag ZO-2 ΔNLS or control vector were separated by using CelLytic NuCLEAR Extraction Kit (Sigma), followed by immunoblotting with indicated antibodies. Molecular masses are indicated in kDa.
(B) Effects of ZO-2 on the localization of YAP2. pDsRed-YAP2 WT was co-transfected with GFP-ZO-2 WT (upper panels) or GFP-ZO-2 ΔNLS (lower panels) in MDCK cells. The localization of each protein was observed. The nucleus was stained with DAPI. Size bar = 20μm.
(C) Graphic representation of results shown in (B). The ratios of nuclear localization of pDsRed-YAP2 WT in the presence of GFP-ZO-2 WT or GFP-ZO-2 ΔNLS are indicated. If the red or green signals were overlapped with blue signal by DAPI, the protein was defined as "localized in the nucleus". Cells expressing both GFP and pDsRed constructs were counted, and cells expressing only either of them were eliminated from this evaluation. This was repeated 3 times independently and ca. 100 cells were observed in each case.
(D) ZO-2 ΔNLS doesn't lose the ability to form a homodimer. Indicated plasmids were co-transfected into HEK293 cells. Cell lysates were immunoprecipitated with Flag antibodies, resolved on SDS-PAGE, and immunoblotted with GFP antibody (upper panel). The middle and lower panels show the expression of transfected proteins. Molecular masses are indicated in kDa.
(E) ZO-2 ΔNLS doesn't lose the ability to bind to YAP2. Indicated portions of human ZO-2 were individually fused to GST. GST 1st PDZ WT contains 1-303 amino acids of human ZO-2. GST 1st PDZ ΔNLS contains 1-104 and 292-303 amino acids and GST 3rd PDZ has 501-600 amino acids, respectively. GST-tagged proteins were purified with Glutathione Sepharose 4B followed by the incubation with purified Flag YAP2 by using anti-Flag M2 affinity gel (Sigma) and 3x Flag peptide. Bound proteins were separated by SDS-PAGE followed by immunoblotting or Coomassie blue staining. Molecular masses are indicated in kDa.

Figure 5 Removal of ZO-2 weakens pro-apoptotic function of YAP2
(A) Indicated RNAi oligos were transfected into HEK293 cells and YAP2 WT was induced by Tetracycline. The cells were cultured in DMEM containing 1% FBS for 96 hours. After the removal of detached cells, attached cells were trypsinized and their numbers were counted. "Number of cells in which YAP2 WT was induced for 96 hours / number of cells in which YAP2 WT was not induced during 96 hour culture" was defined as "Relative changes in cell numbers". The ratio of number of YAP2 WT induced cells to number of non-induced cells in each case is shown. Expression of YAP2, ZO-2, ZO-1, GAPDH and PARP were monitored. Molecular masses are indicated in kDa.
(B) Repression of ZO-2 in HEK 293 cells after RNAi treatment was confirmed by immunostaining. Endogenous ZO-2 was immunostained and observed by a microscope. Size bar = 20μm.
(C) The amount of endogenous YAP in the nucleus of HEK 293 cells was decreased when ZO-2 was downregulated. Cytosol and nuclear fractions of HEK 293 cells were separated by using CelLytic NuCLEAR Extraction Kit (Sigma), followed by immunoblotting with indicated antibodies. Molecular masses are indicated in kDa.

Figure 6 ZO-2 inhibits cell proliferation

(A) ZO-2 enhances pro-apoptotic function of YAP2. HEK293 cells were transfected with indicated plasmids prior to distribution into new plates. The cells were maintained in DMEM with 1% FBS for 96 hours and cell numbers were counted.
"Number of cells after 96 hour culture / number of cells when they were originally plated on the tissue culture dish" was defined as "Growth Rate". Protein expression was monitored by immunoblotting with indicated antibodies. Molecular masses are indicated in kDa.
(B) YAP2 induces cell proliferation in MDCK cells and ZO-2 prevents it. MDCK cells stably expressing indicated plasmids were maintained in DMEM with 0.5% FBS for 96 hours. Growth rate and protein expression were analyzed as described in A. Molecular masses are indicated in kDa.

Figure 1

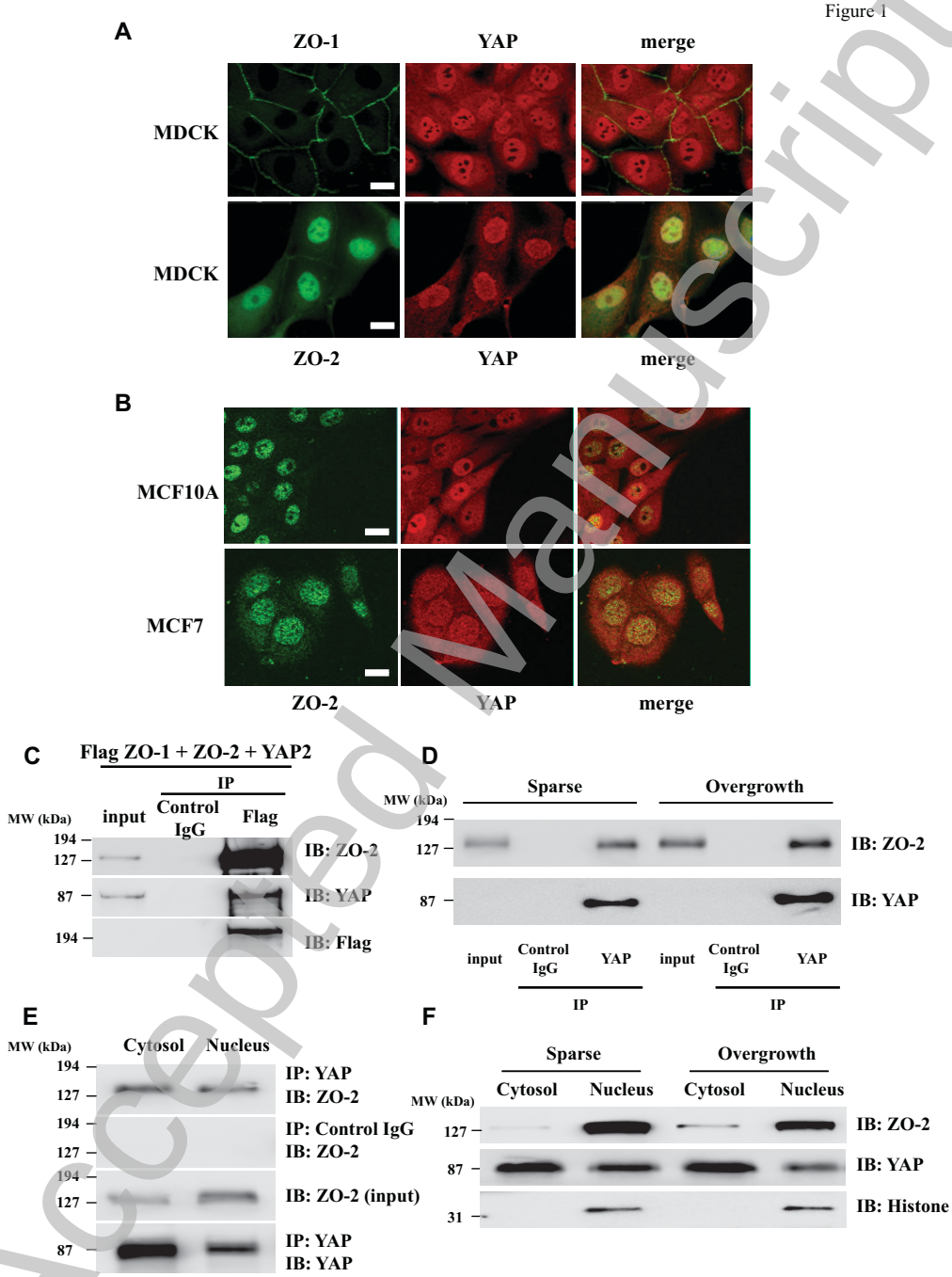

Figure 2

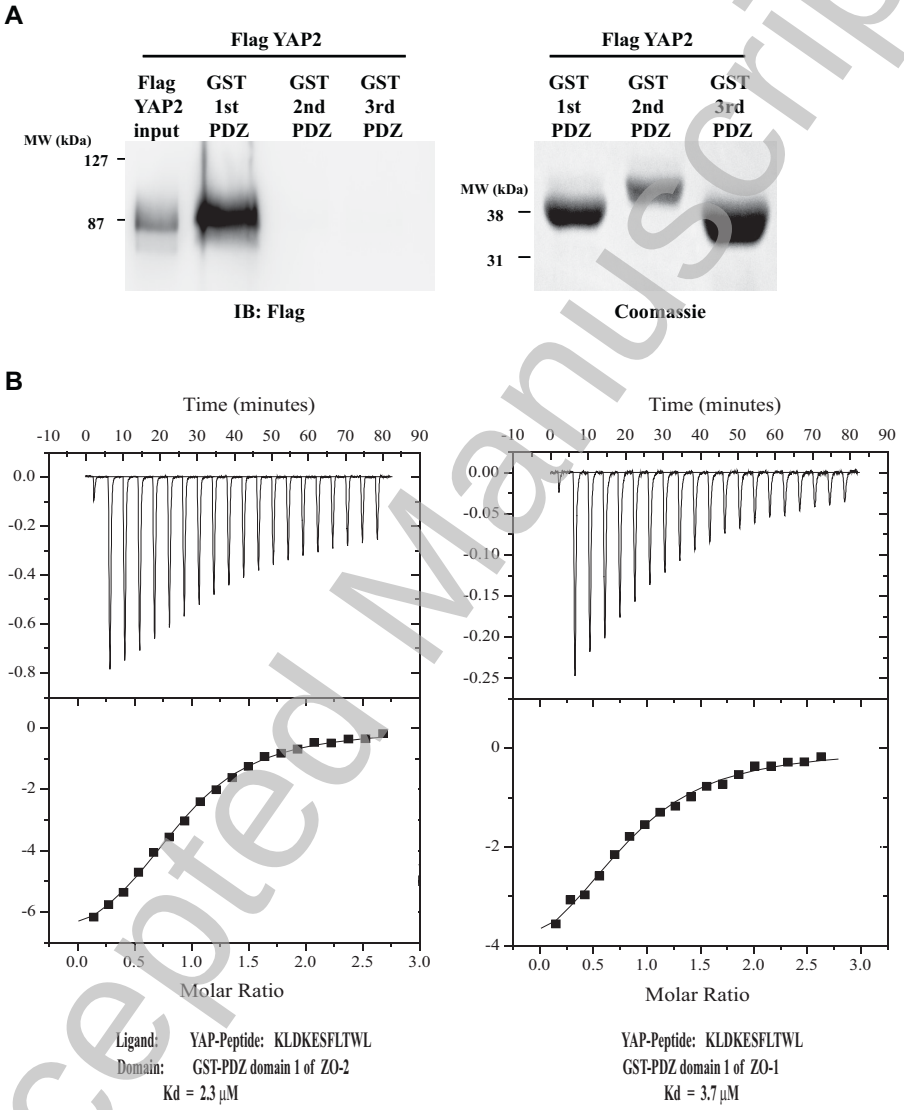

Figure 3

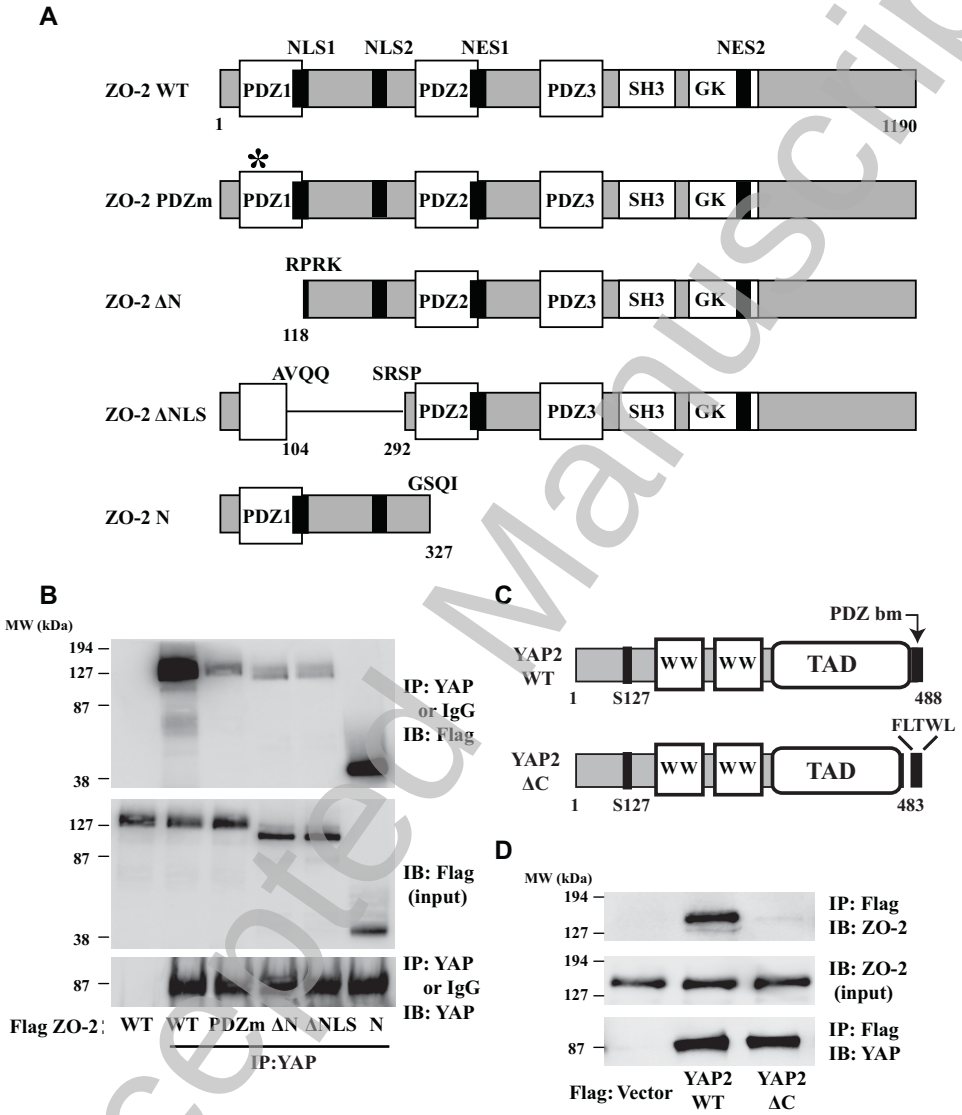

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Figure 4

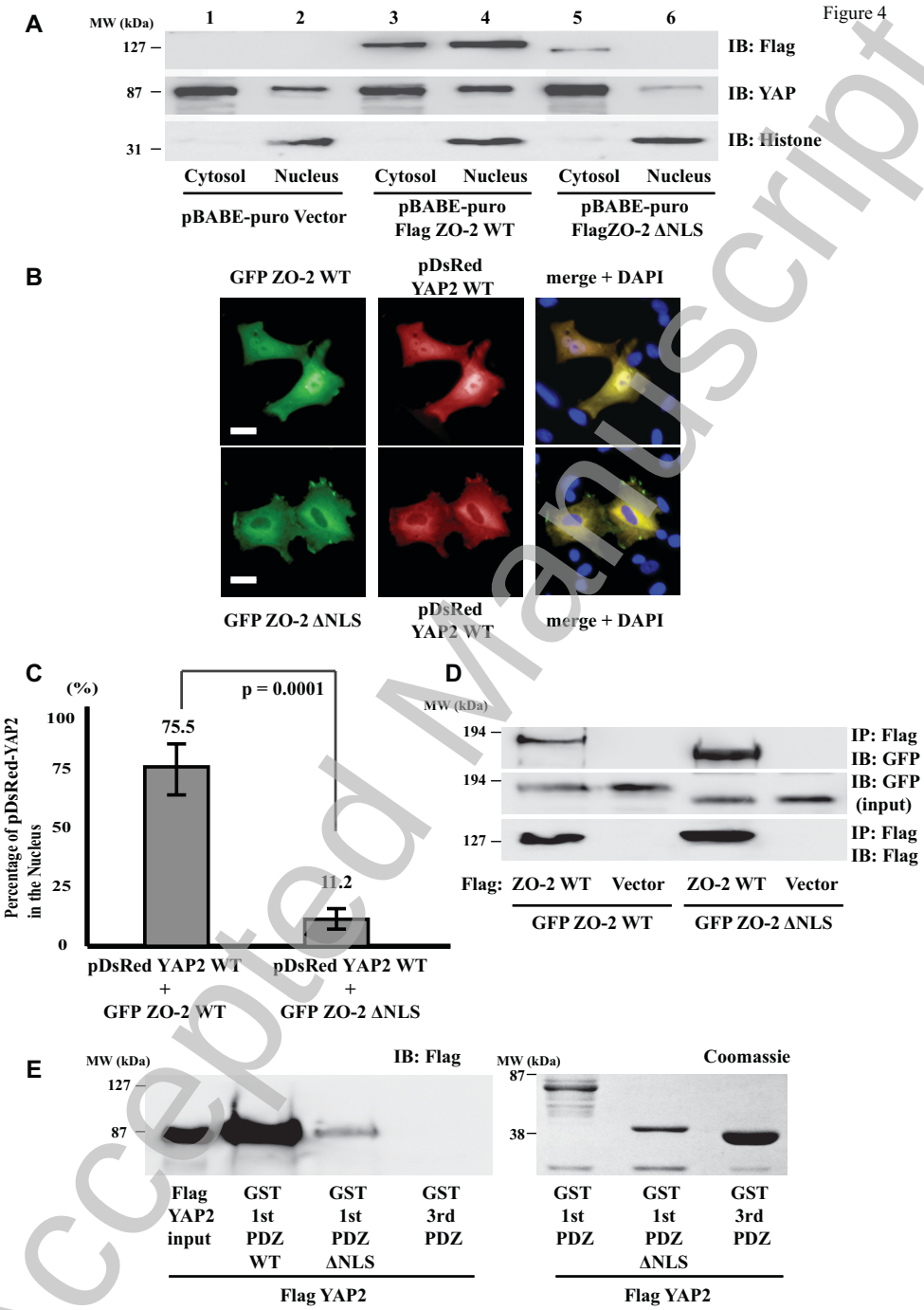

Figure 5

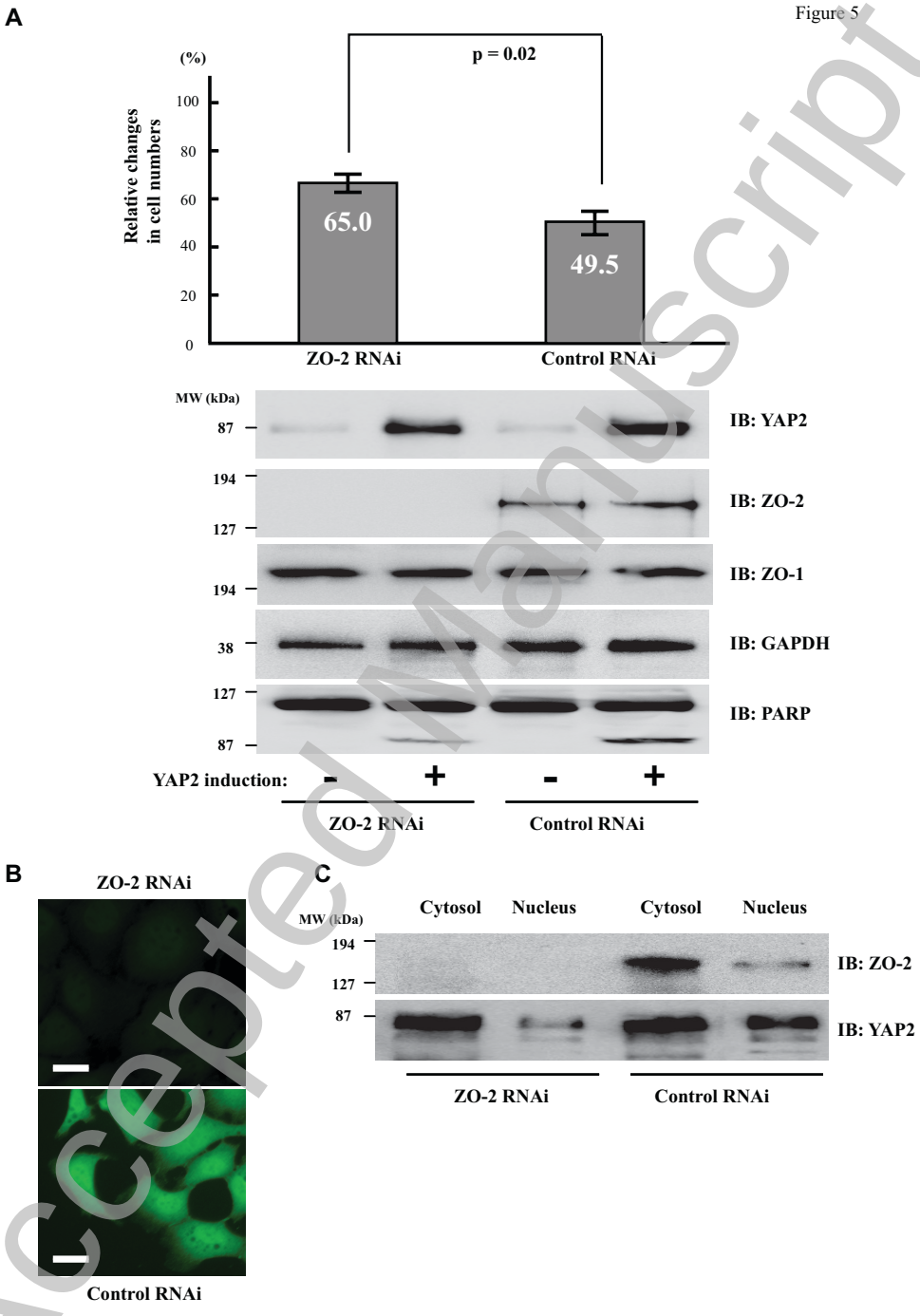

Figure 6