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The Y-box factor ZONAB/DbpA associates with GEF-H1/Lfc and mediates Rho-stimulated transcription

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Epithelial tight junctions recruit different types of signalling proteins that regulate cell proliferation and differentiation. Little is known about how such proteins interact functionally and biochemically with each other. Here, we focus on the Y-box transcription factor ZONAB (zonula occludens 1-associated nucleic-acid-binding protein)/DbpA (DNA-binding protein A) and the Rho GTPase activator guanine nucleotide exchange factor (GEF)-H1/Lbc's first cousin, which are two tight-junction-associated signalling proteins that regulate proliferation. Our data show that the two proteins interact and that ZONAB activity is Rho-dependent. Overexpression of GEF-H1 induces accumulation of ZONAB in the nucleus and activates transcription. Microtubule-affinity regulating kinase/partition-defective-1, another type of GEF-H1-associated signalling protein, remains in the cytoplasm and partially co-localizes with the exchange factor. GEF-H1 and ZONAB are required for expression of endogenous cyclin D1, a crucial RhoA signalling target gene, and GEF-H1-stimulated cyclin D1 promoter activity requires ZONAB. Our data thus indicate that GEF-H1 and ZONAB form a signalling module that mediates Rho-regulated cyclin D1 promoter activation and expression.

Keywords: tight junctions; cyclin D1; Rho GTPases; proliferation; TNFα

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INTRODUCTION

Intercellular junctions regulate epithelial cell proliferation and differentiation using different signalling mechanisms. Tight junctions, the most apical component of the epithelial junctional complex, recruit signalling proteins such as protein kinases, Rho GTPase regulators and transcription factors that can localize to junctions as well as to nuclei (Aijaz *et al*, 2006). However, there is little understanding of how the different junction-associated signalling molecules are interconnected and coordinate cellular responses.

ZONAB (zonula occludens 1 (ZO-1)-associated nucleic-acid-binding protein)/DbpA (DNA-binding protein A) is a Y-box transcription factor that is recruited to tight junctions by binding to the Src homology 3 domain of ZO-1 (Balda & Matter, 2000). ZONAB regulates epithelial proliferation by restricting G1–S phase progression (Balda *et al*, 2003). Its transcriptional activity is important for this regulation because one of the target genes of ZONAB is cyclin D1, which is a key cell-cycle regulator (Sourisseau *et al*, 2006). As ZO-1 binding to ZONAB results in inhibition, tight junction formation suppresses this proliferation-promoting pathway. The reported deregulated expression of ZO-1 and ZONAB in different tumours might, therefore, contribute to the transformed phenotype (Hoover *et al*, 1998; Martin *et al*, 2004; Yasen *et al*, 2005). Binding of activated RalA also inhibits transcriptional activation of ZONAB (Frankel *et al*, 2005). By contrast, the mechanisms that mediate ZONAB activation are poorly understood: Apg-2, a heat-shock protein, can stimulate ZONAB activation indirectly by binding to ZO-1 (Tsapara *et al*, 2006); but mechanisms that activate ZONAB directly are not known.

Another tight-junction-associated signalling component that promotes G1–S phase progression is guanine nucleotide exchange factor (GEF)-H1/Lfc (Benais-Pont *et al*, 2003; Aijaz *et al*, 2005). GEF-H1 is an exchange factor for RhoA, and it has been proposed that its deregulation contributes to carcinogenesis (Birkenfeld *et al*, 2008). In epithelial cells, GEF-H1 activates RhoA signalling, regulating paracellular permeability, junctional dynamics and

proliferation (Benais-Pont *et al*, 2003; Aijaz *et al*, 2005; Ivanov *et al*, 2005). Rho GTPases are central regulators of signalling pathways that guide cell proliferation and differentiation. RhoA signalling regulates G1–S phase transition, and one of the crucial target genes of Rho signalling during this cell-cycle step is the cyclin D1 gene (Coleman *et al*, 2004; Ridley, 2004). However, if and how GEF-H1 participates in the regulation of gene expression is not known.

Cyclin D1 is upregulated during G1 phase and stimulates G1–S phase transition by binding and activating cyclin-dependent kinases 4/6, which contributes to cyclin E–CDK2 activation and inactivation of the cell-cycle inhibitory function of retinoblastoma protein (pRb) by hyperphosphorylation (Fu *et al*, 2004). Cyclin D1 also has a role during other cell-cycle phases that require careful regulation of localization and degradation by phosphorylation and the ubiquitin/proteosome system (Sherr, 2002; Gladden & Diehl, 2005).

Here, we show that two tight-junction-associated signalling proteins, ZONAB and GEF-H1, form a complex and that activation of the transcriptional activity of ZONAB is regulated by Rho. Our results indicate that ZONAB and GEF-H1 form a signalling module that links a Rho activator to a downstream signalling effector and mediates Rho-regulated cyclin D1 expression.

RESULTS

GEF-H1 regulates cyclin D1 expression

ZONAB and Rho GTPase signalling regulate G1–S phase progression and cyclin D1 expression (Balda *et al*, 2003; Coleman *et al*, 2004; Ridley, 2004; Sourisseau *et al*, 2006). As GEF-H1, a GEF for RhoA, regulates epithelial cell proliferation (Aijaz *et al*, 2005), we first tested whether GEF-H1 is also required for cyclin D1 expression. Our experimental system used Madin–Darby canine kidney (MDCK) cells, which allow tetracycline-regulated conditional depletion of ZONAB and GEF-H1 (Fig 1A; Aijaz *et al*, 2005; Sourisseau *et al*, 2006). To monitor the expression levels during G1–S phase progression, cells were arrested in low serum and then stimulated by adding back serum, which results in efficient re-entry of MDCK cells into the cell cycle and effective progression into S phase in 24 h (Balda *et al*, 2003).

Depletion of both ZONAB and GEF-H1 resulted in a strong reduction in cyclin D1 expression in cells that were stimulated with serum after synchronization in low serum (Fig 1). Depletion of GEF-H1 did not affect the expression of ZONAB. Overexpression of both proteins stimulated cyclin D1 expression (Fig 1C,D). Overexpression of GEF-H1, but not ZONAB, was sufficient to stimulate increased cyclin D1 levels in arrested cells (0 h), indicating that ZONAB requires serum stimulation for activity. In confluent cells, expression of cyclin D1 was low in all cell lines (supplementary Fig S1 online). These data show that GEF-H1 regulates cyclin D1 expression in proliferating cells and, similarly to ZONAB, is required for serum-induced cyclin D1 upregulation.

GEF-H1 and ZONAB form a complex

GEF-H1 and ZONAB associate with tight junctions but are also found at other subcellular locations in epithelial cells (Balda & Matter, 2000; Benais-Pont *et al*, 2003). By immunofluorescence, the two proteins co-localize with ZO-1 at intercellular junctions, indicating that the two proteins might exist in a complex (supplementary Fig S2 online). Therefore, we tested whether GEF-H1 and

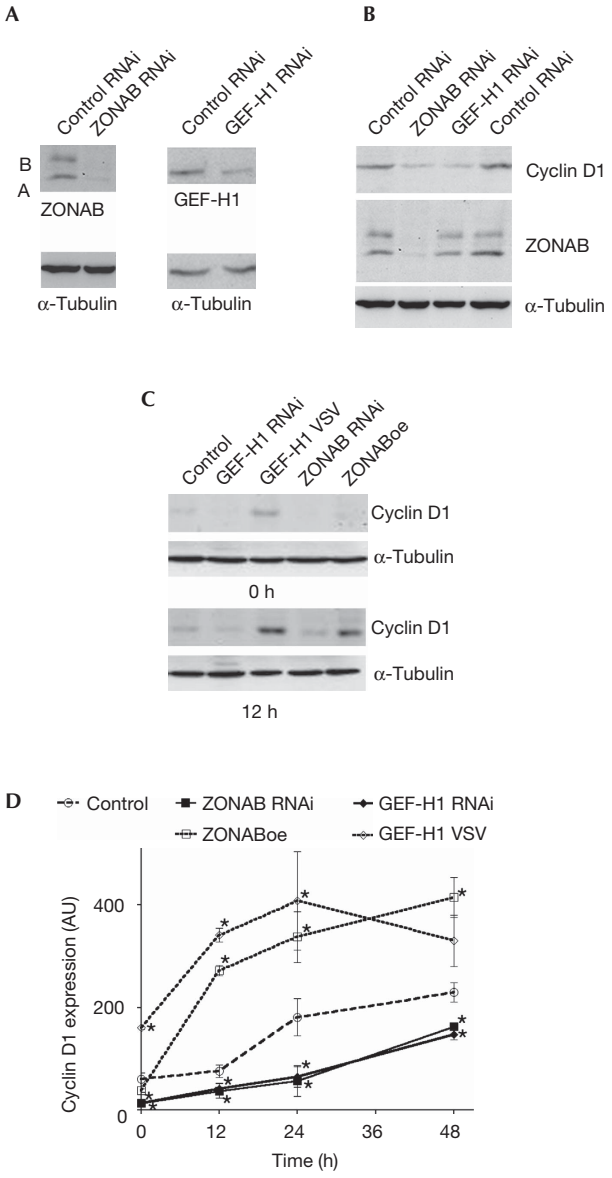

Fig 1 | Regulation of cyclin D1 expression by GEF-H1. (**A**) MDCK cells allowing the tetracycline-regulated depletion of GEF-H1 or ZONAB expression were cultured with the antibiotic for 3 days. Expression of GEF-H1, ZONAB (marked are the two ZONAB isoforms A and B) and, as a loading control, α-tubulin was analysed by immunoblotting. (**B**,**C**) The indicated cell lines were cultured for 3 days as in (**A**), but by reducing the serum to 0.1% for the last 40 h. Serum was then added back for 24 h (**B**), or for 0 h and 12 h (**C**). Expression of cyclin D1 and of the indicated control proteins was then analysed by immunoblotting total cell extracts (GEF-H1-VSV, cells expressing VSV-tagged GEF-H1; ZONABoe, cells overexpressing ZONAB). (**D**) Immunoblots from three independent experiments were analysed by densitometry to quantify cyclin D1 expression after different periods of serum stimulation. (Shown are means ± 1s.d., asterisks mark all values with $P<0.05$ in *t*-tests). GEF-H1, guanine nucleotide exchange factor-H1; MDCK, Madin–Darby canine kidney; RNAi, RNA interference; VSV, vesicular stomatitis virus; ZONAB, zonula occludens 1-associated nucleic-acid-binding protein.

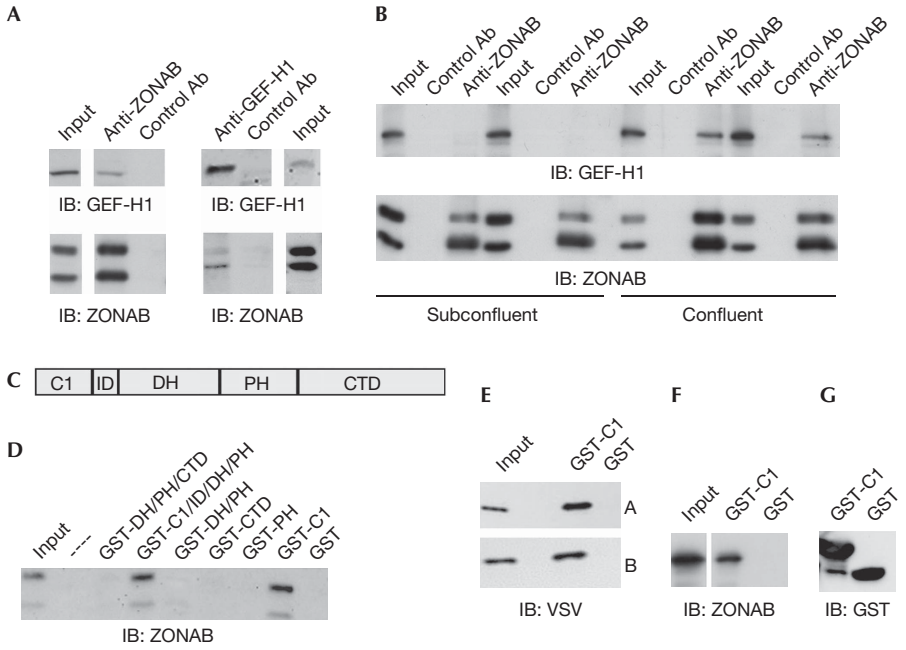

Fig 2 | Association of ZONAB with GEF-H1. (**A**) MDCK cells grown for 4 days were extracted, and GEF-H1 and ZONAB were immunoprecipitated. The immunoprecipitates were analysed by immunoblotting for ZONAB and GEF-H1. The input lane corresponds to 10% of the extract used for the immunoprecipitations. (**B**) Subconfluent (grown for 2 days) and confluent (grown for 7 days) MDCK cells were extracted and subjected to ZONAB immunoprecipitation. Precipitation of GEF-H1 and ZONAB was then monitored by immunoblotting. Duplicates generated from separate lysates for each culture condition are shown. (**C**) Domain structure of GEF-H1. The C1 domain, the ID, DH, PH and CTD are shown. (**D**) MDCK cell extracts were incubated with GST fusion proteins containing the indicated GEF-H1 domains bound to glutathione beads. After washing, the pull-downs were analysed for the presence of ZONAB by immunoblotting. Extracts of COS-7 cells transfected with cDNAs encoding (**E**) VSV-tagged ZONAB isoforms (indicated as A and B) or (**F**) recombinant ZONAB were incubated with beads containing GST or GST–C1 fusion protein. Pull-down was monitored by immunoblotting. (**G**) Immunoblot with anti-GST antibodies of fusion proteins used in pull-downs. cDNA, complementary DNA; CTD, C-terminal domain; DH, Dbl homology domain; GEF-H1, guanine nucleotide exchange factor-H1; GST, glutathione *S*-transferase; IB, immunoblotting; ID, intervening domain; MDCK, Madin–Darby canine kidney; PH, pleckstrin homology domain; VSV, vesicular stomatitis virus; ZONAB, zonula occludens 1-associated nucleic-acid-binding protein.

ZONAB form a complex in MDCK cells by using immunoprecipitation and by monitoring co-precipitation through immunoblotting.

Fig 2A shows that GEF-H1 was detected in ZONAB but not in control immunoprecipitates. Similarly, ZONAB was detected in precipitates with GEF-H1-specific antibodies, although co-precipitation was less efficient. Co-precipitation of GEF-H1 was seen in confluent, but not in proliferating, subconfluent cells that had been cultured for 2 days, indicating that the complex does not exist constitutively (Fig 2B). Similarly, GEF-H1 is less junctional in low confluent than in confluent cells (supplementary Fig S3 online). These data indicate that GEF-H1 and ZONAB can form a complex in epithelial cells and that complex formation depends on cell confluence.

Glutathione *S*-transferase (GST) fusion proteins containing different domains of GEF-H1 were then used to map the binding site for ZONAB (Fig 2C; Aijaz *et al*, 2005). Pull-down experiments with MDCK cell extracts revealed that the C1 domain is sufficient to mediate the interaction with ZONAB (Fig 2D). ZONAB is expressed in two isoforms, ZONAB-A and -B, and both could be pulled down efficiently with the C1 fusion protein from extracts of COS-7 cells transfected with vesicular stomatitis virus (VSV)-tagged ZONAB isoforms (Fig 2E). As both ZONAB-A and -B co-immunoprecipitated with GEF-H1, both seem to be able to form a complex with the exchange factor.

We then used recombinant His-tagged ZONAB to determine whether the interaction could be reconstituted *in vitro*. Fig 2F,G shows that recombinant ZONAB was precipitated by glutathione *S*-transferase (GST)-C1 but not GST alone, indicating that the C1 domain of GEF-H1 can bind directly to ZONAB. ZONAB is thus far the only GEF-H1-interacting protein known to interact with GST-C1, as other proteins require different or further domains (for example, microtubule-affinity regulating kinase/partition-defective-1 (MARK/Par1) needs an additional intervening domain; supplementary Fig S4 online).

GEF-H1 stimulates ZONAB activity

Our next step was to ask whether GEF-H1 can stimulate ZONAB activity. As overexpression of GEF-H1 is sufficient to stimulate RhoA activation (Birkenfeld *et al*, 2008), we first tested whether overexpression stimulates nuclear translocation of ZONAB. Fig 3 shows that overexpression of GEF-H1 did result in nuclear accumulation of ZONAB. The remaining cytoplasmic, dotty staining often seemed to follow the cytoplasmic GEF-H1 staining. GEF-H1 was no longer detected at junctions but, as indicated in

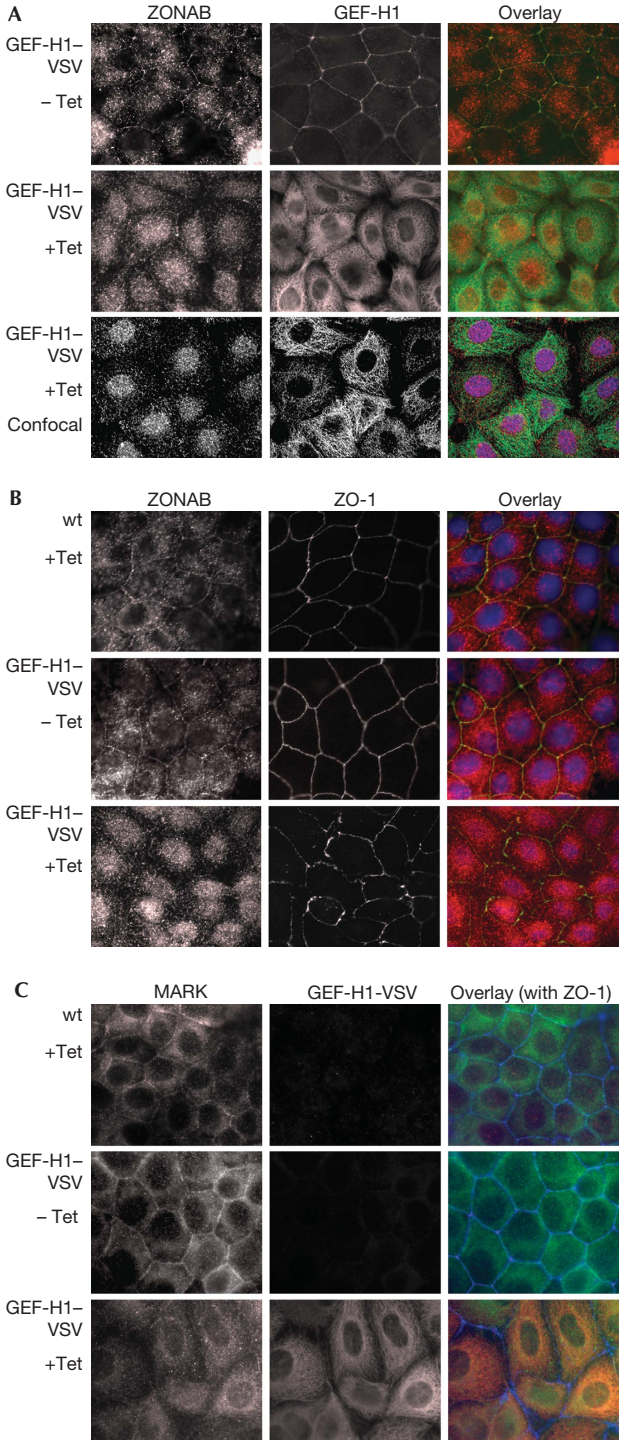

Fig 3 | GEF-H1-induced nuclear accumulation of ZONAB. Wild-type cells and a line for tetracycline-regulated expression of VSV-tagged GEF-H1 (GEF-H1–VSV) were incubated with or without the antibiotic as indicated. The cells were then processed for immunofluorescence by using antibodies against (**A**) ZONAB and GEF-H1, (**B**) ZONAB and ZO-1 (the overlay includes a DNA stain to label the nuclei), and (**C**) MARK/Par1 (mAb), GEF-H1–VSV (by using an antibody against VSV) and ZO-1 (included in the overlay in blue). Epifluorescence images for all stains and a further confocal image in (**A**) (lower row) are shown. GEF-H1, guanine nucleotide exchange factor-H1; mAb, monoclonal antibody; MARK, microtubule-affinity regulating kinase; Tet, tetracycline; VSV, vesicular stomatitis virus; wt, wild type; ZONAB, zonula occludens 1 (ZO-1)-associated nucleic-acid-binding protein.

existing reports (Birkenfeld *et al*, 2008), was cytosolic and partially associated with microtubules. MARK/Par1, another GEF-H1 interaction partner in MDCK cells that was originally identified in a proteomics screen (Brajenovic *et al*, 2004), also dissociated from cell–cell contacts on GEF-H1 overexpression, but remained cytoplasmic and partially co-localized with GEF-H1 (Fig 3C and supplementary Fig S4 online). Induction of GEF-H1 expression induced nuclear translocation of ZONAB without affecting the expression levels of ZONAB and ZO-1 (supplementary Fig S5 online).

We next analysed whether Rho signalling and GEF-H1 regulate the transcriptional activity of ZONAB. We used a reporter gene assay based on two minimal promoters—one driving firefly luciferase expression and the other driving renilla luciferase expression—that differ only by the presence of a ZONAB binding site in one of them (Frankel *et al*, 2005). ZONAB functions as a repressor of the promoter with a functional ZONAB binding site (Balda & Matter, 2000). Expression of dominant-negative RhoA (T19N-RhoA) stimulated the ZONAB-responsive promoter, reflecting ZONAB inhibition (Fig 4A). Accordingly, a constitutively active form (V14-RhoA) increased ZONAB activity, and incubation with membrane-permeable C3 transferase—which inactivates RhoA, B and C—reduced ZONAB activity. As ZONAB is active in low-density, proliferating cells, but not in high-density cells, promoter stimulation reflecting ZONAB inactivation was observed only in low-density cultures. Therefore, the transcriptional activity of ZONAB is regulated by Rho signalling.

Overexpression of GEF-H1 also repressed the promoter, indicating ZONAB activation (Fig 4B). Stimulation did not require the C1 domain, which binds to ZONAB, but overexpression of an active exchange factor domain was sufficient, as expected from the constitutively active RhoA mutant result. This indicates that a direct interaction is not required for regulation when active RhoA levels are very high (that is, expression of constitutively active GEF or RhoA). However, depletion of GEF-H1 inhibited ZONAB activity in low-density cells to a similar extent as did ZONAB depletion, indicating that GEF-H1 is a functionally relevant ZONAB activator (Fig 4C). Expression of the ZONAB-interacting C1 domain inhibited the transcription factor in low-density cells, indicating that it functions as a dominant mutant. As the C1 domain co-localized with endogenous ZONAB but did not affect nuclear accumulation, inhibition might have been due to its prevention of another interaction required for transcription (supplementary Fig S6 online). In addition, expression of the C1 domain did not affect the distribution of GEF-H1 (supplementary Fig S7 online).

ZONAB RNA interference (RNAi) was then combined with the constitutively active GEF-H1 mutant to confirm the involvement of ZONAB in the changes observed in promoter activation. Fig 4D shows that in high-density cells, in which ZONAB is not active, constitutively active GEF-H1 only repressed the promoter in the presence of ZONAB (with or without co-transfection of a control

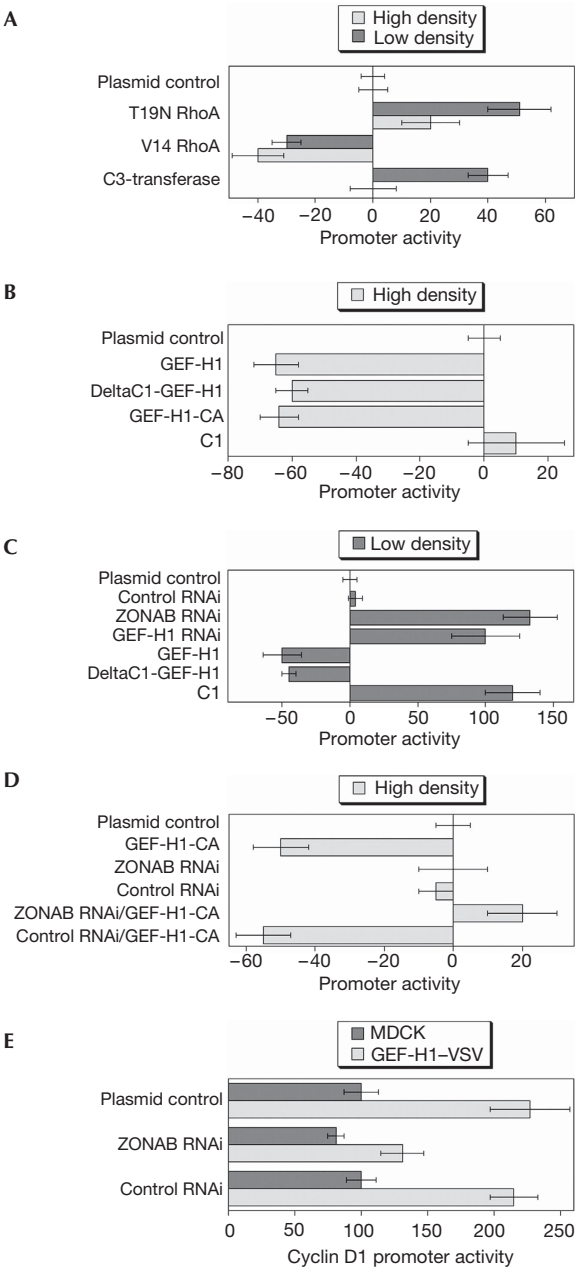

Fig 4 | Rho- and GEF-H1-regulated ZONAB activation. (**A–D**) Low- and high-density MDCK cells were transfected with the ZONAB reporter firefly luciferase construct and the control reporter driving renilla luciferase expression. The used promoter is repressed by ZONAB; therefore, decreased promoter activity reflects increased ZONAB activity. As indicated, expression and RNAi plasmids were co-transfected. In (**A**), C3-transferase refers to samples that were incubated with recombinant TAT–C3 fusion protein. After measuring the two luciferase activities, firefly to renilla luciferase ratios were calculated and changes in percentages compared with control plasmid transfections were calculated. (**E**) Wild-type or GEF-H1–VSV-expressing MDCK cells were plated at high density and transfected with a cyclin D1 promoter/firefly luciferase and a control promoter/renilla luciferase construct together with the indicated RNAi plasmids. The cells were then assayed as above and the cyclin D1 promoter activity, normalized by the measured renilla luciferase activities, was expressed relative to wild-type MDCK cells transfected with a control plasmid. All values represent averages of three independent experiments performed in triplicate. GEF-H1, guanine nucleotide exchange factor-H1; GEF-H1-CA, constitutively active GEF-H1; MDCK, Madin–Darby canine kidney; RNAi, RNA interference; VSV, vesicular stomatitis virus; ZONAB, zonula occludens 1-associated nucleic-acid-binding protein.

RNAi plasmid) but not when the transcription factor was depleted. This confirms the requirement of ZONAB for repression of the promoter by GEF-H1.

ZONAB and GEF-H1 regulate expression of cyclin D1 (Fig 1). To determine whether this involves changes in cyclin D1 promoter activity, we used a firefly luciferase reporter construct containing a full-length cyclin D1 promoter with a ZONAB binding site at position −530 (Albanese *et al*, 1995; Sourisseau *et al*, 2006). Fig 4E shows that this promoter was stimulated by overexpression of GEF-H1. However, when the ZONAB RNAi plasmids were co-transfected, stimulation was strongly reduced. Co-transfection of the control RNAi plasmid did not influence the promoter. ZONAB is thus required for GEF-H1-stimulated cyclin D1 promoter activation.

DISCUSSION

Epithelial junctions recruit various signalling proteins, and several of them are thought to regulate proliferation and differentiation. Unravelling the biochemical and functional networks that these proteins form is therefore important in order to understand the signalling mechanisms they are involved in. The data presented here show that GEF-H1 and ZONAB form a signalling module that mediates Rho-stimulated transcription.

The transcriptional activity of ZONAB is Rho-regulated as both dominant-negative RhoA and C3 transferase inhibit its activity. How Rho activation stimulates ZONAB is not yet clear. Inhibition of Rho signalling by inhibition of Rho-associated kinase (ROCK) and myosin, or transfection of dominant-negative Dia, did not inhibit ZONAB activity (data not shown). We could not find evidence for direct binding of ZONAB to RhoA. Therefore, it might be that a yet-to-be-identified Rho effector stimulates ZONAB activation. Alternatively, it is possible that inactive GEF-H1 and ZONAB form a complex in the absence of a stimulus that localizes at tight junctions and is thus primarily observed in confluent cultures. GEF-H1-mediated Rho activation then leads to a dissociation of the complex and, hence, activation of ZONAB. Given the absence of GEF-H1 from junctions in overexpressing cells, Rho activation might also lead to an inactivation of the mechanisms that recruit GEF-H1 complexes to the junction.

GEF-H1 has already been linked to Rho/ROCK/myosin-regulated junctional dynamics and dissociation (Aijaz *et al*, 2006; Samarin *et al*, 2007). It thus seems that GEF-H1 functions as a junction-associated Rho signalling hub that activates Rho and, subsequently, different Rho effector pathways that then drive junctional rearrangements as well as transcriptional responses. Recently, GEF-H1 has also been found to be activated in response

to inflammatory stimuli (Fukazawa *et al*, 2008; Kakiashvili *et al*, 2009), which is also observed in MDCK cells (supplementary Fig S8 online). However, at least one such stimulator, TNFα, does not stimulate the transcriptional activity of ZONAB (data not shown), suggesting that additional signalling mechanisms act on ZONAB to modulate its activation. One possibility is that RalA binding inhibits TNFα-induced ZONAB activation: RalA binds to and inhibits ZONAB and is activated in response to TNFα (Sugihara *et al*, 2002; Frankel *et al*, 2005).

Rho signalling regulates transcription of genes by different mechanisms. Perhaps the best studied one is the regulation of serum response factor, a transcription factor that is activated by a co-activator in which nuclear accumulation is regulated by Rho GTPases through actin polymerization (Miralles *et al*, 2003). Another type of Rho regulation is represented by NF-κB, a transcription factor that is modulated by Rho GTPases and Rho kinases either directly or indirectly by phosphorylation of upstream regulators (Perkins, 2007). To our knowledge, ZONAB is the first case of a Rho-regulated transcription factor that associates with a Rho GEF. Thus, GEF-H1 and ZONAB form a new type of signalling module that spatially links a Rho activator to an effector pathway to transmit signals to the nucleus.

METHODS

Cell lines, expression constructs, RNAi and antibodies. MDCK cells for tetracycline-regulated GEF-H1 expression, and RNAi of GEF-H1 and ZONAB were described previously (Aijaz *et al*, 2005; Sourisseau *et al*, 2006). GEF-H1 constructs fused to VSV and GST were described by Aijaz *et al* (2005) and ZONAB expression and RNAi plasmids by Balda *et al* (2003). The cDNAs encoding the four HA-tagged MARKs were provided by Gerard Drewes (Cellzome AG, Heidelberg, Germany). See supplementary information online for a list of antibodies used.

Immunofluorescence and protein analysis. Fixation with methanol, and processing and analysis of cells for immunofluorescence were performed as described (Balda *et al*, 2003). Immunoprecipitations, immunoblotting, fusion protein production and pull-down assays were described by Aijaz *et al* (2005). Recombinant C-TAK1 was from Millipore (Billerica, MA, USA). See supplementary information online for sources of antibodies used and details of protein analysis, including cell culture.

Reporter assays. MDCK cells were plated into 96-well dishes at a low (2,000 cells/well) or high (12,000 cells/well) concentration the day before transfection. To measure ZONAB activity, two plasmids were co-transfected using the calcium phosphate method: one with a minimal promoter with a ZONAB binding site driving firefly luciferase expression, and the other with an identical promoter but with a mutant ZONAB binding site driving renilla luciferase expression (Balda & Matter, 2000; Frankel *et al*, 2005). All expression and RNAi plasmids that were co-transfected were mixed with the reporter plasmids before forming the precipitate; total DNA concentrations were kept constant with empty vectors. After 2 days, the two luciferases were measured sequentially using the dual luciferase assay kit (Promega Corp, Madison, WI, USA). The activity of the cyclin D1 promoter was assayed as described previously (Sourisseau *et al*, 2006).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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