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The Heat-Shock Protein Apg-2 Binds to the Tight Junction Protein ZO-1 and Regulates Transcriptional Activity of ZONAB

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The tight junction adaptor protein ZO-1 regulates intracellular signaling and cell proliferation. Its Src homology 3 (SH3) domain is required for the regulation of proliferation and binds to the Y-box transcription factor *ZO*-1-associated *n*ucleic *a*cid *b*inding protein (ZONAB). Binding of ZO-1 to ZONAB results in cytoplasmic sequestration and hence inhibition of ZONAB's transcriptional activity. Here, we identify a new binding partner of the SH3 domain that modulates ZO-1–ZONAB signaling. Expression screening of a cDNA library with a fusion protein containing the SH3 domain yielded a cDNA coding for Apg-2, a member of the heat-shock protein 110 (Hsp 110) subfamily of Hsp70 heat-shock proteins, which is overexpressed in carcinomas. Regulated depletion of Apg-2 in Madin-Darby canine kidney cells inhibits G_1/S phase progression. Apg-2 coimmunoprecipitates with ZO-1 and partially localizes to intercellular junctions. Junctional recruitment and coimmunoprecipitation with ZO-1 are stimulated by heat shock. Apg-2 competes with ZONAB for binding to the SH3 domain in vitro and regulates ZONAB's transcriptional activity in reporter gene assays. Our data hence support a model in which Apg-2 regulates ZONAB function by competing for binding to the SH3 domain of ZO-1 and suggest that Apg-2 functions as a regulator of ZO-1–ZONAB signaling in epithelial cells in response to cellular stress.

INTRODUCTION

Tight junctions (TJs) constitute the most apical intercellular junction in epithelial cells. They regulate selective paracellular diffusion and restrict the intermixing of apical and basolateral membrane components (Cereijido *et al*., 2000; Tsukita *et al*., 2001; Anderson *et al*., 2004). TJs have also been linked to the regulation of epithelial proliferation, polarization, and differentiation (Zahraoui *et al*., 2000; Ohno, 2001; Balda and Matter, 2003). TJs consist of complex protein networks containing different types of transmembrane proteins linked to multiple adaptor proteins that interact with each other as well as the actin cytoskeleton (D'Atri and Citi, 2002; Gonzalez-Mariscal *et al*., 2003). This cytoskeleton-linked scaffold recruits different types of signaling proteins, which regulate junction assembly and transmit signals from the junction to the cell interior (Matter and Balda, 2003).

ZO-1, the first identified TJ protein, is a member of the membrane-associated guanylate kinases and contains three PDZ domains, one Src homology 3 (SH3) domain, a domain homologous to yeast guanylate kinase as well as a large C-terminal domain that binds actin filaments (Stevenson *et al*., 1986; Fanning and Anderson, 1999; Tsukita *et al*., 2001). These domains engage in protein–protein interactions with multiple junctional components, including membrane proteins, other adaptors, F-actin, and signaling proteins. One of the interacting signaling proteins is ZONAB, a Y-box transcription factor that binds to the SH3 domain of ZO-1 (Balda and Matter, 2000).

Y-box transcription factors are multifunctional proteins that can bind DNA as well as RNA and regulate transcription as well as translation (Matsumoto and Wolffe, 1998; Kohno *et al*., 2003). *ZO*-1-associated *n*ucleic *a*cid *b*inding protein (ZONAB) is the canine homologue of human DbpA (Sakura *et al*., 1988). ZONAB/DbpA has been linked to the regulation of transcription of the erbB-2 proto-oncogene as well as to mRNA stability (Balda and Matter, 2000; Coles *et al*., 2004). In epithelial cells, ZONAB regulates cell proliferation and gene expression in a cell density-dependent manner because of its interaction with ZO-1(Balda and Matter, 2000; Balda *et al*., 2003). ZONAB localizes to the nucleus and forming junctions in proliferating cells and becomes restricted to the cytoplasm in mature monolayers when the ZO-1 concentration has reached its maximum and proliferation ceases. ZONAB regulates G_1/S phase progression and interacts with the cell division kinase CDK4, resulting in codistribution of the protein kinase and ZONAB (Balda *et al*., 2003). Thus, inhibition of nuclear accumulation of ZONAB by ZO-1 also reduces the nuclear pool of CDK4, which contributes to the inhibition of G_1/S phase progression by up-regulation of ZO-1 expression.

Although ZONAB is transcriptionally active during proliferation and becomes inhibited by binding to ZO-1 or RalA when cells reach confluence (Balda and Matter, 2000; Frankel *et al*., 2005), little is known about stimuli that activate ZONAB. Here, we identified the heat-shock protein Apg-2 as a new binding partner of ZO-1. Apg-2, a member of the Hsp110 family of heat-shock proteins, is overexpressed in carcinomas and is therefore thought to play a role

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Abbreviations used: Hsp, heat-shock protein; TJ, tight junction; ZONAB, *ZO*-1-associated *n*ucleic *a*cid *b*inding protein.

during proliferation (Kaneko *et al*., 1997; Nakatsura *et al*., 2001; Hayashi *et al*., 2002; Gotoh *et al*., 2004). Heat-shock proteins function in protein folding as well as regulation of intracellular signaling mechanisms, and a single type of heat-shock protein can regulate various processes at different subcellular locations (Picard, 2004). By combining in vitro binding assays with functional assays in Madin-Darby canine kidney (MDCK) cells, we found that the interaction of Apg-2 with ZO-1 is stimulated by heat shock and that Apg-2 functions as an activator of ZONAB by competing for binding to the SH3 domain of ZO-1.

MATERIALS AND METHODS

Isolation of Apg-2, RNA Interference (RNAi) and cDNA Constructs

For the isolation of Apg-2, a glutathione *S*-transferase (GST) fusion protein containing the third PDZ and the SH3 domain of ZO-1 was biotinylated and then used to probe an MDCK expression library (Cicchetti and Baltimore, 1995; Balda and Matter, 2000). A single clone was isolated corresponding to almost the entire mRNA of Apg-2. The 5′ end was cloned by 5′-rapid amplification of cDNA ends (RACE) (Balda and Matter, 2000). The cDNA coding for the full-length protein was assembled in pcDNA4/TO (Invitrogen, Paisley, United Kingdom) without or with a vesicular stomatitis virus (VSV) tag at the C terminus. The same vector was used to express a cDNA resistant to the *z*2 RNAi plasmid (see below), which had been generated by converting the targeted sequence 5′-AAGTTCTGGCCACTGCATTTG-3′ to 5′-AAGTCTTAGCAACGGCCTTTG-3′ by the introduction of silent mutations.

Histidine $(His)_6$-tagged Apg-2 fusion proteins were generated with pRSET-A by inserting sequences coding for residues 43–840 (Apg-2), 1–419 (ATPase domain), 393–636 (peptide binding domain; PBD), 620–840 (C-terminal domain; CTD). The His_6-tagged ATPase domain of heat-shock cognate 70 (Hsc70) was also produced with pRSET-A. The cDNAs encoding the SH3 domains of ZO-2 (amino acid residues 586–665) and ZO-3 (amino acid residues 464–548) were amplified by PCR from an MDCK expression library and cloned into pGEX-4T-3. ZO-1, ZONAB, and other SH3 domain fusion proteins were generated as described previously (Balda *et al*., 1996a; Balda and Matter, 2000).

The mU6pro-T vector, which contains a modified U6 promoter with a tetracycline operator at its 3′ end, was used for the expression of Apg-2-directed RNA hairpins (Yu *et al*., 2002; Balda *et al*., 2003; Aijaz *et al*., 2005). The sequences z2, 5′-AAGTTCTGGCCACTGCATTTG-3′, and z5, 5′-AAACAAGGAGGACCAGTATGA-3′, of Apg-2 were targeted. Vectors for the constitutive depletion of ZONAB and their use in reporter assays were described previously (Balda *et al*., 2003; Frankel *et al*., 2005). For regulated depletion and expression, MDCK II cells were cotransfected with the above-described plasmids and pcDNA6/TR (Invitrogen) using the calcium phosphate method as described previously (Matter *et al*., 1992).

Antibodies, Immunoprecipitation, and Pull-Down Assays

Rabbit antibodies against Apg-2 were raised against a C-terminal, NH_2-PSDSDKKLPEMDID-COOH, and an N-terminal, NH_2-MSVVGIDLGFQSC-COOH, peptide. In guinea pigs, a recombinant GST fusion protein containing residues 764–840 of Apg-2 was used as antigen. Sera were affinity purified using the respective antigens. ZO-1 and ZONAB antibodies were described previously (Anderson *et al*., 1988; Balda and Matter, 2000; Benais-Pont *et al*., 2003). Antibody P5D4 was used for the VSV-epitope and 1A2 for α-tubulin (Kreis, 1987). Mouse anti-Hsp70 (SPA-810) and rat anti-Hsc70 (SPA-815) were obtained from Stressgen (San Diego, CA). The mouse anti-His_6-tag antibody was from Sigma-Aldrich (Dorset, United Kingdom).

For immunoprecipitations, MDCK cells were extracted with 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, and a cocktail of protease and phosphatase inhibitors (Gumbiner *et al*., 1991; Balda *et al*., 1993). One 14-cm tissue culture plate was used per immunoprecipitate, and 25% of final immunoprecipitates were loaded per gel. ZO-1 was immunoprecipitated with R40.76 bound to protein G-Sepharose (Anderson *et al*., 1988; Balda and Matter, 2000). For immunoblots of total cell extracts, cells were directly lysed in SDS-PAGE sample buffer. For pull-down assays, His_6-tagged fusion proteins in phosphate-buffered saline (PBS) containing 1% Triton X-100, 1 mM dithiothreitol and a cocktail of protease inhibitors were preabsorbed with inactive beads for 15 min and then incubated with glutathione-Sepharose beads coated with equal amounts of GST or the indicated GST fusion proteins for 2 h at 4°C. For the competition experiment, equal amounts of His_6-ZONAB A were incubated with glutathione-Sepharose beads coated with either GST or GST-SH3 for 30 min at 4°C. Increasing amounts of His_6-ATPase were then added, and the mixes were incubated for an additional 2 h at 4°C. For all immunoblots, the ECL detection system (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) was used and images were acquired with a Fuji Las-1000 imager.

Immunofluorescence

Cells grown on coverslips were fixed in methanol (5 min at −20°C) with or without a preextraction of 1 min on ice with 0.1% Triton X-100 in 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 200 mM sucrose, 10 mM HEPES, pH 7.1 (Balda *et al*., 1996b). After rehydration with PBS, the samples were blocked and processed for immunofluorescence as described using fluorescein isothiocyanate (FITC)-, Cy3-, and Cy5-conjugated secondary antibodies generated in donkeys (Jackson ImmunoResearch Laboratories, West Grove, PA) (Balda *et al*., 1996b). Confocal images were obtained with a Zeiss LSM510, and epifluorescence images were obtained with a Leica DM1 RB microscope equipped with a Hamamatsu ORCA285 camera. In both cases, 63×/1.4 oil immersion objectives were used. The Zeiss image acquisition software was used for the confocal images and the Hamamatsu interface to acquire epifluorescence images with Adobe Photoshop (Adobe Systems, Mountain View, CA). Mean intensities were measured with the Adobe Photoshop Histogram function over areas that were kept at a constant size. Five nuclear and five cytoplasmic areas were measured to determine the nuclear-to-cytoplasmic ratio for each image, and 10 images were analyzed for each condition. Image brightness and contrast were adjusted with Adobe Photoshop.

Report Gene Assay, Bromodeoxyuridine Incorporation, and Apoptosis Assay

A promoter pair that differs only by the presence of a ZONAB binding site was used for dual luciferase reporter assays to measure ZONAB activation. A promoter with a ZONAB binding site was used to drive firefly luciferase expression and a promoter with an inactivated binding site but otherwise identical sequence was used to express *Renilla* luciferase (Frankel *et al*., 2005). The plasmids were cotransfected by calcium phosphate together with the indicated expression and RNAi vectors (Balda and Matter, 2000; Frankel *et al*., 2005). Ratios of the two luciferase activities were then calculated and compared between the different samples. For the heat-hock experiment, the cells were incubated for 2 h at 43°C and then transferred back to 37°C for an additional 2 h after which the luciferase assay was performed. Because heat shock resulted in complete inactivation of luciferase, this protocol allowed us to measure luciferase synthesized in response to stress.

Bromodeoxyuridine incorporation was used to quantify entry into S phase. Cells were synchronized by serum starvation in 0.1% fetal calf serum (FCS)-containing medium (Balda *et al*., 2003). Entry into S phase was then stimulated with medium containing 5% FCS and bromodeoxyuridine before fixation and staining with anti-bromodeoxyuridine antibody (Balda *et al*., 2003). Apoptosis was determined by fluorescent detection of active caspase-3 using the CaspaseACE FITC-VAD-FMK in situ marker (Promega, Southampton, United Kingdom) as described previously (Balda *et al*., 2003).

RESULTS

Identification of Apg-2 as a ZO-1 Interacting Protein

The SH3 domain of ZO-1 is crucial for the function of ZO-1 in the regulation of epithelial gene expression via the transcription factor ZONAB (Balda *et al*., 2003). To search for proteins that regulate these activities, we screened an MDCK expression library with a biotinylated GST fusion protein containing the third PDZ and the SH3 domain of ZO-1. This resulted in the isolation of a clone that contained almost the entire open reading frame of Apg-2, a member of the Hsp110 family (Kaneko *et al*., 1997; Nonoguchi *et al*., 1999; Yagita *et al*., 1999), coding for amino acids 43 to the C terminus (Figure 1A). Cloning of the 5′ end of the mRNA using a 5′-RACE protocol confirmed the high conservation of the Apg-2 N terminus. Overall, canine Apg-2 is >94% identical to the human and mouse proteins and 80% identical to *Xenopus* Apg-2.

Apg-2 has the typical Hsp70-homologous ATPase and peptide binding domains followed by a unique C-terminal domain (Figure 1A). We generated two polyclonal antibodies against the C-terminal domain: a rabbit peptide antibody against the last 14 residues of the protein and a guinea pig antibody against a recombinant protein containing a larger C-terminal fragment (residues 764–840). In immunoblots of total MDCK cell extracts, the rabbit anti-peptide antibody recognized a band of ~116 kDa that comigrated with exogenously expressed VSV-tagged canine Apg-2 (Figure 1B).

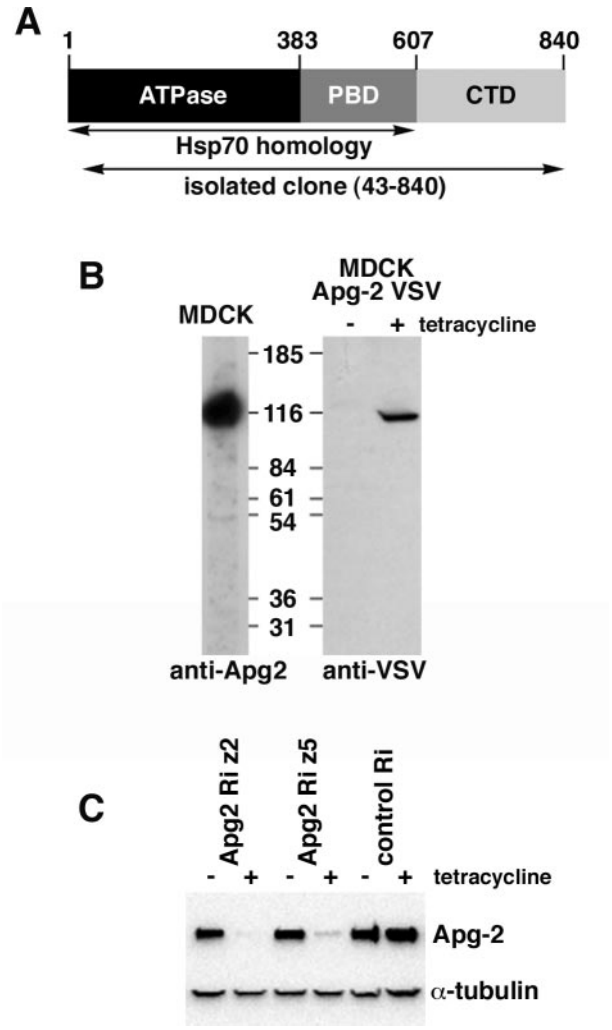

Figure 1. Domain structure and expression of Apg-2. (A) Domain structure of Apg-2. The ATPase, peptide binding (PBD), and C-terminal (CTD) domains are indicated (canine Apg-2: GenBank/EMBL/DDBJ accession no. AY911512). (B) Expression of Apg-2. For detection of endogenous Apg-2, total MDCK cell extracts were immunoblotted with a rabbit antibody against the C terminus of Apg-2. Regulated expression of full-length Apg-2-VSV was analyzed by immunoblotting with an anti-VSV antibody. (C) Regulated depletion of Apg-2. MDCK cells stably transfected with the Apg-2-directed regulated RNAi plasmids (z2 and z5) and a control RNAi clone were plated without or with tetracycline. After 3 d, the cells were harvested in SDS gel sample buffer, and expression of Apg-2 and α-tubulin was analyzed by immunoblotting.

The same result was obtained with the guinea pig antibody (our unpublished data).

We next generated cell lines permitting the tetracycline-regulated depletion of Apg-2 by RNA interference. We targeted two different sequences of the Apg-2 mRNA using a previously described vector with a modified U6 promoter containing a tetracycline operator (Aijaz *et al*., 2005). Figure 1C shows that induction of both RNA duplexes resulted in efficient depletion of Apg-2, whereas expression of a control RNA duplex did not affect the expression levels of the Hsp. Expression of ZO-1 was not affected by depletion of Apg-2 (our unpublished data). These depletion results support the specificity of the anti-Apg-2 antibodies.

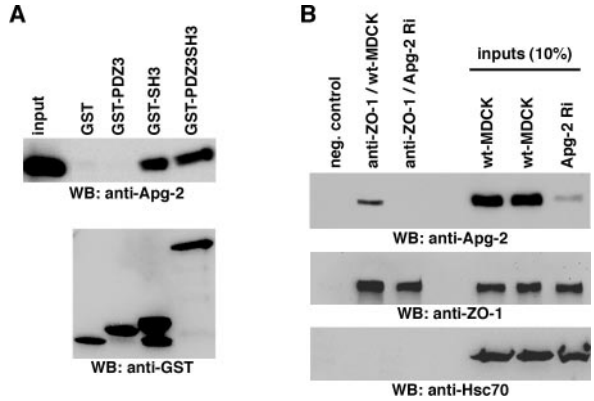

Figure 2. In vitro and in vivo interaction of Apg-2 with ZO-1. (A) Apg-2 binds to the SH3 domain of ZO-1 in vitro. Glutathione beads carrying equal amounts of GST fusion proteins of PDZ3SH3, PDZ3, and SH3 domains of ZO-1 or GST were incubated with His_6-Apg-2. Pull-downs were probed with anti-Apg-2 (C terminus) and anti-GST antibodies. (B) In vivo association of Apg-2 and ZO-1. Wild-type MDCK cell extracts were immunoprecipitated with a control antibody (neg. control) or anti-ZO-1 antibody (anti-ZO-1/wt-MDCK). As an additional control, ZO-1 was also precipitated from extracts of tetracycline-treated Apg-2 Ri z2 cells (anti-ZO-1/Apg-2 Ri). The precipitates and total cell extracts (inputs) were analyzed by immunoblotting using antibodies against Apg-2, ZO-1, and Hsc70.

To confirm the expression screening result, we performed pull-down assays using GST ZO-1 fusion proteins and His_6-tagged Apg-2. Because the ZO-1 fusion protein used for the isolation of Apg-2 contained two different domains, the third PDZ (PDZ3) and the SH3 domain, fusion proteins containing either one or both of these domains were generated. Figure 2A shows that the GST fusion protein containing PDZ3 and the SH3 domain efficiently precipitated His_6-Apg2, confirming that Apg-2 is able to interact directly with ZO-1. The SH3 domain alone was sufficient to precipitate recombinant Apg-2, whereas the PDZ domain was not, indicating that the SH3 domain of ZO-1 alone can bind Apg-2 in vitro.

We next investigated whether Apg-2 can interact with ZO-1 in vivo. MDCK cell extracts were immunoprecipitated with anti-ZO-1 or control antibodies, and the presence of Apg-2 in the immunoprecipitates was monitored by immunoblotting. Apg-2 was specifically detected in the immunoprecipitates of ZO-1 from wild-type cell extracts but not control precipitates or ZO-1 precipitates from Apg-2-depleted cell extracts (Figure 2B). Hsc70, an Hsp70 family member expressed at high levels in the cytosol, was not detected in the immunoprecipitates. This indicates that Apg-2 can indeed associate with ZO-1 in vivo. However, only low levels of Apg-2 seem to be associated with ZO-1 under normal conditions.

Apg-2 has chaperone activity; thus, it is possible that it binds to partially unfolded SH3 domain (Gotoh *et al*., 2004). We therefore mapped the interacting domain in Apg-2 by repeating the pull-down assays with His_6-tagged recombinant proteins corresponding to the three main domains of Apg-2 (Figure 1A). Figure 3A shows that only a recombinant protein containing the ATPase domain of the heat-shock protein was precipitated by the SH3 domain of ZO-1 but none of the other two proteins. Apg-2 therefore binds with its ATPase domain to the SH3 domain of ZO-1. Because ZO-1 does not bind to the PBD, it is unlikely that ZO-1 is an Apg-2 substrate.

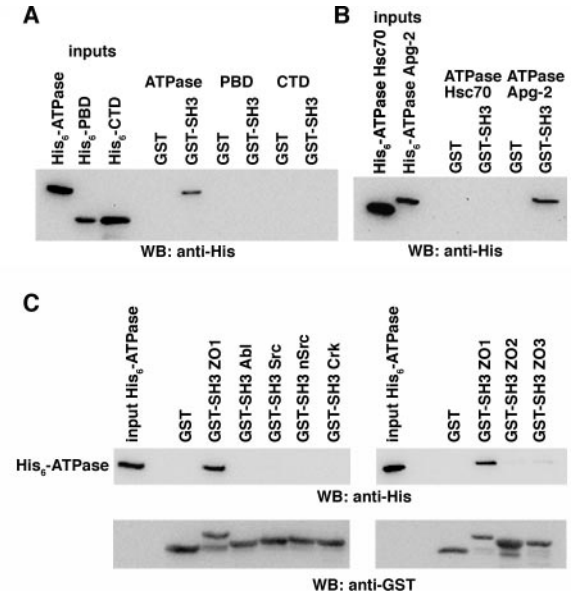

Figure 3. Binding of the SH3 domain of ZO-1 to the ATPase domain of Apg-2. (A and B) Glutathione beads carrying either GST-SH3 or GST were incubated with His_6-ATPase, His_6-PBD, or His_6-CTD (A); or with His_6-ATPase domains derived from Apg-2 or Hsc70 (B). (C) Apg-2 His_6-ATPase domain was incubated with GST fusion proteins containing the indicated SH3 domains. In all panels, pull-down was assayed by immunoblotting with an antibody against His_6.

Because the ATPase domains of different members of the Hsp70 family are conserved to each other, we tested whether the interaction with ZO-1 is conserved as well. We repeated the pull-down experiment using the recombinant ATPase domain of Hsc70, a widely expressed Hsp. Figure 3B shows that no interaction was observed between the SH3 domain of ZO-1 and the ATPase domain of Hsc70.

SH3 domains are found in many different types of proteins including ZO-2 and ZO-3, two junctional proteins homologous to ZO-1. To further test the specificity of the interaction between the ATPase domain of Apg-2 and the SH3 domain of ZO-1, we repeated the pull-down experiment using GST fusion proteins containing different SH3 domains. Figure 3C shows that neither the SH3 domain of Abl, cSrc, nSrc, nor Crk was able to pull down the ATPase domain. We could also not detect an interaction between the ATPase domain and fusion proteins containing Nck and Grb2 (our unpublished data). Moreover, pull-downs generated with the SH3 domains of ZO-2 and ZO-3 contained very small amounts of the ATPase, suggesting that they are also not good interaction partners (Figure 3C).

These observations suggest that Apg-2 can interact with ZO-1 in vitro as well as in epithelial cells and that this interaction is mediated by the ATPase domain of the Hsp and the SH3 domain of the TJ protein.

Heat Shock Stimulates the Apg-2–ZO-1 Interaction

We next used immunofluorescence combined with confocal microscopy to determine the distribution of Apg-2 in MDCK cells. Figure 4A shows that Apg-2 was present throughout the cytosol as well as the nucleus in cells grown at 37°C. Only a small fraction of Apg-2 was detected at the cell periphery, which was more evident when cells were briefly extracted with Triton X-100 before fixation, suggesting that only a small fraction of Apg-2 is junction associated. This staining was specific because it was not observed in cells in which Apg-2 was depleted by RNA interference (Figure 4B).

When cells were heat shocked at 43°C, however, junctional Apg-2 became more evident, and much of the nuclear pool was found in nucleoli (Figure 4A). The nucleolar local-

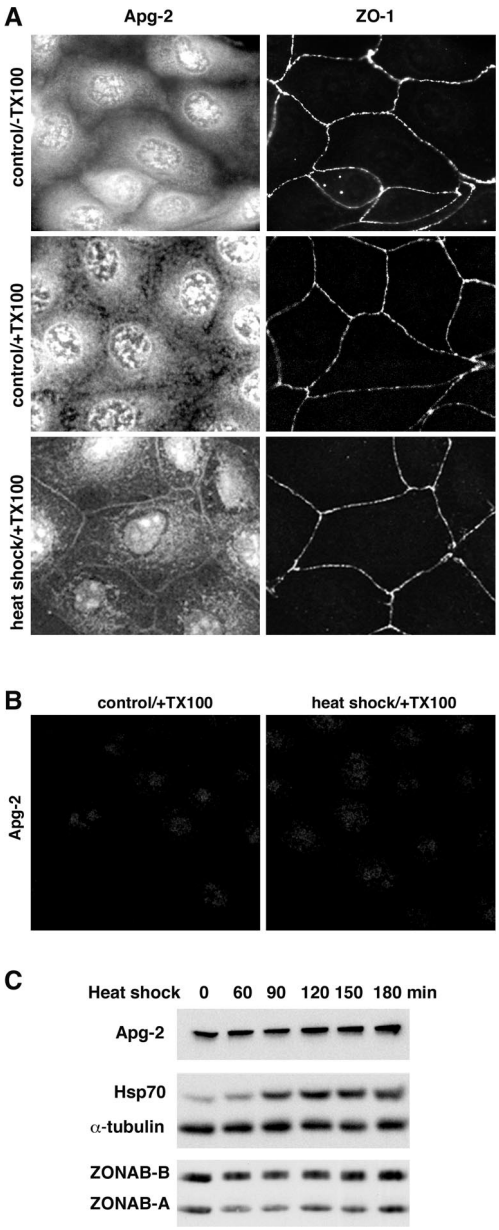

Figure 4. Localization of Apg-2 in normal and heat-shocked MDCK cells. (A) MDCK cells were cultured on coverslips and then either fixed directly (control) or first incubated at 43°C for 2 h (heat shock). Cells were preextracted with Triton X-100 before fixation as indicated. The samples were then processed for indirect immunofluorescence using antibodies against Apg-2 and ZO-1. (B) Apg-2-depleted MDCK cells (Apg-2 Ri z2) were processed as described for the samples in A and were then stained for Apg-2. Panels A and B show confocal sections that were taken with identical microscope settings. (C) MDCK cells were incubated either at 37°C for 3 h (0 min) or at 43°C for the indicated times and were then lysed and expression of Apg-2, α-tubulin, Hsp70, and ZONAB was analyzed by immunoblotting.

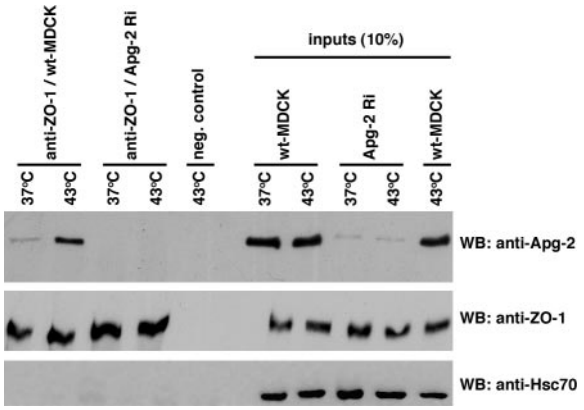

Figure 5. Stimulation of the Apg-2–ZO-1 interaction by heat shock. Wild-type (wt-MDCK) or Apg-2-depleted MDCK cells (Apg-2 Ri z2) were either kept at 37°C or heat shocked for 2 h at 43°C. The cells were then extracted, and ZO-1 was immunoprecipitated as described in Figure 2B. Precipitates and total extracts were then immunoblotted for Apg-2, ZO-1, and Hsc70.

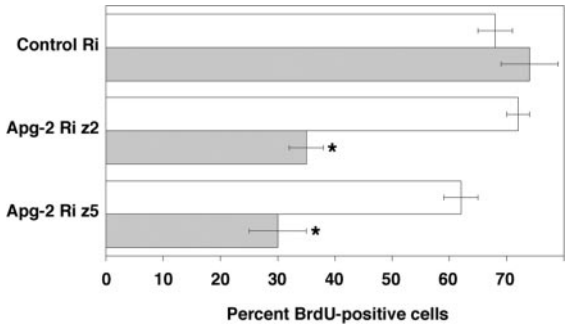

Figure 6. Regulation of G_1/S phase transition by Apg-2. Control and Apg-2 RNAi cell lines were cultured on coverslips without (white bars) or with tetracycline (gray bars), and synchronized in G_0/G_1 phase by serum starvation. Entry into S phase was then stimulated by adding serum and monitored by bromodeoxyuridine labeling. Replicating cells were quantified by counting (10 different fields per sample, 3 samples per condition; * denotes statistically significant [$p < 0.05$] differences using *t* tests).

ization was confirmed by double staining for Apg-2 and nucleolin (our unpublished data). Heat shock did not result in a significant increase in Apg-2 expression, and only expression of Hsp70 was clearly up-regulated (Figure 4C). This is in agreement with previously published reports in other cell types according to which heat shock does not affect the expression levels of Apg-2 (Kaneko *et al*., 1997; Nonoguchi *et al*., 1999). Thus, heat shock induces a redistribution of Apg-2, which includes an apparent increase in the junctional pool.

The apparent increase in junctional Apg-2 suggested that heat shock might stimulate the interaction with ZO-1. To test this, the coimmunoprecipitation was repeated with cell extracts of control and heat shocked cells. Figure 5 shows that increased amounts of Apg-2 coprecipitated with ZO-1 from heat-shocked cell extracts. The increase was approximately fivefold, resulting in coprecipitation of ~7% of Apg-2 present in the cell extract derived from heat-shocked cells. Again, no band was detected in control precipitates and ZO-1 precipitates from Agp-2-depleted cells. Hsc70 could not be detected in these precipitates. The amount of immunoprecipitated ZO-1 was ~30% of the total pool present in cell extracts and was not affected by the heat shock or Apg-2 depletion. These data indicate that heat shock indeed stimulates the Apg-2–ZO-1 interaction.

Apg-2 Regulates G_1/S Phase Transition

ZO-1 functions as an inhibitor of G_1/S phase transition in MDCK cells. The SH3 domain is required and sufficient for this activity (Balda *et al*., 2003). Therefore, we tested whether depletion of Apg-2 affects G_1/S phase transition as well. Control RNAi and Apg-2 RNAi cells, cultured without or with tetracycline, were arrested in G_0/G_1 by serum starvation (Balda *et al*., 2003). Entry into S phase was then stimulated by the addition of serum, and replicating cells were labeled by adding bromodeoxyuridine. After 7 h, cells were fixed and stained with anti-bromodeoxyuridine antibody, and labeled cells were counted.

Figure 6 shows that induction of either one of the Apg-2-directed RNA duplexes resulted in an inhibition of G_1/S phase transition. Control cells were not affected by tetracycline, and Apg-2 RNAi cells proliferated normally in the absence of tetracycline. Depletion of Apg-2 did not induce apoptosis because we could neither detect fragmented nuclei nor active caspase-3. Normal expression of Apg-2 is thus required for efficient G_1/S phase progression.

Apg-2 Regulates ZONAB Signaling

Because both Apg-2 and ZONAB bind to the SH3 domain of ZO-1, we tested whether they compete with each other for binding to the SH3 domain using His_6-ZONAB and His_6-ATPase domain. Figure 7A shows that His_6-ZONAB was efficiently precipitated by the GST-SH3 fusion protein as reported previously (Balda and Matter, 2000). When increasing concentrations of His_6-ATPase domain were added to the reaction, the amounts of recovered His_6-ZONAB decreased, indicating that the ATPase domain of Apg-2 was able to displace ZONAB from the SH3 domain. These observations indicate that Apg-2 competes with ZONAB for binding to the SH3 domain of ZO-1.

ZO-1 binding to the transcription factor ZONAB results in cytoplasmic sequestration, and, hence, inhibition. Because heat shock stimulates the ZO-1–Apg-2 interaction; we tested whether heat shock induces an increase in nuclear ZONAB. Figure 7B shows that in control cells little ZONAB was nuclear. In heat-shocked cells, nuclear ZONAB staining was increased. Nevertheless, there was also still junctional staining left, suggesting that only a fraction of ZONAB translocated into the nucleus. The increased nuclear staining was not because of higher expression levels as heat shock did not induce ZONAB expression (Figure 4C). In cells in which Apg-2 expression was reduced by RNAi during the last 2 d of culture, the appearance of nuclear ZONAB in response to heat shock was strongly reduced (Figure 7B). These observations suggest that Apg-2 promotes the nuclear accumulation of ZONAB.

We next tested whether Apg-2 regulates ZONAB function. We used a ZONAB-specific luciferase-based reporter assay in which ZONAB functions as a transcriptional repressor (Frankel *et al*., 2005). Figure 8A1 shows that cotransfection of Apg-2 resulted in reduction of the promoter activity in low-density cells, suggesting that ZONAB was stimulated. In agreement, depletion of Apg-2 by transfection of either one of the RNAi plasmids stimulated luciferase expression, indicating reduced ZONAB activity. The effect of the z2 Apg-2 RNAi plasmid could be counteracted by cotransfecting an Apg-2 cDNA that had been rendered resistant (Agp-2 H z2; see Figure 8B for test of resistance). When

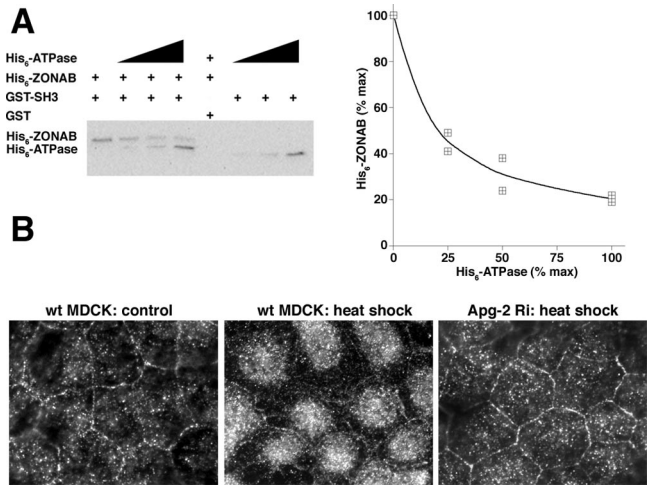

Figure 7. Apg-2 and ZONAB compete for binding to the SH3 domain. (A) GST-SH3 samples conjugated to glutathione beads were incubated with or without His_6-ZONAB for 30 min. Increasing amounts of His_6-ATPase were then added and the reactions were incubated for 2 h (final concentrations: His_6-ZONAB, 1 μg/ml; His_6-ATPase, 0, 1, 2, and 4 μg/ml). As negative control, GST was incubated with His_6-ZONAB, and the highest concentration of His_6-ATPase. Pull-downs were assayed using antibodies against ZONAB and the N terminus of Apg-2. The quantification was obtained by densitometric scanning of two independent experiments with different batches of recombinant proteins. (B) Wild-type and Apg-2 RNAi cells were grown for 4 d on coverslips. Apg-2 was depleted by adding tetracycline during the last 2 d of culture. The cells were either left at 37°C (control) or heat shocked for 2 h before processing for immunofluorescence. The cells were then preextracted with Triton X-100, fixed in methanol, and the distribution of ZONAB was monitored by indirect immunofluorescence and epifluorescence microscopy. The distribution of ZONAB was not affected in tetracycline-treated control RNAi cells (our unpublished data). Quantification of fluorescence intensities showed that heat shock induced an increase in the ratio of nuclear to cytoplasmic fluorescence from 1.1 ± 0.2 to 2.4 ± 0.4 in control cells, whereas in heat-shocked Apg-2-depleted cells, the ratio remained at 1.2 ± 0.3 (10 images were quantified for each condition averaging 5 cells per image).

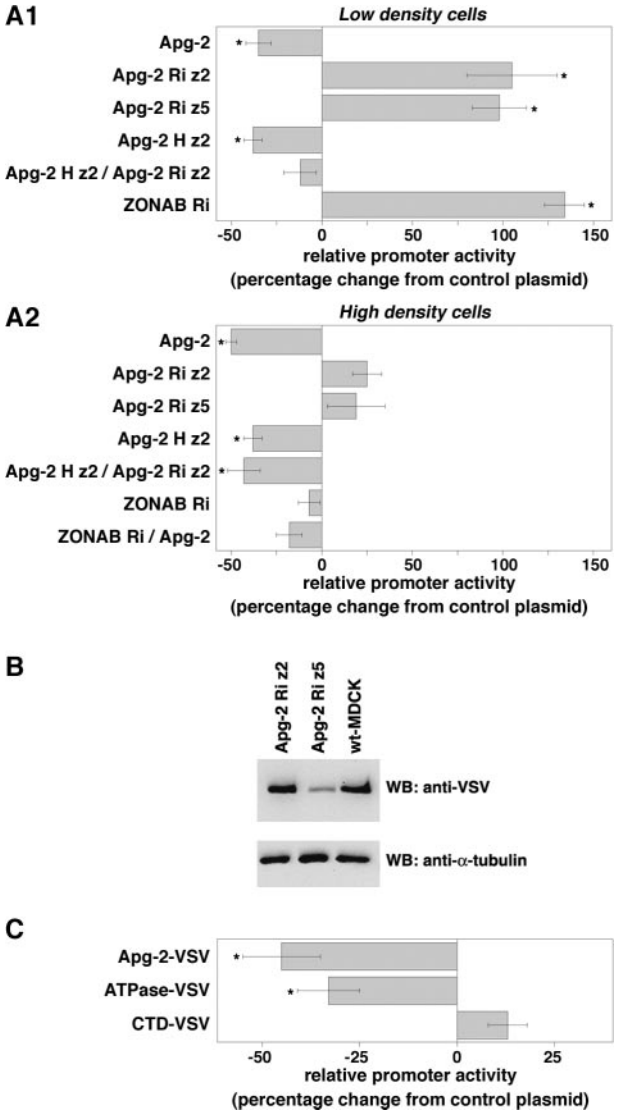

Figure 8. Regulation of transcriptional activation of ZONAB. (A) MDCK cells at low (A1) or high (A2) density were cotransfected with a plasmid containing a minimal promoter with a ZONAB binding site driving firefly luciferase expression, a plasmid containing an identical promoter sequence but with an inactivated ZONAB binding site driving *Renilla* luciferase expression together with plasmids resulting in either Apg-2- or ZONAB-specific RNAi, and/or Apg-2 overexpression. Apg-2 H z2 marks an Apg-2 construct that is resistant to RNAi induced by the z2 plasmid. The ratios between the two luciferase activities were calculated and results expressed as percentage of change from control plasmid transfections (shown are means ± 1 SD). (B) Wild-type MDCK cells and Apg-2 RNAi cell lines z2 and z5 were incubated with tetracycline and transfected with a plasmid driving the expression of VSV-tagged Apg-2 resistant to the z2 RNAi plasmid. Total cell extracts were blotted with antibodies against the VSV epitope and α-tubulin. (C) Cells grown to high density were transfected and ZONAB activity was assayed as in A. Plasmids coding for VSV-tagged constructs representing either full-length Apg-2, the ATPase domain, or the C-terminal (CTD-VSV) domain were cotransfected (* denotes statistically significant [$p < 0.05$] differences using *t* tests).

ZONAB was depleted by RNAi, the reporter was stimulated even more strongly as expected. Transfection of a control RNAi construct had no effect.

In high-density cells, in which ZONAB is transcriptionally inactive (Balda and Matter, 2000; Frankel *et al*., 2005), overexpression of Apg-2 resulted in a more pronounced inhibition of the promoter than in low confluent cells, and depletion of neither Apg-2 nor ZONAB had significant effects on promoter activity (Figure 8A2). Furthermore, simultaneous Apg-2 overexpression and ZONAB depletion did not affect promoter activity, indicating that ZONAB expression was required for Apg-2 to inhibit the promoter. This confirms that inhibition by Apg-2 reflects activation of ZONAB. These observations indicate that Apg-2 modulates ZONAB function and support a model according to which Apg-2 regulates ZONAB activation by competing for binding to the SH3 domain of ZO-1.

If Apg-2 binding to the SH3 domain of ZO-1 is responsible for activation of ZONAB, expression of the ATPase domain alone should be sufficient for ZONAB activation because it mediates the interaction of Apg-2 with ZO-1. Figure 8C shows that expression of the ATPase domain alone indeed inhibited the ZONAB-regulated promoter similar to full-length Apg-2. In contrast, expression of the C-terminal domain did not result in an inhibition of the promoter. The effect of the peptide binding domain could not be tested because we detected only very low levels of expression of

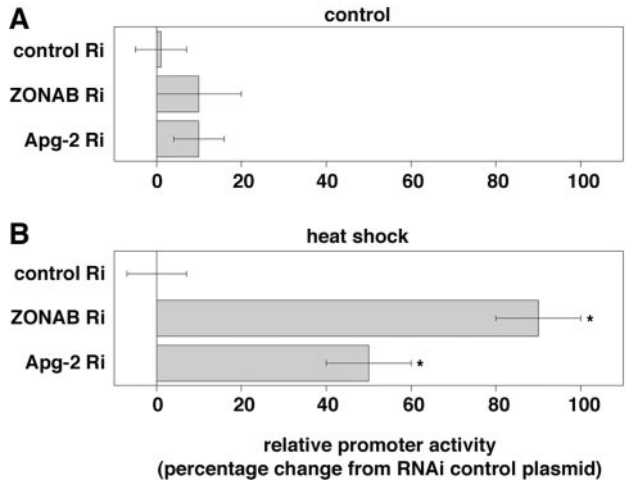

Figure 9. Heat shock induces ZONAB activity. High-density cells were transfected with the same reporter plasmids as in Figure 8A together with a control RNAi plasmid or RNAi constructs against ZONAB or Apg-2. Cells were either incubated continuously at 37°C (A, control) or incubated at 43°C for 2 h and then allowed to recover for 2 h at 37°C (B, heat shock) before measuring the luciferases. Values are given as percentage change from control transfections performed at the same conditions. Shown are means ± 1 SD (* marks statistically significant [$p < 0.05$] differences using *t* tests).

such a construct in MDCK cells. These results indicate that the ATPase domain of Apg-2 is sufficient for stimulating the transcriptional activity of ZONAB.

If binding of Apg-2 to ZO-1 results in ZONAB activation, as suggested by the increased nuclear pool (Figure 7B), one would expect that heat-shock induction stimulates the transcription factor. Therefore, we repeated the reporter assays comparing control and heat-shocked high-density cells. As previously, ZONAB and Apg-2 depletion in nonshocked (control) cells had no effect on the promoter activity, suggesting that ZONAB was transcriptionally inactive (Figure 9A). Because luciferase becomes inactivated by the incubation at 43°C, we allowed the cells to recover for 2 h at 37°C before the luciferase assay and then compared the measured values to those obtained with lysates from control transfections incubated in parallel. On heat shock, depletion of ZONAB stimulated the promoter activity compared with control transfections, indicating that the repressor had become activated (Figure 9B). Similarly, depletion of Apg-2 also stimulated the promoter, suggesting that promoter repression in heat shocked cells required normal Apg-2 expression. These results indicate that heat shock induces ZONAB activation and suggest that Apg-2 is involved in this process.

DISCUSSION

The data presented here identify the Hsp110 family member Apg-2 as a new interaction partner of ZO-1 that regulates the function of ZO-1 in the control of the transcription factor ZONAB. Our observations indicate that Apg-2 plays a role in the regulation of epithelial proliferation and the response to heat shock and that the ZO-1–ZONAB signaling pathway becomes activated during the cellular stress response in epithelial cells. Our results suggest a model according to which heat shock-induced binding of Apg-2 to ZO-1 stimulates dissociation of ZONAB from the junctional adaptor followed by its nuclear translocation and activation of its transcriptional activity.

Apg-2 binds to the SH3 domain of ZO-1. This interaction seems to be specific because no significant binding was observed with several other SH3 domains. However, the interaction between the Hsp and the junctional protein does not occur constitutively but is regulated. Only little Apg-2 is associated with ZO-1 under control conditions. On heat shock, however, Apg-2 is redistributed, not up-regulated, resulting in accumulation in nucleoli and at intercellular junctions. The junctional accumulation is likely to be because of the increased association with ZO-1, which does not exclude that it might also bind to other junctional components, perhaps involving other regions than the ATPase domain. For example, it is conceivable that Apg-2 stabilizes tight junctions during stress conditions. Since the ATPase domain interacts with ZO-1, it is possible that such a stabilizing function involves the peptide binding domain of the heat-shock protein.

How heat shock induces the Apg-2 redistribution is not known. Because ZO-1 binds to the ATPase domain, it is possible that ATP binding or hydrolysis regulates the interaction between Apg-2 and ZO-1. However, we have so far not been able to detect a difference in the in vitro binding to ZO-1 of Apg-2 loaded with either ATP or ADP (our unpublished data). Whether Apg-2 has any specific binding partners in nucleoli is also not clear. Nevertheless, the ATPase domain is sufficient to mediate localization to both junctions as well as nucleoli in response to heat shock (our unpublished data), suggesting that an interaction mediated by the ATPase domain also occurs in nucleoli. Because the interaction between Apg-2 and the SH3 domain of ZO-1 can be reconstituted with recombinant proteins, it seems unlikely that a posttranslational modification is directly required for binding in vivo. However, it is possible that an inactivating modification needs to be removed or that an interaction that prevents junctional recruitment needs to be dissociated.

Binding of Apg-2 to the SH3 domain of ZO-1 competes with the interaction between ZONAB and ZO-1, resulting in stimulation of the transcriptional activity of the transcription factor. Because ZONAB activation has been related to proliferation, and, in particular, to G_1/S phase progression, it is possible that the herein observed requirement of Apg-2 for efficient G_1/S phase progression is in part because of the inhibition of ZONAB function by Apg-2 depletion. It is unlikely, however, that this is the only reason for the observed effect on G_1/S phase progression. For example, there is a considerable nuclear pool of Apg-2, suggesting that it might also have nuclear interaction partners that are relevant for proliferation. It is thus possible that Apg-2 affects proliferation by modulating different cellular mechanisms and signaling pathways.

Such a model of Apg-2 function would not be without precedent because heat-shock proteins are often multifunctional and differentially interact with different partners depending on their subcellular localization. For example, the same isoforms of Hsp90 function in the cytoplasm, the nucleus, and even extracellularly (Picard, 2004). Hsp90 binds to a variety of different proteins at different subcellular sites and thereby regulates different signaling pathways, gene expression, and proliferation (Pratt and Toft, 2003). However, also Hsp70 family members associate with signaling proteins and have been linked to the regulation of proliferation, and the proliferative state of a cell often affects not only their expression but also their localization (Helmbrecht *et al*., 2000). Many heat-shock proteins are thus multifunctional in terms of the interactions they engage in as well as

the types of cellular processes they modulate at different subcellular and extracellular locations.

The finding that heat shock induces activation of the transcriptional activity of ZONAB indicates that the ZO-1–ZONAB pathway not only functions during proliferation but also during the cellular response to certain stresses. Although it is currently not known whether other types of stress also affect the transcriptional activity of ZONAB, it is possible that conditions that interfere with junctional integrity, for example, such as reduced availability of energy or oxidative stress also induce ZONAB activation (Welsh *et al*., 1985; McAbee and Weigel, 1987; Bacallao *et al*., 1994; Ebnet *et al*., 2001; Kale *et al*., 2003; Bailey *et al*., 2004). It will therefore be important to determine whether and how other stress conditions affect Apg-2 localization and expression, and how this affects ZONAB activity. Furthermore, stress conditions such as shear stress and oxidative stress are known to activate β-catenin signaling (Norvell *et al*., 2004; Essers *et al*., 2005; Harris and Levine, 2005). Hence, cross-talk between ZONAB and other stress-induced signaling pathways such as the mitogen-activated protein kinase pathways or β-catenin signaling will have to be analyzed.

YB-1/DbpB, another Y-box factor, is activated in response to genotoxic stress and participates in DNA repair (Holm *et al*., 2002; Kohno *et al*., 2003). Thus, Y-box factors do not just regulate transcription in response to cellular stress but play a more general role. The nucleic acid binding domain of Y-box factors is a cold shock domain, an evolutionarily well conserved type of nucleic acid binding domain that also exists in bacteria in cold shock-induced proteins (Matsumoto and Wolffe, 1998). Although the nucleic acid binding domain is the only structural conservation between Y-box factors and bacterial cold shock proteins, the function of these proteins in the cellular stress response of bacteria and eukaryotes is intriguing.

Environmental stress often induces pathways that are important for proliferation and that become activated in carcinogenesis. Both Apg-2 (Figure 6) and ZONAB (Balda *et al*., 2003) are required for normal proliferation and regulate entry into S phase. In hepatocellular carcinomas, Apg-2 as well as the human ZONAB homologue DbpA are often overexpressed (Hayashi *et al*., 2002; Gotoh *et al*., 2004), suggesting that ZONAB signaling becomes activated. This is further supported by the isolation of both proteins as overexpressed markers in pancreatic cancer cells (Nakatsura *et al*., 2001). It will thus be important to determine the role of the Apg-2–ZO-1–ZONAB signaling pathway in the development and progression of different types of cancers and to evaluate this pathway as a possible target for cancer therapy.

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