Functional interaction between the ZO-1-interacting transcription factor ZONAB/DbpA and the RNA processing factor symplekin

Emma Kavanagh[1,*], Michael Buchert[2,*], Anna Tsapara[1], Armelle Choquet[2], Maria S. Balda[1,‡], Frédéric Hollande[2] and Karl Matter[1,‡]

[1]Division of Cell Biology, Institute of Ophthalmology, University College London, London, UK
[2]Institut de Génomique Fonctionnelle, Département d'Oncologie Cellulaire et Moléculaire, CNRS UMR5203/INSERM U661/Universités Montpellier I&II, Montpellier, France

*These authors contributed equally to this work
‡Authors for correspondence (e-mail: m.balda@ucl.ac.uk; k.matter@ucl.ac.uk)

Accepted 11 October 2006
Journal of Cell Science 119, 5098-5105 Published by The Company of Biologists 2006
doi:10.1242/jcs.03297

Summary

Epithelial tight junctions participate in the regulation of gene expression by controlling the activity of transcription factors that can interact with junctional components. One such protein is the Y-box transcription factor ZONAB/DbpA that binds to ZO-1, a component of the junctional plaque. Symplekin, another nuclear protein that can associate with tight junctions, functions in the regulation of polyadenylation and thereby promotes gene expression. Here, we addressed the question of whether these two proteins interact and whether this is of functional relevance. We demonstrate that ZONAB/DbpA and symplekin form a complex in kidney and intestinal epithelial cells that can be immunoprecipitated and that exists in the nucleus. The interaction between ZONAB/DbpA and symplekin can be reconstituted with recombinant proteins. In reporter gene assays in which ZONAB/DbpA functions as a repressor, symplekin functionally interacts with ZONAB/DbpA, indicating that symplekin can also promote transcriptional repression. RNAi experiments indicate that symplekin depletion reduces the nuclear accumulation and the transcriptional activity of ZONAB/DbpA in colon adenocarcinoma cells, resulting in inhibition of proliferation and reduced expression of the ZONAB/DbpA-target gene cyclin D1. Our data thus indicate that symplekin and ZONAB/DbpA cooperate in the regulation of transcription, and that they promote epithelial proliferation and cyclin D1 expression.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/24/5098/DC1

Key words: Y-box factor, erbB-2, Adenocarcinoma, Cyclin D1, Cell cycle, Epithelia

Introduction

Tight junctions are the most apical component of the epithelial junctional complex. They are critical for epithelial barrier function as they form the paracellular diffusion barrier (Anderson et al., 2004; Cereijido et al., 2004; Schneeberger and Lynch, 2004). Tight junctions are composed of several transmembrane proteins that are linked to a cytoplasmic plaque and the actin cytoskeleton (Aijaz et al., 2006; Tsukita et al., 2001). This cytoplasmic plaque consists of a protein network formed by adaptor proteins with multiple protein/protein interaction motifs, cytoskeletal linkers and signalling proteins such as protein kinases and phosphatases (Gonzalez-Mariscal et al., 2003; Matter and Balda, 2003). These junction-associated protein complexes also interact with dual localisation proteins that can localise to both the junction and the nucleus (Balda and Matter, 2003). Several of these junctional components have been linked to the regulation of epithelial proliferation, differentiation and polarisation (Matter et al., 2005).

ZONAB was originally described in canine kidney epithelial cells and is a Y-box transcription factor. ZONAB is one of the tight junction-associated dual localisation proteins: it localises to junctions where it binds to the SH3 domain of the adaptor protein ZO-1, and to the nucleus where it regulates transcription. Its distribution is regulated by the cell density as it localises to both junctions and nuclei in low density, proliferating cells, and becomes restricted to the cytoplasm in high-density cells (Balda and Matter, 2000). This distribution is also reflected in its transcriptional activity, as ZONAB is transcriptionally active in proliferating cells but inactive in non-proliferating cells. In the canine kidney cell line, MDCK, ZONAB is required for normal rates of proliferation and regulates G1/S phase transition (Balda et al., 2003). ZONAB affects cell cycle progression by two distinct mechanisms: it regulates the nuclear accumulation of the cell division kinase CDK4 via a direct interaction and regulates expression of genes encoding cell cycle regulators such as PCNA and cyclin D1 (Balda et al., 2003; Sourisseau et al., 2006). In 3D cultures of MDCK cells, normal ZO-1 and ZONAB activities are required for epithelial cyst formation, suggesting that the Y-box factor also regulates epithelial differentiation (Sourisseau et al., 2006). As ZO-1 and ZONAB are also able to associate with other types of intercellular junctions, such as gap junctions, in cells that lack tight junctions, it is possible that ZO-1/ZONAB

signalling is also of functional importance in other cell types than epithelia (Ciolofan et al., 2006; Giepmans and Moolenaar, 1998; Itoh et al., 1993; Li et al., 2004; Penes et al., 2005; Toyofuku et al., 1998). ZONAB's human homologue, DbpA, is over-expressed in different types of cancer tissues (Hayashi et al., 2002; Nakatsura et al., 2001). It is thus possible that ZONAB/DbpA over-expression contributes to carcinogenesis and epithelial dedifferentiation in vivo. We will use the name ZONAB for the canine and the human protein in this article.

Symplekin is a ubiquitously expressed protein that is enriched in the nucleus and associates with tight junctions in polarised epithelial cells (Keon et al., 1996). Symplekin interacts with components of the polyadenylation machinery and participates in 3′-mRNA processing and polyadenylation in the nucleus as well as the cytoplasm (Barnard et al., 2004; Hofmann et al., 2002; Kolev and Steitz, 2005; Takagaki and Manley, 2000). Symplekin has been shown to interact with the transcription factor HSF1 in response to heat shock, which promotes Hsp70 expression (Xing et al., 2004).

As both symplekin and ZONAB can associate with tight junctions, we tested whether the two proteins interact. Our experiments show that ZONAB and symplekin indeed form a stable complex that can be immunoisolated and that this interaction is of functional importance for the transcriptional activity of ZONAB. As the two proteins cooperate to repress a minimal ZONAB-responsive promoter, these data indicate that symplekin can also promote transcriptional repression. In addition, the down-regulation of ZONAB transcriptional activity induced by symplekin depletion was correlated with reduced transcription of the ZONAB target gene cyclin D1 and with decreased proliferation, suggesting that symplekin and ZONAB cooperate to regulate cell proliferation in epithelial cells.

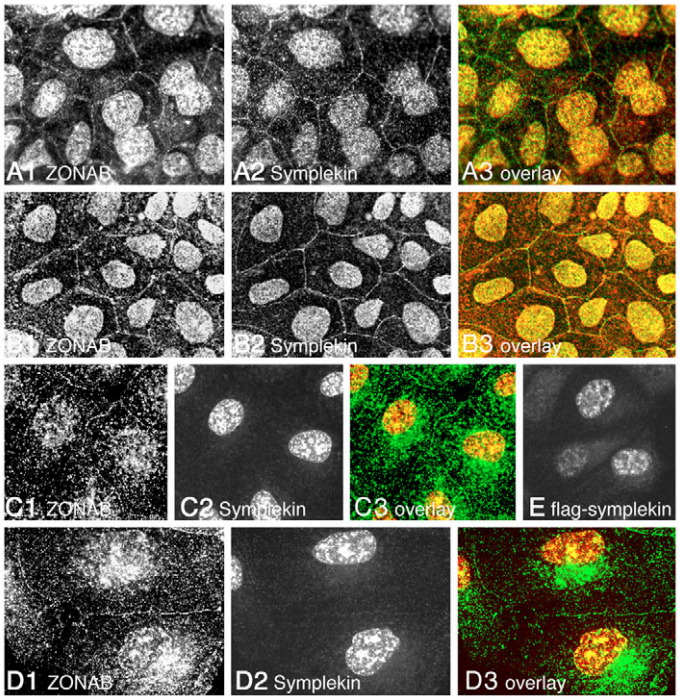

Fig. 1. Localisation of symplekin and ZONAB in epithelial cells. HT29-16E cells (A,B), wild-type MDCK cells (C,D), or MDCK cells stably transfected with a cDNA construct encoding flag-tagged full-length symplekin (E), were fixed and permeabilised and then stained with either anti-ZONAB and symplekin antibodies (A-D) or anti-flag antibodies (E). A, C, D and E are epifluorescence images and B shows images taken with a confocal microscope. Note, junctional staining for symplekin was often absent (C) and only observed occasionally (D) in MDCK cells.

Results

ZONAB and symplekin form a complex

ZONAB and symplekin can both localise to the nucleus and tight junctions in epithelial cells. We first confirmed this co-distribution in the human intestinal cell line HT-29 by immunofluorescence. For the immunolocalisation as well as all subsequent experiments with HT-29 cells, clone 16E was used as it forms polarised monolayers and functional tight junctions (Bertrand et al., 1998; Lesuffleur et al., 1991). Symplekin co-localises with the tight junction marker occludin in this cells (see Figs S1 and S2 in supplementary material), suggesting that symplekin associates with tight junctions in this cell line as previously described for other epithelial cells (Keon et al., 1996). Fig. 1A,B show that both ZONAB and symplekin stainings were observed at intercellular junctions as well as in the nucleus, where they mostly co-localised, as shown by epifluorescence and confocal microscopy. In MDCK cells, ZONAB staining was also observed at intercellular junctions as well as in the nucleus of low-density cells (Fig. 1C). However, while symplekin staining was very intense in the nucleus, junctional staining was only rarely observed and, when present, was very weak (Fig. 1D). Similarly, transfected flag-tagged symplekin exhibited a predominantly nuclear distribution (Fig. 1E), suggesting that symplekin resides primarily in the nucleus in MDCK cells, and that the amount of symplekin associated with junctions depends on the analysed cell type. The intensity of the nuclear staining for ZONAB varied in both cell lines, which is likely to be due to the proliferation-dependence of the nuclear localisation of ZONAB. In addition, the nuclear staining of both proteins was not uniform but appeared to be freckled, and their nuclear patterns appeared to overlap, suggesting that ZONAB and symplekin partially co-localise in the nucleus.

To determine whether the partial co-localisation of ZONAB and symplekin reflects the existence of a complex that contains the two proteins, we tested whether symplekin co-immunoprecipitates with ZONAB from detergent extracts of MDCK cells and the human intestinal cell line Caco-2.

Fig. 2A shows that ZONAB was efficiently precipitated from both types of cell extracts. Immunoblots with anti-symplekin antibodies revealed that symplekin was also precipitated, although not as efficiently as ZONAB. Thus, symplekin and ZONAB exist in a complex in both types of epithelial cells. As previously demonstrated, the junctional adaptor protein ZO-1 was also found in the ZONAB immunoprecipitate (Balda and Matter, 2000).

This could reflect the existence of a complex containing all three proteins, or imply that ZONAB antibodies precipitated two different complexes, one containing ZO-1 and the other symplekin. The latter possibility appears to be more likely as we could not detect symplekin in ZO-1 precipitates derived from MDCK and Caco-2 cells (not shown). The almost complete absence of symplekin at junctions in MDCK cells

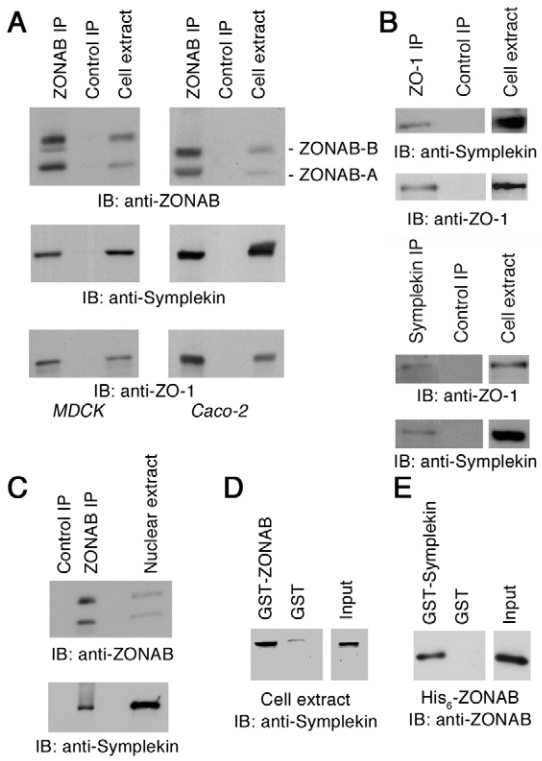

Fig. 2. Association of ZONAB with symplekin. (A) Detergent extracts of MDCK and Caco-2 cells were precipitated with anti-ZONAB immunobeads or control beads. After washing, the precipitates were analysed by immunoblotting with anti-ZONAB, anti-symplekin and anti-ZO-1 antibodies. (B) Detergent extracts of HT29-16E cells were precipitated using an anti-symplekin antibody, an anti-ZO-1 antibody, or pre-immune serum (control IP) and protein G agarose beads. After washing, the precipitates were analysed by immunoblotting with anti-symplekin and anti-ZO-1 antibodies. (C) A fraction enriched in nuclei was purified by density centrifugation, solubilised and the presence of ZONAB/symplekin complexes was assayed by co-immunoprecipitation as in A. (D) MDCK cell extracts were loaded on beads carrying either a GST-ZONAB fusion protein or GST alone. After washing, the presence of symplekin was analysed by immunoblotting. (E) Recombinant His_6-tagged ZONAB was loaded onto beads with bound GST-symplekin or GST. After washing, pull down of recombinant ZONAB was tested by immunoblotting. All extract and input lanes represent 10% of total inputs.

also argues against the presence of a protein complex containing ZONAB, ZO-1 and symplekin in these cells. Nevertheless, ZO-1 and symplekin could be immunoprecipitated with one another from HT29-16E cell extracts, suggesting that the junctional pool in this cell type is either more stably associated with the ZO-1/ZONAB complex, or is larger than in MDCK cells (Fig. 2B). The more important junctional localisation of symplekin in HT29-16E cells observed by immunofluorescence (Fig. 1A,B), supports the latter possibility. Importantly, a ZONAB/symplekin complex could also be recovered from a MDCK fraction enriched in nuclei (Fig. 2C), suggesting that the complex indeed exists in the nucleus.

We next used recombinant proteins to test whether symplekin and ZONAB can interact directly. Symplekin could be pulled down from total cell extracts using a glutathione S-transferase (GST)-ZONAB fusion protein, but not with GST alone, supporting the conclusion that symplekin and ZONAB can interact, and suggesting that this interaction can be reconstituted (Fig. 2D). If recombinant His_6-tagged ZONAB and GST-symplekin were mixed in vitro, they also interacted, suggesting that complex formation is the result of a direct interaction between ZONAB and symplekin (Fig. 2E).

These data indicate that ZONAB and symplekin form complexes in epithelial cells that, at least to some extent, occur in the nucleus and that are likely to be based on a direct ZONAB/symplekin interaction.

Symplekin modulates the transcriptional activity of ZONAB

The biochemical analysis suggests that symplekin is associated with ZONAB in the nucleus; hence, it could be that symplekin modulates the transcriptional activity of ZONAB. To test this, we made use of a ZONAB-specific reporter assay that is based on two luciferase reporter plasmids that contain a minimal version of the erbB-2 promoter, which is repressed by ZONAB (Balda and Matter, 2000; Frankel et al., 2005). The first plasmid contains a minimal promoter with a functional ZONAB-binding site that drives firefly luciferase expression; the second is a renilla luciferase plasmid that contains an identical promoter with the exception that the inverted CCAAT box required for ZONAB binding had been substituted. Therefore, differential regulation of these two promoters reflects differences caused by the ZONAB-binding site. Importantly, ZONAB functions as a repressor of this promoter; hence, increases in ZONAB activity are reflected in reduced firefly luciferase expression.

We first tested whether symplekin affects the promoter in MDCK cells by co-transfecting the luciferase plasmids with increasing amounts of symplekin expression vector (the total DNA concentration was kept constant by supplementing the transfections with empty expression vector). Fig. 3A shows that increased repression of the promoter was observed with increasing concentrations of symplekin expression vector after 1 as well as 2 days of transfection. These data suggest that increased expression of symplekin results in an activation of the transcriptional suppressor ZONAB.

We next co-transfected the two proteins to test whether a functional interaction could be observed. Fig. 3B shows that overexpression of ZONAB represses the promoter as previously published (Balda and Matter, 2000). Similarly, symplekin also repressed the promoter as observed in Fig. 3A. Co-transfection resulted in the strongest suppression, suggesting that ZONAB and symplekin synergise in the regulation of transcription. Analogous results were obtained when the luciferases were measured 1 or 2 days after transfection.

To determine whether the effect of symplekin on the promoter required ZONAB, we made use of an RNAi construct that depletes ZONAB expression in MDCK cells and that has previously been shown to inactivate ZONAB in the type of reporter assays used here (Balda et al., 2003; Frankel et al., 2005; Tsapara et al., 2006). Fig. 3C shows that transfection of symplekin again repressed the promoter and ZONAB depletion resulted in a weak stimulation, which was not statistically significant, as expected, since the cells were close to

confluence and, hence, contained only a small amount of active ZONAB. If symplekin was transfected together with the ZONAB RNAi plasmid, no suppression of the promoter was observed, indicating that ZONAB expression is required for symplekin to stimulate repression of the reporter plasmid. These data indicate that symplekin regulates the activity of a promoter with a ZONAB-binding element and that this effect requires ZONAB expression.

Symplekin depletion hampers ZONAB function in human intestinal adenocarcinoma cells

To determine whether symplekin only stimulates or is required for ZONAB transcriptional activity, symplekin expression was depleted by RNA interference in the human intestinal adenocarcinoma cell line HT29-16E, using a tetracycline-regulated shRNA construct that contains a modified RNA polymerase III promoter with a tetracycline operator at its 3′ end. An HT29-16E clone stably expressing the tetracycline repressor was transfected with either an RNAi plasmid targeting symplekin or a control RNAi plasmid.

We first tested depletion of symplekin and expression of ZONAB and ZO-1 in such cells by immunoblotting. Fig. 4A shows that symplekin expression was reduced by about 70% by the addition of tetracycline for 4 days in symplekin RNAi cells but not in a control clone. Expression of ZO-1 was not affected. However, total ZONAB expression was reduced as the level of the heavier B isoform was lower in symplekin-depleted cells. Reduced ZONAB-B expression was due to translational or posttranslational regulation as no difference in ZONAB-B mRNA expression was observed (Fig. 4B). The reduced ZONAB expression was also apparent in immunofluorescence experiments in which staining for symplekin and ZONAB were weaker in symplekin-depleted cells (Fig. 5). The remaining ZONAB staining in symplekin-

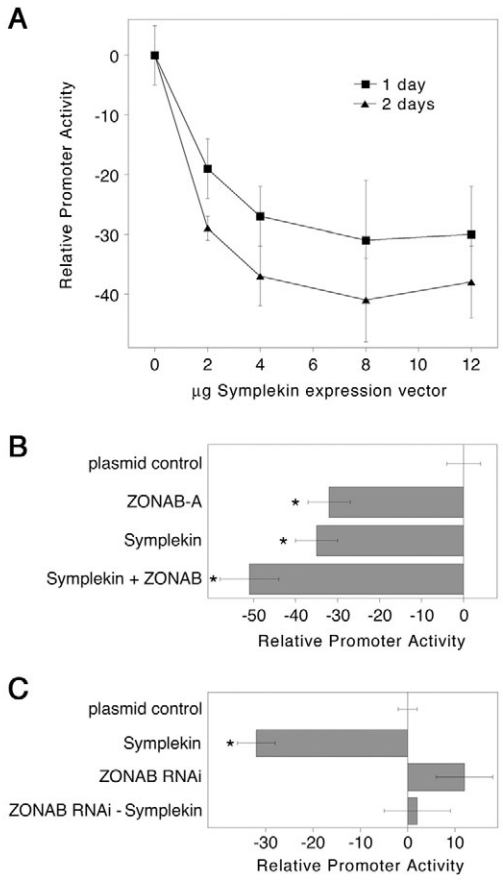

Fig. 3. Symplekin modulates transcriptional activity of ZONAB. MDCK cells at 90% density were transfected with the ZONAB-specific reporter constructs together with the indicated expression and RNAi plasmids. (A) Cells were transfected with increasing amounts of a symplekin expression vector. The total DNA concentration was kept constant by adding corresponding amounts of empty expression vector. Luciferases were assayed either 1 or 2 days after transfection. Because ZONAB functions as a repressor in this assay, reduced promoter activity represents increased ZONAB activity. (B,C) The luciferase constructs were transfected together with the indicated expression and RNAi plasmids and the luciferases were assayed 1 day after transfection. Analogous results were obtained when the transfections were left for 2 days. Note that normal ZONAB expression is required for symplekin to affect the promoter (C). Values are derived from representative experiments performed in triplicates. (*Values that are statistically significantly different from control transfections using a *t*-test.)

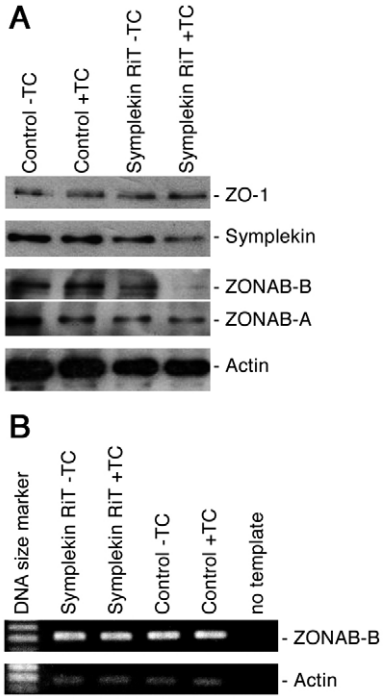

Fig. 4. Downregulation of symplekin in HT29-16E cells. (A) Regulated depletion of symplekin in the intestinal adenocarcinoma cell line HT29-16E. Clones derived from transfections with either control plasmid or the symplekin RNAi plasmid were analysed after 4 days of culture in tetracycline. The cells were lysed and the expression of the indicated proteins was analysed by immunoblotting. Actin was blotted as a loading control. Note that partial depletion of symplekin led to reduced expression of ZONAB-B. (B) Expression of ZONAB mRNA in HT29-16E cells. Control and symplekin RNAi HT29-16E cells were grown for 4 days in the presence or absence of tetracycline. Total RNA was purified and equal amounts were used for the synthesis of the first strand cDNA according to the manufacturer's instructions (Invitrogen). The level of ZONAB-B mRNA was analysed using semi-quantitative RT-PCR.

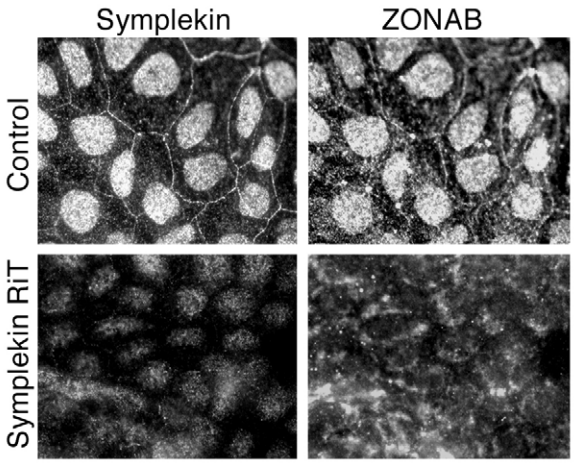

Fig. 5. Localisation of symplekin and ZONAB in HT29-16E cells. Control and symplekin-depleted HT29-16E cells were fixed and stained for symplekin and ZONAB. Note that in non-depleted cells, much of the stainings for both proteins was nuclear. In depleted cells, ZONAB was primarily in the cytosol and there was only a small amount of nuclear and junctional staining.

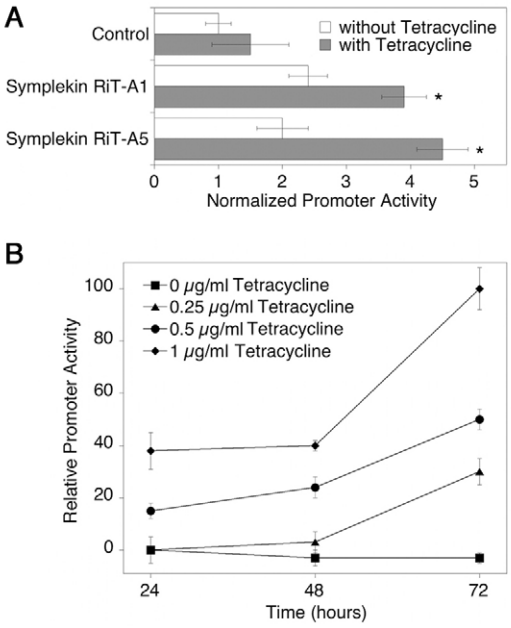

Fig. 6. Symplekin depletion inhibits ZONAB function. (A) A control HT29-16E clone and two clones expressing symplekin shRNA were grown without (white bars) or with tetracycline (grey bars) and then transfected with the ZONAB-specific luciferase reporter plasmids. Note the stimulation of the promoter upon symplekin depletion, indicating inhibition of the transcriptional activity of ZONAB. Values represent the mean ± s.e.m. of triplicates from three experiments (*P<0.05, Student's t-test compared with untreated clones). (B) Symplekin RiT-A5 clone and a control clone were grown in the absence or presence of the indicated concentrations of tetracycline for the indicated duration. Cells were transfected with equal amounts of reporter plasmids. 24 hours later, cells were lysed and luciferases were assayed. Ratios of the two luciferase activities were then calculated and compared between the different samples. Values are the mean ± s.e.m. of a representative experiment performed in quadruplicate. The results obtained with the symplekin RiT-A5 clone are expressed relative to the activity in the respective (untreated or tetracycline-treated) control sample.

depleted cells was mostly cytosolic, suggesting that the normal ZONAB distribution in the nucleus and at cell junctions is regulated by symplekin in HT29-16E cells.

Fig. 5 shows that nuclear ZONAB staining was mostly excluded from the nucleus in symplekin-depleted cells, suggesting that transcriptional activity of ZONAB is reduced in the absence of symplekin. In order to test this hypothesis, we used the above described reporter assay plasmids to determine whether the activity of the ZONAB-specific promoter was affected by symplekin depletion. Fig. 6A shows that addition of tetracycline did not significantly affect relative luciferase expression in the control cell line. In two different symplekin RNAi clones, however, tetracycline induced a twofold stimulation of the promoter, indicating that depletion of symplekin induced an inactivation of ZONAB. The effect on the ZONAB promoter depended on both the tetracycline concentration and the time of shRNA induction (Fig. 6B). In both symplekin RNAi clones, luciferase expression was already higher than in control cells in the absence of tetracycline. This is probably due to a leaky repression of the RNAi plasmid, which results in diminished expression in the absence of the antibiotic (Fig. 4A: compare minus tetracycline lanes).

In various epithelial cell lines, ZONAB interacts with the cell cycle kinase CDK4 and increases the expression of cell cycle regulators such as cyclin D1; ZONAB function therefore results in a stimulation of proliferation (Balda et al., 2003; Sourisseau et al., 2006). Hence, in view of symplekin's stimulatory effect on ZONAB activity, one would expect that depletion of symplekin reduces proliferation of HT29-16E cells. To test this, control and symplekin RNAi cells were grown in the presence or absence of tetracycline. After a 4-day treatment with tetracycline, a significant reduction of cyclin D1 expression was detected by qRT-PCR in cells expressing symplekin shRNA when compared with controls (Fig. 7A). Cell numbers, quantified by absorbance at 595 nm after crystal violet staining, were reduced by 50% in symplekin-depleted clones (Fig. 7B). In addition, TUNEL assays demonstrated that this reduction was not due to increased apoptosis (Fig. 7C). These data indicate that depletion of symplekin indeed reduces proliferation of HT29-16E cells.

Our data indicate that symplekin regulates ZONAB-mediated transcriptional repression not only in MDCK cells but also in the intestinal adenocarcinoma cell line HT29-16E. Moreover, symplekin depletion reduces proliferation of HT29-16E cells and regulates expression of ZONAB and cyclin D1.

Discussion

Several proteins have been demonstrated to localise to tight junctions as well as the nucleus, but very little is known about if and how these proteins cooperate in the regulation of nuclear processes. We show here that two such proteins, symplekin and ZONAB not only physically interact, but that this interaction is important for the transcriptional activity of ZONAB and that symplekin, like ZONAB, regulates cell proliferation and expression of the cyclin D1 gene.

Symplekin is a ubiquitously expressed protein that is found

in the nucleus of most cells but, in epithelial cells, also localises to the junctional complex (Keon et al., 1996). Symplekin interacts with the nuclear and cytoplasmic mRNA processing and polyadenylation machinery and is thought to promote gene expression by positively affecting polyadenylation and, hence, mRNA stability and expression (Hofmann et al., 2002; Kolev and Steitz, 2005; Takagaki and Manley, 2000). This positive role of symplekin in gene expression is supported by the finding that it interacts with HSF1 and thereby promotes polyadenylation and expression of Hsp70 (Xing et al., 2004). As symplekin seems to be a common component of the polyadenylation machinery, it is reasonable to think that reduced polyadenylation in symplekin-depleted cells contributes to slowing down the cell cycle. Nevertheless, the present work shows that symplekin cooperates with ZONAB even in the context of a promoter that is repressed, indicating that it can regulate ZONAB activity and, hence, epithelial cell proliferation independent of its function in polyadenylation.

The depletion of symplekin resulted in reduced expression of the ZONAB-B isoform at the protein level. As the mRNA expression was not affected, symplekin must affect expression of ZONAB-B at the translational or posttranslational level. It is currently not known whether this is a direct or indirect effect of the absence of symplekin. For example, we have previously observed that the ZONAB-B isoform is more markedly upregulated during proliferation than ZONAB-A; hence, it is possible that symplekin depletion reduced expression of the ZONAB-B indirectly by inhibition of proliferation (Balda and Matter, 2000; Sourisseau et al., 2006). However, the mechanisms that control expression of the ZONAB isoforms during proliferation have not yet been identified. Nevertheless, it is likely that the reduced total ZONAB expression in symplekin-depleted cells contributed to the observed reduction in transcriptional activity. However, as overexpression of symplekin in MDCK cells did not affect ZONAB protein levels (not shown), the synergising effect of symplekin on ZONAB activity was not due to increased expression of ZONAB-B.

ZONAB is a transcription factor that has been demonstrated to be a transcriptional repressor of the erbB-2 gene in MDCK as well as human mammary epithelial cells (Balda and Matter, 2000; Sourisseau et al., 2006). Nevertheless, symplekin promotes the transcriptional function of ZONAB on a promoter that is repressed by the Y-box factor. Hence, symplekin can also support transcriptional repression, indicating that symplekin can cooperate with both transcriptional activators and suppressors. In the case of transcriptional activators, symplekin is thought to promote their function by recruiting the machinery for mRNA processing. In the case of ZONAB, it is not clear how it cooperates in gene repression. One possibility is that symplekin promotes the assembly and/or trafficking of functional ZONAB complexes as the Y-box factor was largely excluded from the nucleus and strongly reduced at cell junctions in symplekin-depleted cells. The molecular mechanisms by which symplekin regulates the ZONAB distribution are not clear and might depend on additional factors as ZONAB efficiently associates with junctions in MDCK cells but only very little junctional symplekin can be detected. Moreover, we did not observe increased nuclear ZONAB upon overexpression of symplekin in MDCK cells (not shown). An alternative possible mechanism by which ZONAB and symplekin cooperate in gene repression is that ZONAB prevents the interaction of symplekin with other transcription factors bound to the same promoter, resulting in suppression of transcription. It is thus possible that the interaction of ZONAB with symplekin affects different mechanisms important for the

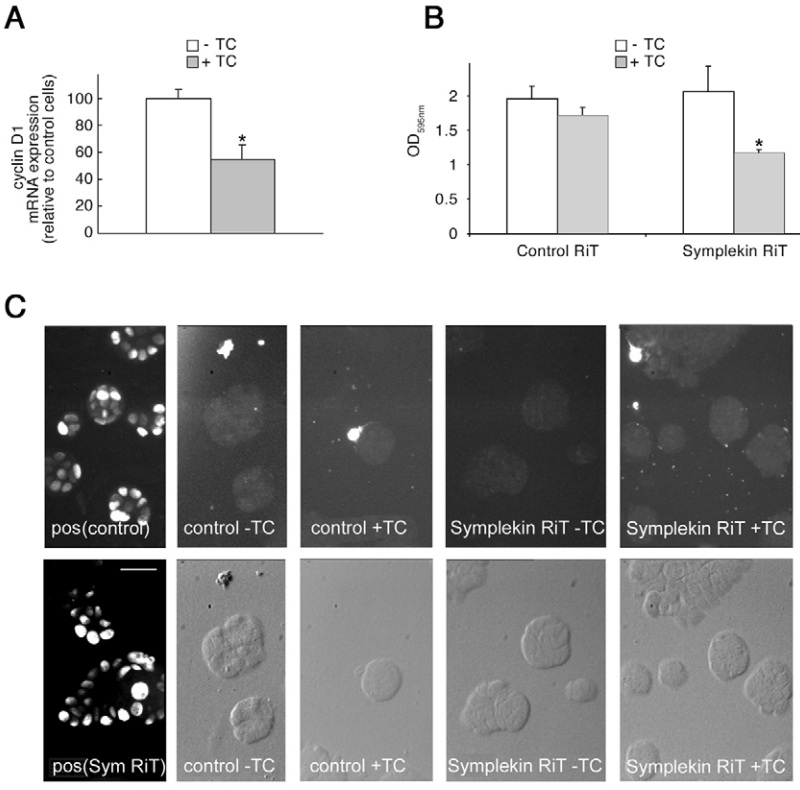

Fig. 7. Symplekin depletion inhibits cyclin D1 expression and intestinal cell proliferation. Control and Symplekin RiT cells were grown for 4 days with (grey bars) or without (white bars) addition of tetracycline. (A) Cyclin D1 mRNA expression was quantified using qRT-PCR from total RNA, relative to the expression of GAPDH mRNA. Values are the average from three experiments performed on two symplekin-depleted clones. Since addition of tetracycline in control clones resulted in a slight increase of cyclin D1 mRNA expression, results are expressed relative to the expression in their respective (untreated or tetracycline-treated) control (*P<0.05 compared with untreated cells, Student's t-test). (B) Proliferation was determined by measuring absorbance at 595 nm after cells were stained with Crystal Violet and solubilised in 1% SDS. Shown is a representative experiment performed in quadruplicates (*P<0.05 compared with untreated clones, Student's t-test). (C) Apoptosis was measured using the TUNEL assay (Roche Diagnostics) in Sym RiT cells before (–TC) and after (+TC) treatment with tetracycline, as well as DNase-treated positive controls (pos). Bar, 20 μm.

transcriptional function of ZONAB, ranging from a direct role in transcriptional regulation to the regulation of ZONAB trafficking and stabilisation.

ZONAB is a Y-box factor, a family of proteins that are multifunctional and can interact with DNA as well as RNA. It is hence possible that the interaction between symplekin and ZONAB is not only of functional consequence for transcription but also for RNA processing, stability, or localisation. It is intriguing that ZONAB is mostly cytoplasmic in non-proliferating cells, suggesting that it might also have a function in interphase epithelial cells. As both symplekin and ZONAB localise to the junctional complex, it is possible that a ZONAB/symplekin complex mediates such a cytoplasmic function and, perhaps, that it might be involved in localising specific RNAs to sites of cell-cell adhesion.

ZONAB has been directly linked to the regulation of epithelial proliferation, which involves on one hand, an interaction with the cell division kinase CDK4 and, on the other hand, transcriptional regulation of genes encoding proteins important for the cell cycle (Balda et al., 2003; Sourisseau et al., 2006). The role of ZONAB in proliferation seems to be of pathological relevance as its human homologue, DbpA, is over-expressed in hepatocellular carcinomas as well as in cells isolated from pancreatic cancers (Hayashi et al., 2002; Nakatsura et al., 2001). As ZONAB is important for the proliferation of various epithelial cell types (Balda et al., 2003; Sourisseau et al., 2006) (M.S.B. and K.M., unpublished), ZONAB might be a useful target for new anti-cancer therapies. It is thus important to identify cellular factors that regulate ZONAB function such as symplekin, which, in HT29-16E adenocarcinoma cells, is also required to maintain normal proliferation rates.

Materials and Methods

Cell lines

MDCK (strain 2) and Caco-2 cells were cultured as previously described, in DMEM containing 10% foetal calf serum (FCS) (Matter et al., 1992; Matter et al., 1989). A mucus-secreting subclone of HT-29 cells, clone 16E (HT29-16E), which is known to form functional tight junctions, was grown in DMEM containing 10% FCS (Lesuffleur et al., 1991).

RNAi and cDNA constructs

Plasmids for the expression and RNAi-induced depletion of ZONAB were as previously described (Balda et al., 2003; Balda and Matter, 2000). A plasmid for the generation of glutathione S-transferase (GST)-symplekin was obtained from W. Franke (Heidelberg, Germany) (Keon et al., 1996). A full length symplekin plasmid with an N-terminal flag epitope was constructed in pcDNA3 and was obtained from B. H. Keon (San Ramon, USA). For the regulated depletion of symplekin, the pTER vector (a gift from M. van de Wetering, Utrecht, Netherlands) was used (Yu et al., 2002). The sequence 5′-TCTGGTCCTCATCAGCATG-3′ of human symplekin was targeted. For the regulated depletion by RNAi, the pTER constructs were transfected into HT29-16E cells stably expressing the Tet-repressor protein, which were kindly provided by P. Blache (Montpellier, France).

Antibodies, immunoprecipitation and pull down assays

ZO-1 and ZONAB antibodies have been described previously (Anderson et al., 1988; Balda and Matter, 2000; Benais-Pont et al., 2003), and the anti-symplekin antibodies were obtained from BD Transduction Laboratories (clone 25; for immunoblotting) and from Progen Biotechnik (clone E150; for immunofluorescence). The mouse anti-His_6-tag and anti-flag antibodies were from Sigma-Aldrich.

For immunoprecipitations, MDCK and Caco-2 cells were grown in 14 cm plates, harvested in phosphate-buffered saline (PBS) and then frozen at –80°C. For immunoprecipitations, the cells were defrosted on ice and then solubilised in PBS containing 0.5% Triton X-100 and a cocktail of protease and phosphatase inhibitors (Balda et al., 1993). One 14 cm tissue culture plate was used per immunoprecipitate, and 20% of final immunoprecipitates were loaded per gel. The use of previously frozen cells resulted in a more efficient solubilisation of ZONAB/symplekin complexes than when fresh cells were used. Covalently conjugated anti-ZONAB antibody was then used for the precipitations as described previously (Balda and Matter, 2000). Nuclear fractions were prepared by sucrose density centrifugation (Balda and Matter, 2000), and were processed for immunoprecipitations as the defrosted cells. For pull down assays, MDCK cell extract or His_6-tagged ZONAB in PBS containing 1% Triton X-100, 1 mM DTT and a cocktail of protease inhibitors were pre-absorbed with inactive beads for 15 minutes and then incubated with glutathione-Sepharose beads coated with equal amounts of GST or the indicated GST fusion proteins for 2 hours at 4°C.

Immunofluorescence

Cells grown on coverslips were fixed in methanol (5 minutes at –20°C) with or without a pre-extraction of 1 minute 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., 1996). After re-hydration with PBS, the samples were blocked and processed for immunofluorescence as described using FITC- and Cy3-conjugated secondary antibodies generated in donkeys (Jackson Immunochemicals, Inc.) (Balda et al., 1996). Epifluorescence images were obtained with a Leica DM1 RB microscope equipped with a 63×/1.4 oil immersion objective and a Hamamatsu ORCA285 camera. Confocal images were taken with a Bio-Rad confocal microscope equipped with a 60× oil immersion lens. The image acquisition software supplied by the manufacturer was used to obtain the images, the brightness and contrast of which were then adjusted using Adobe Photoshop.

Reporter gene 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 co-transfected 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. Control plasmid transfections were used to standardise the experiments.

For time course and dosage experiments, Symplekin RiT-A5 clone and a control clone were seeded at 0.5×10^6, 1×10^6 or 2×10^6 cells/well into 6-well plates. Cells seeded at 0.5×10^6 were treated, or not, with increasing concentrations of tetracycline for 72 hours. Cells seeded at 1×10^6 were treated for 48 hours with tetracycline, and cells seeded at 2×10^6 were treated for 24 hours before transfection. Equal amounts of ZONAB reporter plasmids were transfected into each well. Cells were trypsinized 6 hours after transfection, counted and equal amounts of cells were seeded for each condition into 48 well plates to eliminate effects of variations in cell density. Cells were lysed 24 hours later, and luciferases were assayed.

Reverse transcription-PCR

For conventional RT-PCR, cDNA equivalent to 125 ng of total RNA was PCR amplified in a Mastercycler *personal* (Eppendorf) using the following conditions and primers: initial denaturation at 94°C for 3 minutes followed by 30 cycles (for actin) or 35 cycles (for ZONAB-B) of 94°C for 45 seconds, 60°C for 30 seconds, 72°C for 54 seconds; final extension was for 10 minutes at 72°C; Primers for ZONAB-B, 5′-GCT GGG GAG GAG GAG GA-3′ and 5′-CTG TTG GAG TGG GGT AAG AC-3′; Primers for actin, 5′-CGG GAA ATC TGC GTG ACA T-3′ and 5′-AAG GAA GGC TGG AAG AGT GC-3′

For quantitative (q)RT-PCR of cyclin D1 mRNA, 2.5 μg of total RNA was pretreated with DNAse RQ1 (Promega) for 30 minutes at 37°C and used for reverse transcription with M-MLV reverse transcriptase (Invitrogen). Quantitative PCR was carried out using the LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics, Meylan, France), under the following conditions:

GAPDH amplification: denaturation for 10 minutes at 95°C, amplification for 50 cycles: 10 seconds at 95°C, 6 seconds at 70°C, 13 seconds at 72°C. The melting curve was 0 seconds at 95°C, 30 seconds at 80°C, 0 seconds at 95°C and cooling was for 2 minutes at 40°C.

Cyclin D1 amplification: denaturation for 10 minutes at 95°C, amplification for 50 cycles with: 10 seconds at 95°C, 6 seconds at 70°C, 11 seconds at 72°C. The melting curve was 0 seconds at 95°C, 30 seconds at 80°C, 0 seconds at 95°C and cooling was for 2 minutes at 40°C.

Primers used for amplification of selected genes were: GAPDH sense: 5′-GGTGGTCTCCTCTGACTTCAACA-3′, antisense: 5′-GTTGCTGTAGCCAAA-TTCGTTGT-3′; cyclin D1: sense 5′-CCGTCCATGGGGAAGATC-3′, antisense: 5′-ATGGCCAGCGGGAAGAC-3′.

This research was supported by The Wellcome Trust (063661 and 066100), BBSRC and MRC (to M.S.B. and K.M.), by grants from INSERM (CRES no. 4CR04G), Association pour la Recherche sur le Cancer (ARC no. 3563), and GEFLUC (to F.H.), and by a postdoctoral

fellowship from ARC to M.B. We would like to thank Werner Franke and Brigitte H. Keon for symplekin plasmids and antibodies, M. De Wetering for his generous gift of inducible shRNA vectors, and P. Blache for the HT-29 clone16E cells expressing the Tet-repressor protein.

References

Aijaz, S., Balda, M. S. and Matter, K. (2006). Tight junctions: molecular architecture and function. *Int. Rev. Cytol.* **248**, 261-298.

Anderson, J. M., Stevenson, B. R., Jesaitis, L. A., Goodenough, D. A. and Mooseker, M. S. (1988). Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.* **106**, 1141-1149.

Anderson, J. M., Van Itallie, C. M. and Fanning, A. S. (2004). Setting up a selective barrier at the apical junction complex. *Curr. Opin. Cell Biol.* **16**, 140-145.

Balda, M. S. and Matter, K. (2000). The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. *EMBO J.* **19**, 2024-2033.

Balda, M. S. and Matter, K. (2003). Epithelial cell adhesion and the regulation of gene expression. *Trends Cell Biol.* **13**, 310-318.

Balda, M. S., González-Mariscal, L., Matter, K., Cereijido, M. and Anderson, J. M. (1993). Assembly of tight junctions: the role of diacylglycerol. *J. Cell Biol.* **123**, 293-302.

Balda, M. S., Whitney, J. A., Flores, C., González, S., Cereijido, M. and Matter, K. (1996). Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J. Cell Biol.* **134**, 1031-1049.

Balda, M. S., Garrett, M. D. and Matter, K. (2003). The ZO-1 associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. *J. Cell Biol.* **160**, 423-432.

Barnard, D. C., Ryan, K., Manley, J. L. and Richter, J. D. (2004). Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. *Cell* **119**, 641-651.

Benais-Pont, G., Punn, A., Flores-Maldonado, C., Eckert, J., Raposo, G., Fleming, T. P., Cereijido, M., Balda, M. S. and Matter, K. (2003). Identification of a tight junction-associated guanine nucleotide exchange factor that activates Rho and regulates paracellular permeability. *J. Cell Biol.* **160**, 729-740.

Bertrand, C. A., Durand, D. M., Saidel, G. M., Laboisse, C. and Hopfer, U. (1998). System for dynamic measurements of membrane capacitance in intact epithelial monolayers. *Biophys. J.* **75**, 2743-2756.

Cereijido, M., Contreras, R. G. and Shoshani, L. (2004). Cell adhesion, polarity, and epithelia in the dawn of metazoans. *Physiol. Rev.* **84**, 1229-1262.

Ciolofan, C., Li, X. B., Olson, C., Kamasawa, N., Gebhardt, B. R., Yasumura, T., Morita, M., Rash, J. E. and Nagy, J. I. (2006). Association of connexin36 and zonula occludens-1 with zonula occludens-2 and the transcription factor zonula occludens-1-associated nucleic acid-binding protein at neuronal gap junctions in rodent retina. *Neuroscience* **140**, 433-451.

Frankel, P., Aronheim, A., Kavanagh, E., Balda, M. S., Matter, K., Bunney, T. D. and Marshall, C. J. (2005). RalA interacts with ZONAB in a cell density-dependent manner and regulates its transcriptional activity. *EMBO J.* **24**, 54-62.

Giepmans, B. N. and Moolenaar, W. H. (1998). The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. *Curr. Biol.* **8**, 931-934.

Gonzalez-Mariscal, L., Betanzos, A., Nava, P. and Jaramillo, B. E. (2003). Tight junction proteins. *Prog. Biophys. Mol. Biol.* **81**, 1-44.

Hayashi, J., Kajino, K., Umeda, T., Takano, S., Arakawa, Y., Kudo, M. and Hino, O. (2002). Somatic mutation and SNP in the promoter of dbpA and human hepatocarcinogenesis. *Int. J. Oncol.* **21**, 847-850.

Hofmann, I., Schnolzer, M., Kaufmann, I. and Franke, W. W. (2002). Symplekin, a constitutive protein of karyo- and cytoplasmic particles involved in mRNA biogenesis in Xenopus laevis oocytes. *Mol. Biol. Cell* **13**, 1665-1676.

Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T. and Tsukita, S. (1993). The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell Biol.* **121**, 491-502.

Keon, B. H., Schafer, S., Kuhn, C., Grund, C. and Franke, W. W. (1996). Symplekin, a novel type of tight junction plaque protein. *J. Cell Biol.* **134**, 1003-1018.

Kolev, N. G. and Steitz, J. A. (2005). Symplekin and multiple other polyadenylation factors participate in 3′-end maturation of histone mRNAs. *Genes Dev.* **19**, 2583-2592.

Lesuffleur, T., Kornowski, A., Augeron, C., Dussaulx, E., Barbat, A., Laboisse, C. and Zweibaum, A. (1991). Increased growth adaptability to 5-fluorouracil and methotrexate of HT-29 sub-populations selected for their commitment to differentiation. *Int. J. Cancer* **49**, 731-737.

Li, X., Olson, C., Lu, S., Kamasawa, N., Yasumura, T., Rash, J. E. and Nagy, J. I. (2004). Neuronal connexin36 association with zonula occludens-1 protein (ZO-1) in mouse brain and interaction with the first PDZ domain of ZO-1. *Eur. J. Neurosci.* **19**, 2132-2146.

Matter, K. and Balda, M. S. (2003). Signalling to and from tight junctions. *Nat. Rev. Mol. Cell. Biol.* **4**, 225-236.

Matter, K., McDowell, W., Schwarz, R. T. and Hauri, H.-P. (1989). Asynchronous transport to the cell surface of intestinal brush border hydrolases is not due to differential trimming of N-linked oligosaccharides. *J. Biol. Chem.* **264**. 13131-13139.

Matter, K., Hunziker, W. and Mellman, I. (1992). Basolateral sorting of LDL receptor in MDCK cells: the cytoplasmic domain contains two tyrosine-dependent targeting determinants. *Cell* **71**, 741-753.

Matter, K., Aijaz, S., Tsapara, A. and Balda, M. S. (2005). Mammalian tight junctions in the regulation of epithelial differentiation and proliferation. *Curr. Opin. Cell Biol.* **17**, 453-458.

Nakatsura, T., Senju, S., Yamada, K., Jotsuka, T., Ogawa, M. and Nishimura, Y. (2001). Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. *Biochem. Biophys. Res. Commun.* **281**, 936-944.

Penes, M. C., Li, X. and Nagy, J. I. (2005). Expression of zonula occludens-1 (ZO-1) and the transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB)-MsY3 in glial cells and colocalization at oligodendrocyte and astrocyte gap junctions in mouse brain. *Eur. J. Neurosci.* **22**, 404-418.

Schneeberger, E. E. and Lynch, R. D. (2004). The tight junction: a multifunctional complex. *Am. J. Physiol.* **286**, C1213-C1228.

Sourisseau, T., Georgiadis, A., Tsapara, A., Ali, R. R., Pestell, R. G., Matter, K. and Balda, M. S. (2006). Regulation of PCNA and cyclin D1 expression and epithelial morphogenesis by the ZO-1 regulated transcription factor ZONAB/DbpA. *Mol. Cell. Biol.* **26**, 2387-2398.

Takagaki, Y. and Manley, J. L. (2000). Complex protein interactions within the human polyadenylation machinery identify a novel component. *Mol. Cell. Biol.* **20**, 1515-1525.

Toyofuku, T., Yabuki, M., Otsu, K., Kuzuya, T., Hori, M. and Tada, M. (1998). Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes. *J. Biol. Chem.* **273**, 12725-12731.

Tsapara, A., Matter, K. and Balda, M. S. (2006). The heat shock protein Apg-2 binds to the tight junction protein ZO-1 and regulates transcriptional activity of ZONAB. *Mol. Biol. Cell* **17**, 1322-1330.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell. Biol.* **2**, 286-293.

Xing, H., Mayhew, C. N., Cullen, K. E., Park-Sarge, O. K. and Sarge, K. D. (2004). HSF1 modulation of Hsp70 mRNA polyadenylation via interaction with symplekin. *J. Biol. Chem.* **279**, 10551-10555.

Yu, J. Y., DeRuiter, S. L. and Turner, D. L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 6047-6052.

Journal of Cell Science