The EMBO Journal (2005) 24, 54–62 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
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THE EMBO JOURNAL

RalA interacts with ZONAB in a cell density-dependent manner and regulates its transcriptional activity

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Ral proteins are members of the Ras superfamily of small GTPases and are involved in signalling pathways for actin cytoskeleton remodelling, cell cycle control, cellular transformation and vesicle transport. To identify novel RalA effector proteins, we used the reverse Ras recruitment system and found that RalA interacts with a Y-box transcription factor, ZO-1-associated nucleic acid-binding protein (ZONAB), in a GTP-dependent manner. The amount of the RalA–ZONAB complex increases as epithelial cells become more dense and increase cell contacts. The RalA–ZONAB interaction results in a relief of transcriptional repression of a ZONAB-regulated promoter. Additionally, expression of oncogenic Ras alleviates transcriptional repression by ZONAB in a RalA-dependent manner. The data presented here implicate the RalA/ZONAB interaction in the regulation of ZONAB function.
The EMBO Journal (2005) **24,** 54–62. doi:10.1038/sj.emboj.7600497; Published online 9 December 2004
Subject Categories: signal transduction; chromatin & transcription
Keywords: cell–cell contact; RalA; signal transduction; transcription; ZONAB

Introduction

Ral proteins are members of the Ras superfamily of small GTPases. Although the exact roles that Ral signalling plays in cell function are poorly understood, Ral proteins have been reported to be required for the activation of phospholipase D1 (PLD1) (Jiang *et al*, 1995; Frankel *et al*, 1999; Voss *et al*, 1999), Src kinase (Goi *et al*, 2000), the transcription factor NF-κB (Henry *et al*, 2000) and Jun kinase (de Ruiter *et al*, 2000). Most recently, Ral has been shown to be involved in vesicle trafficking through association with the exocyst complex via an interaction with Sec5 (Moskalenko *et al*, 2002; Sugihara *et al*, 2002). RalA also binds in a GTP-dependent manner to Filamin (an actin-binding protein) (Ohta *et al*, 1999) and Ral-binding protein-1 (RalBP1; a Rac/CDC42 GTPase accelerating protein) (Cantor *et al*, 1995), suggesting that these molecules are effectors of Ral signalling. Studies using RalA mutants that are selectively uncoupled from individual effectors suggest that there are additional effector molecules likely to mediate Ral functions (Luo *et al*, 1998; Lu *et al*, 2000).

In order to identify additional RalA effectors, we performed a yeast two-hybrid screen using a novel system, the reverse Ras recruitment system (rRRS) (Hubsman *et al*, 2001). This system was used because it enabled us to employ a RalA bait localised to membranes rather than in the nucleus as used in conventional two-hybrid analysis. A number of studies suggest that Ral may function in membrane-associated events because Ral proteins have been shown to be associated with components of the exocyst complex, localise to endocytic vessels, associate with secretory granules and synaptic vesicles, and Ral is localised at the plasma membrane in lipid raft compartments (Kinsella *et al*, 1991; Bielinski *et al*, 1993; Volknandt *et al*, 1993; Xu *et al*, 2003). Surprisingly, we identified the ZO-1-associated nucleic acid-binding protein (ZONAB), a Y-box transcription factor that binds to the SH3 domain of ZO-1 (Balda and Matter, 2000), as a binding partner that binds to RalA in a GTP-dependent manner. ZONAB regulates the expression of genes with inverted CCAAT boxes such as the proto-oncogene ErbB-2 in a cell density-dependent manner (Balda and Matter, 2000) and was also recently found to associate with the cell division kinase 4 (CDK4) and play a role in regulating epithelial cell proliferation and cell density (Balda *et al*, 2003). We present evidence that RalA and ZONAB colocalise at intercellular junctions and that RalA binding regulates ZONAB function in a cell density-dependent manner providing a novel mechanism whereby cytosolic small GTPases can regulate the function of a transcription factor.

Results and discussion

Interaction of RalA and ZONAB

rRRS (Hubsman *et al*, 2001) employs a 'bait' that is naturally localised to membrane compartments in yeast permitting the detection of protein–protein interactions that occur at membranes. To test the feasibility of this assay in searching for Ral interacting proteins, we determined whether it would detect RalA binding to a known effector, RalBP1. Bait plasmids encoding different RalA mutants were constructed under the control of the methionine promoter, while the Ral-binding domain (RBD) of RalBP1 was fused in-frame to the

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Received: 23 June 2004; accepted: 5 November 2004; published online: 9 December 2004

C-terminal domain of Ras (Ras-RalBP1-RBD). CDC25-2 yeast transformants expressing the 'activated' version of RalA (G23V) and Ras-RalBP1-RBD were examined for their ability to grow at the restrictive temperature, indicative of protein–protein interaction. These transformants exhibited efficient growth at the restrictive temperature, indicating interaction between RalA and RalBP1 at the plasma membrane. No growth was observed when a 'dominant inhibitory' RalA mutant (S28N) constitutively in the GDP-bound state was expressed with Ras-RalBP1-RBD (Figure 1A). This result suggests that the binding of RalA to RalBP1 observed using the rRRS system is GTP dependent. No interaction of RalA was observed with the regulatory region of Pak2, an effector of CDC42/Rac/Chp signalling showing that the Ral-RalBP1 interaction observed in this system was specific (Figure 1A). The 'activated' version of RalA (G23V) was then used as bait to screen a rat pituitary cDNA library. The screens represented coverage of about 35% of the library. From these screens, four clones were isolated that bound specifically to RalA. One clone (clone 4), which bound to RalA-GTP but not to RalA-GDP (Figure 1A), was identical in sequence to the rat Y-box-binding protein (RYB A) (Ito *et al*, 1994). Specificity of binding to RalA-GTP was demonstrated by showing that clone 4 did not bind to GTP-bound versions of other small GTPases such as Cdc42, Rac1 or Chp (Figure 1A). Interestingly, the RYB A protein showed very strong homology with the canine protein ZONAB. ZONAB was initially identified in MDCK cells via an interaction with the SH3 domain of the tight junction protein ZO-1 and like RalA can localise to the plasma membrane (Balda and Matter, 2000). We examined whether ZONAB would associate with RalA in a GTP-dependent manner. Using the rRRS system, we observed that ZONAB binds to RalA in a GTP-dependent manner mediated by the ZONAB cold shock domain (CSD) (Figure 1B and C). This is the first report of a small GTPase binding to a CSD sequence, which has been previously described as being involved in DNA binding, and which ZONAB requires for DNA binding *in vitro* (Schnuchel *et al*, 1993; Kloks *et al*, 2002) (E Kavanagh, MS Balda and K Matter, unpublished results). To demonstrate that the interaction between RalA and ZONAB is direct and not mediated by a yeast protein binding to mammalian RalA in a GTP-dependent manner, we investigated whether the RalA–ZONAB interaction could be detected using recombinant proteins expressed in *Escherichia coli*. Figure 1D shows that recombinant glutathione *S*-transferase (GST)-ZONAB pulls down recombinant S-His-RalA only when it is in the GTP-bound state. Similar results were obtained using S-His-RalA recombinant RalA to pull down GST-ZONAB (data not shown). Thus the interaction is direct and not mediated by another protein.

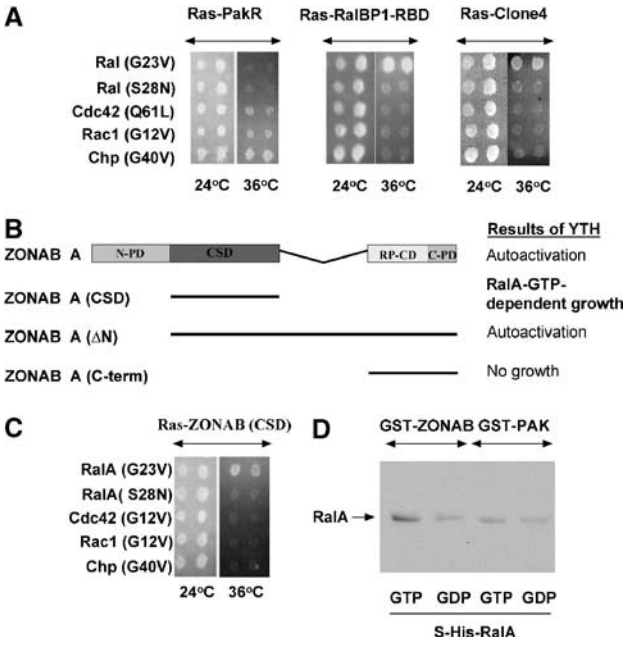


Figure 1 RalA binds to ZONAB in a GTP-dependent manner via the ZONAB CSD. (**A**) Specificity test for RalA (bait) and Ras-Clone4 (prey). DNA plasmid isolated from candidate clone #4 was subjected to restriction digest and the library plasmid was identified. The plasmid was used to cotransfect Cdc25-2 cells with the Met expression vector encoding the original bait, RalA (G23V), the Met expression vector (pMet) and the Met expression vector encoding full-length activated Rac, Cdc42, Chp and the (S28N) version of RalA, which is constitutively in the GDP-bound form. Transformants were selected and used to replica plate onto appropriate plates containing galactose and lacking methionine and incubated at 36°C. Growth at 36°C indicates a positive two-hybrid interaction. (**B**) Identification of RalA-binding domain in ZONAB. Deletion analyses were performed on ZONAB to generate the indicated Ras-ZONAB fusion proteins. The growth of CDC25-2 transformants expressing the corresponding proteins is indicated. (**C**) Only the ZONAB CSD binds RalA in a GTP-dependent manner. Transformants expressing the indicated small GTPases and the Ras-CSD were tested for their ability to confer growth at 36°C as described in (A). (**D**) Recombinant GST-ZONAB-A binds specifically to S-His-RalA-GTP *in vitro*. GST-ZONAB-A or the control GST-Pak (N-term regulatory domain) was bound to glutathione–Sepharose beads followed by incubation with either S-His-RalA-GTP or S-His-RalA-GDP. Data shown are representative of three separate experiments.

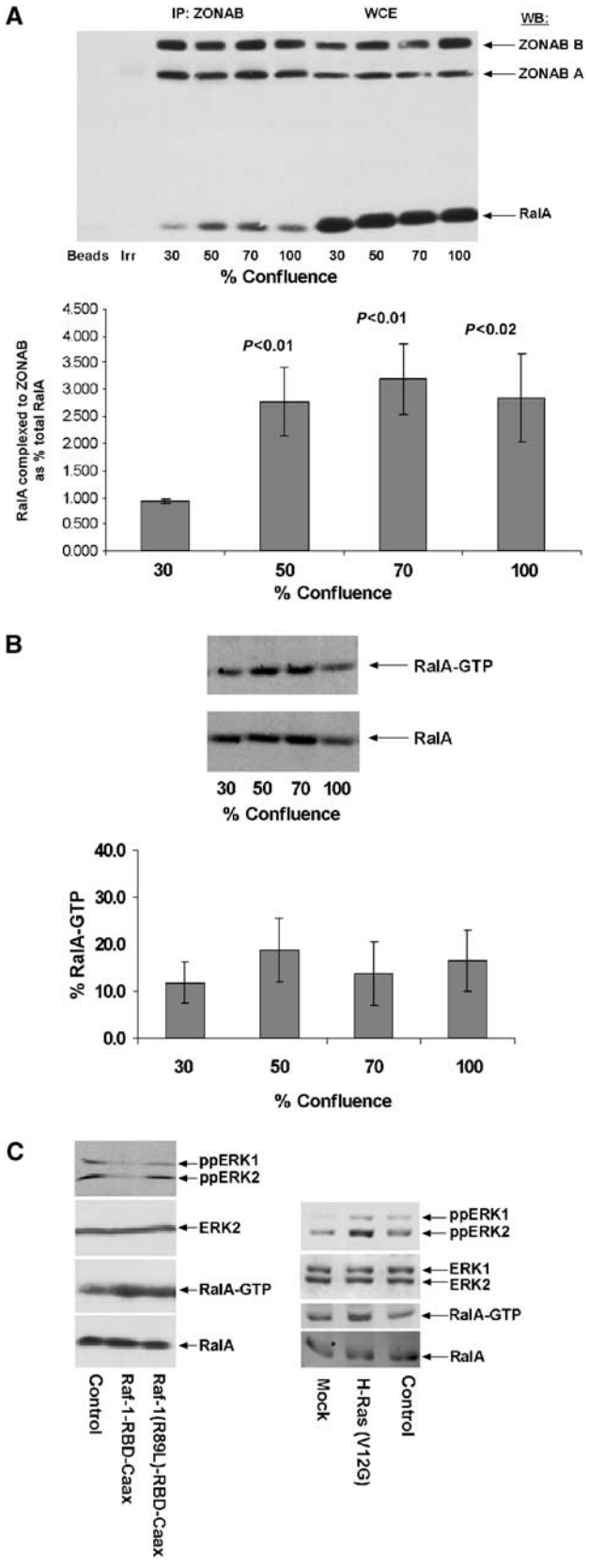
Endogenous RalA and ZONAB associate in a density-dependent manner

To determine whether the interaction between RalA and ZONAB could be seen with endogenous proteins, we carried out co-immunoprecipitation experiments. We used an antibody against ZONAB capable of immunoprecipitating both isoforms of ZONAB and previously shown to co-immunoprecipitate complexes of ZONAB with ZO-1 (Balda and Matter, 2000). Figure 2A shows that endogenous RalA was co-immunoprecipitated with endogenous ZONAB.

This is the first time that we are aware of immunoprecipitation of endogenous Ral with endogenous effector proteins, including RalBP1 and Sec5. Previously, co-immunoprecipitation has only been achieved with overexpression systems (Cantor *et al*, 1995; Ohta *et al*, 1999; Moskalenko *et al*, 2002; Sugihara *et al*, 2002). Strikingly, the amount of this complex changes with cell density. At low cell densities, when cells are highly proliferative, the amount of complex is low. However, as cell density increases and cells form a monolayer and stop proliferating, the amount of complex increases. This change in the amount of complex does not appear to reflect a change in ZONAB levels as cells become denser since Figure 2A shows that the amount of ZONAB in total cell lysates does not change. While it has been previously reported that the levels of ZONAB in sparse and dense cells are the same (Balda and Matter, 2000), a transient increase in ZONAB level has been observed as MDCK cells proliferate, but the conditions used in that study are somewhat different from those used here.

Since binding of ZONAB to RalA is dependent on RalA being GTP bound, one possible mechanism for this change in the amount of the complex is that there is an increase in the level of RalA-GTP as cells become dense. Therefore, we determined the levels of RalA-GTP using a 'pull-down' assay. Figure 2B shows that as cells become denser, the level of RalA-GTP does not change. Therefore, the change in the amount of complex is not mediated by changes in the amount of Ral-GTP and must have some other basis.

Activation of RalA is mediated by Ral guanine nucleotide exchange factors (GEFs), many of which have been shown to be regulated by Ras-GTP suggesting that the presence of RalA-GTP could be a consequence of Ras activation. However, the activation of RalA does not seem to be mediated by Ras-GTP since infection with an adenovirus expressing the Ras-binding domain (RBD) of Raf-1 to block Ras-GTP action did not affect RalA-GTP levels while it did significantly reduce activation of the Raf–Mek–ERK pathway (Figure 2C). We conclude therefore that activation of RalA to the GTP-bound state in nontransformed MDCK cells is mediated either by a RalGEF that lacks a Ras association (RA) domain such as RalGEF2 (de Bruyn *et al*, 2000) or by other signals impinging on exchange factors with RA domains (Hofer *et al*, 1998). While the activation of RalA to the GTP-bound state in nontransformed MDCK cells appears to be independent of Ras signalling, as expected expression of oncogenic Ras increases the level of RalA-GTP and phosphorylated ERK (Figure 2C). These data are therefore consistent with both Ras-dependent and -independent signalling to RalA-GTP in MDCK cells.



Localisation of RalA and ZONAB

To further characterise the interaction of RalA and ZONAB, we looked at the localisation of RalA, ZONAB and ZO-1. ZONAB and ZO-1 are localised to the tight junctions in confluent MDCK cells (Stevenson *et al*, 1986; Balda and Matter, 2000). Previous studies show RalA to be present in several membrane compartments. For example, in neurons, RalA seems to be present in vesicles that shuttle along the axon, and has been reported to be involved in synaptic release (Polzin *et al*, 2002). Interestingly, under conditions of low cell density, when cells proliferate, we find that RalA is absent from the plasma membrane and displays a punctate

Figure 2 RalA/ZONAB complex formation is dependent on cell density. (**A**) MDCK cells were seeded at different densities to reach the indicated degrees of confluence on the same day and then harvested. Cleared lysates were prepared from equal numbers of MDCK cells at each cell density and incubated with Sepharose beads with covalently bound polyclonal antibody against ZONAB. The immunoprecipitates were analysed by immunoblotting with antibodies against RalA and ZONAB. Six experiments were performed; in each, the amount of RalA bound to ZONAB as a proportion of total RalA was measured by quantitative Western blotting. Data are presented as the means with standard deviations. The *t*-test showed that the amount of RalA bound to ZONAB differs at confluences above 30% ($P<0.02$). (**B**) MDCK cells were treated as above, and cell lysates were incubated with the Ral-binding domain (RBD) of RalBP1 immobilised on beads. Five experiments were performed; the amount of RalA-GTP bound was analysed by Western blotting. Equal amounts of cells representing 1/20 of the total were subjected to Western blot analysis for total endogenous RalA. % RalA-GTP was determined from the ratio of RalA-GTP/total RalA and is represented in the histogram below. (**C**) Representative autoradiograms showing levels of RalA-GTP and phospho-ERK in 100% confluent MDCK cells either infected with Raf-1-RBD-Caax adenovirus compared to control (pAd-CMV-Myc) or Raf-1 (R89L)-RBD-Caax infected cells or transiently transfected with H-Ras (V12G) or control empty vector.

staining pattern especially around the perinuclear region, which appears to be in discrete vesicles (Figure 3A). These vesicles do not appear to be lysosomes, endosomes or associated with Golgi since they do not label with giantin, LAMP, EEA1 or transferrin (Honing and Hunziker, 1995; Gut *et al*, 1998; Folsch *et al*, 2001; Hopkins *et al*, 2003). In low-density cells, ZONAB is localised to the membrane and nucleus with some nonvesicular cytoplasmic staining (Figure 3A; Balda and Matter, 2000). Note that because of differences in fixation protocols required for RalA, ZO-1 and ZONAB in low-density cells, ZONAB staining (shown in Figure 3A, lower panel) is presented using Triton X-100/ethanol fixation rather than methanol, which is required to preserve RalA staining. These results show that at low density, RalA and ZONAB are localised in different cellular compartments. Upon increased cell density and cell cycle arrest, we find that RalA is located predominately at the plasma membrane (Figure 3B). Other examples of redistribution of small GTPases have been described: RhoA redistributes from the cytoplasm to the plasma membrane in response to extracellular signals (Takaishi *et al*, 1995; Mattii *et al*, 2000) and Rac has been shown to become activated and localise to the plasma membrane as cells make adhesions to the extracellular matrix (Nakagawa *et al*, 2001; Del Pozo *et al*, 2002), but this striking redistribution of RalA with changes in cell density is the first time we are aware of RalA showing a change of cellular localization.

The redistribution of RalA to the plasma membrane in dense cells leads to colocalisation of RalA with ZONAB at the plasma membrane (Figure 3B). Because ZONAB localises with ZO-1 at tight junctions (Balda and Matter, 2000), we wished to determine whether RalA is localised at tight junctions in dense cells. Since we found that the optimal fixation protocol of Triton X-100/ethanol to demonstrate ZONAB localisation at tight junctions does not preserve RalA localisation, we used methanol fixation and ZO-1 staining as a marker of tight junctions. Figure 3B shows that in an *xz* series, RalA is localised all along the basolateral membrane but is localised with ZO-1 at tight junctions. These results suggest that RalA and ZONAB may colocalise at tight junctions. Interestingly, expression of oncogenic Ras in low-density cells results in increased RalA localisation at the plasma membrane (Figure 3C), demonstrating that the localisation of RalA can be modulated by intracellular signalling events. As described below, oncogenic Ras signalling through RalA has consequences for the effects of ZONAB on transcription (Figure 4D).

From these results, we conclude that the density-dependent formation of the RalA–ZONAB complex is paralleled by a change in localisation of RalA to membrane sites where ZONAB is localized, suggesting that plasma membrane localisation is an important determinant of the RalA–ZONAB interaction.

Why active RalA accumulates at the plasma membrane as MDCK cells become confluent is not clear. Several studies show that small GTPases are required for epithelial junction integrity, although in some contexts GTPase signalling can disrupt cell–cell junctions (Jou *et al*, 1998; Wojciak-Stothard *et al*, 2001; Gopalakrishnan *et al*, 2002; Sahai and Marshall, 2002). Assembly of functional tight junctions is critical for the normal behaviour of epithelial cells. Epithelial tight junctions mediate cell adhesion and form the intramembrane and paracellular diffusion barriers. Recent results also indicate that tight junctions may play a central role in processes that

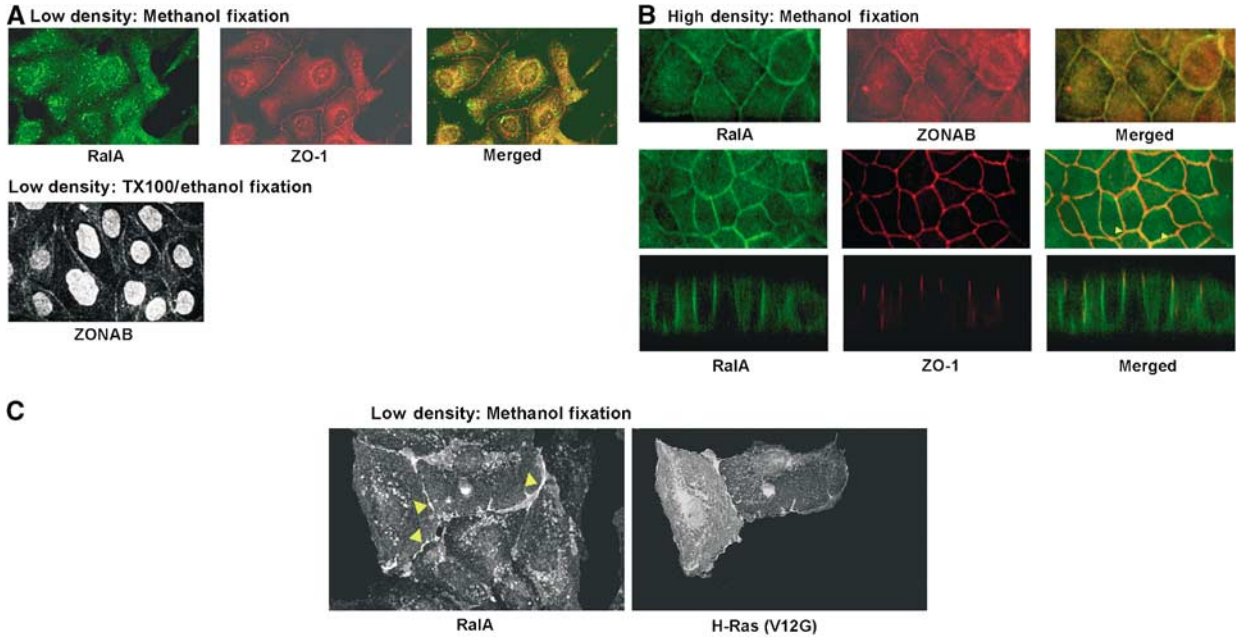


Figure 3 RalA subcellular localisation is dependent on cell density and oncogenic Ras activation. (**A**) Upper panel: MDCK cells at low cell density were fixed with methanol and stained for RalA and ZO-1. At low cell density, RalA is present in small intracellular vesicles in the cytoplasm. Lower panel: cells were fixed with Triton X-100/ethanol and stained for ZONAB. (**B**) Upper panel: cells at high density were fixed and stained for RalA and ZONAB to show that RalA partially colocalises with ZONAB at high cell density. Lower panel: cells at high density were fixed and stained for RalA and ZO-1 and *xz* series prepared to demonstrate colocalisation of RalA at tight junctions. Data shown are representative of at least three separate experiments. (**C**) H-Ras (G12V) was transiently transfected into low-density MDCK cells and stained for endogenous RalA and Ras. The arrows show that RalA is only localised at the plasma membrane in cells expressing oncogenic Ras.

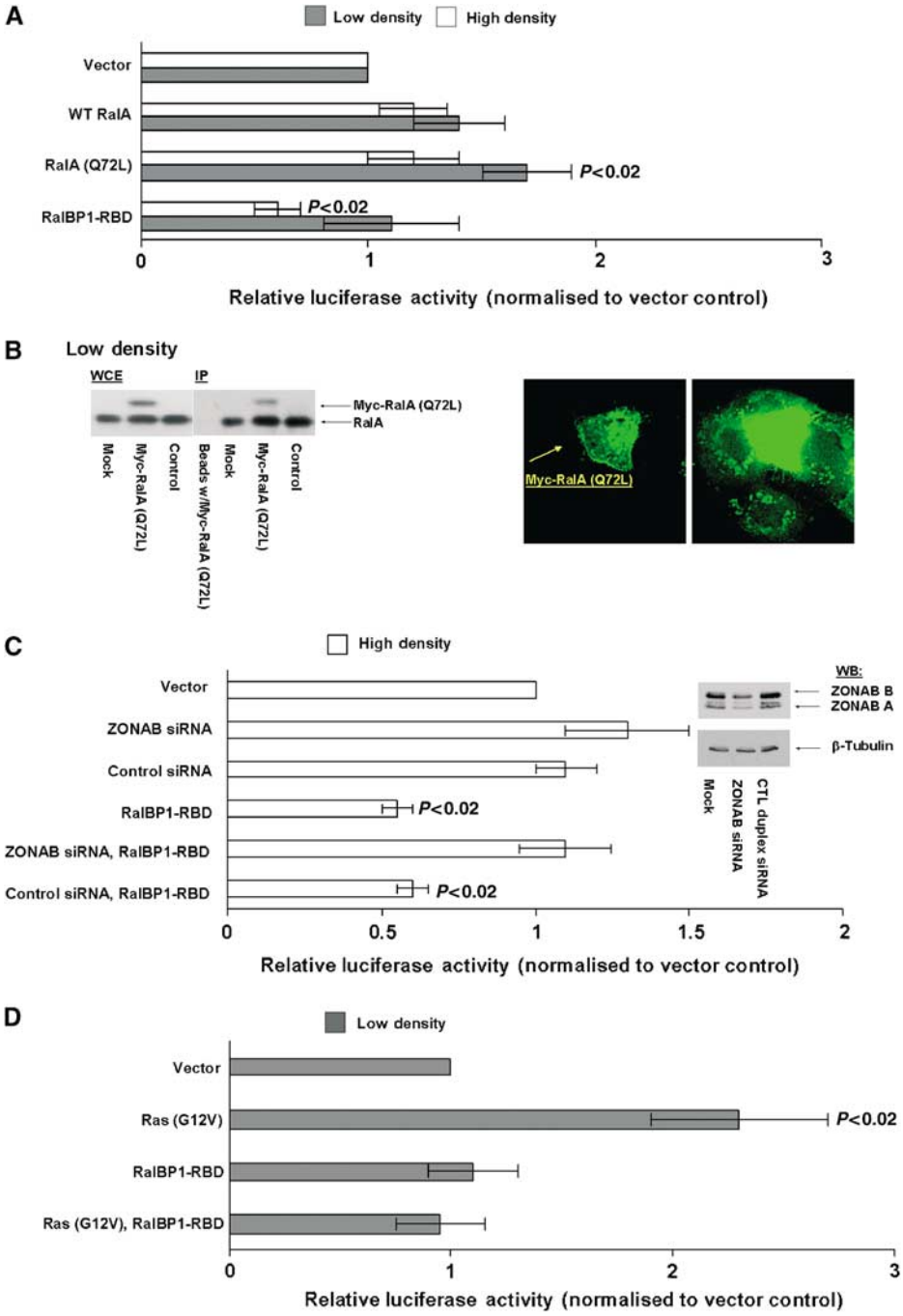


Figure 4 Regulation of ZONAB-mediated transcriptional repression by RalA. Low- or high-density MDCK cells were cotransfected with plasmids carrying a short version of the human ErbB-2 promoter with an intact ZONAB-binding site driving the expression of firefly luciferase and a control plasmid carrying a mutant version of the same promoter, in which the ZONAB-binding site had been inactivated, driving the expression of *Renilla* luciferase. (**A**) These reporter plasmids were transfected together with empty expression vector (vector) or the indicated vectors resulting in the expression of myc-tagged wild-type RalA, activated RalA (Q72L) or the Ral-binding domain (RBD) of RalBP1. Expression of the luciferases was assayed with a dual-luciferase assay system. The ratios obtained (firefly luciferase divided by *Renilla* luciferase) were normalised to those obtained by cotransfecting empty vector. (**B**) Myc-RalA (Q72L) was transiently transfected into low-density MDCK cells and stained for endogenous RalA or harvested for immunoprecipitation with the ZONAB antibody as described in Figure 2A. **(C**) The reporter plasmids were transfected into high-density cells, together with an empty expression vector (vector) or the indicated vectors resulting in the expression of either an siRNA against ZONAB, the control duplex, or an expression vector for the Ral-binding domain (RBD) of RalBP1 either alone or in combination with both the siRNA against ZONAB or control duplex. Expression of the luciferases was assayed with a dual-luciferase assay system. Inset: Extracts of cells transfected with siRNA to ZONAB, or control as above were immunoblotted with anti-ZONAB and anti-β-tubulin antibodies. (**D**) The reporter plasmids were transfected into low-density cells together with either an active H-Ras allele (G12V), the Ral-binding domain (RBD) of RalBP1 or both of these constructs. The values in all panels represent means $\pm$ 1 s.d. of at least three independent experiments performed in triplicate. *P*-values obtained from *t*-tests mark experimental conditions that resulted in significant changes from control samples.

regulate epithelial proliferation and differentiation (Balda *et al*, 2003; Matter and Balda, 2003). Therefore, we explored the possibility that there is a requirement for RalA in tight junction integrity through introducing the dominant-negative Ral28N version of RalA. However, in agreement with a recent report, we could detect no change in two measures of junctional selective permeability, ZO-1 localisation and biotin permeability (data not shown), although a more subtle effect not revealed by these assays cannot be ruled out (Moskalenko *et al*, 2002).

RalA regulates the transcriptional activity of ZONAB

ZONAB has been shown to act as a transcriptional repressor regulating the activity of the ErbB-2 promoter via binding to an inverted CCAAT box. This regulation has been shown to depend on cell density (Balda and Matter, 2000): at low cell density, a pool of ZONAB is nuclear and represses transcription, while at high cell densities, relocation of ZONAB from the nucleus relieves transcriptional repression. In view of our observations that ZONAB can bind to RalA-GTP, and that the amount of this complex increases as cells become dense, we investigated whether RalA plays a role in the regulation of the transcriptional activity of ZONAB. To address this question, we used a reporter gene assay based on a pair of luciferase reporter constructs in which the expression of firefly luciferase was driven by a short version of the ErbB-2 promoter containing a functional ZONAB-binding site (ErbB-2: S) while *Renilla* luciferase was driven by an identical promoter harbouring a mutated ZONAB-binding site (Balda and Matter, 2000). Hence, manipulations that resulted in changes in the ratio of firefly to *Renilla* luciferase activity directly reflected effects on the ZONAB-binding site in the ErbB-2 promoter. These reporter constructs were cotransfected with either an empty expression vector (vector control), wild type or an activated version of RalA (Q72L), or a construct containing the Ral-binding domain (RBD) of RalBP1. This RalBP1-RBD construct behaves as a dominant interfering mutant by sequestering endogenous RalA-GTP and preventing it from forming complexes with effectors (Hofer *et al*, 1998; Wolthuis *et al*, 1998; Rosario *et al*, 2001).

Figure 4A shows that transfection of an active RalA mutant (Q72L) into cells cultured at low density, under which ZONAB functions as a transcriptional repressor (Balda and Matter, 2000), led to elevated expression of firefly luciferase, indicating a relief of ZONAB transcriptional repression. Figure 4B shows that this relief of repression is correlated with the formation of a ZONAB–RalA72L complex. Note that in these experiments transfection efficiency is 10–20% so that the level of the Ral72L–ZONAB complex in transfected cells will be much higher than that of the endogenous RalA–ZONAB complex with the consequence that more ZONAB will be bound to RalA-GTP in these cells. Interestingly, we found that unlike the endogenous RalA in low-density cells, exogenous RalA72L is localised at the plasma membrane (Figure 4B).

In contrast, under conditions of high cell density, when ZONAB does not repress transcription and there are increased levels of the RalA–ZONAB complex, expression of the RalBP1-RBD construct led to decreased transcription. These data indicate that interfering with Ral signalling leads to increased transcriptional repression. To demonstrate that this effect of expression of RalBP1-RBD is mediated by increased repression by ZONAB, we cotransfected an expression vector for an siRNA targeting ZONAB (Balda *et al*, 2003) with RalBP1-RBD. Figure 4C shows that this siRNA relieved the effects of RalBP1-RBD in increasing transcriptional repression whereas the control duplex plasmid did not. The RalBP1-RBD construct did not affect promoter activity in cells at low confluence (Figure 4A), which have low levels of the RalA–ZONAB complex, supporting the conclusion that the effect of expression of RalBP1-RBD is due to inhibiting RalA–ZONAB interactions. Importantly, since expression of RalBP1-RBD and siRNA targeting ZONAB interferes with the function of endogenous proteins, these results show that this signalling pathway operates under normal physiological conditions and not just on overexpression of active RalA. These reporter assays therefore demonstrate that the RalA–ZONAB complex plays a central role in the relief of transcriptional repression by endogenous ZONAB.

These results argue that RalA plays a role in the density-dependent regulation of gene expression by ZONAB. As we have shown that expression of oncogenic Ras leads to plasma membrane localisation of RalA in low-density cells (Figure 3C) and to RalA activation (Figure 2C), we examined whether oncogenic Ras would relieve transcriptional repression by ZONAB. Figure 4D shows that transfection of H-Ras (G12V) into MDCK cells at low density, where ZONAB would normally repress the ErbB-2 promoter, led to elevated transcriptional activity of the promoter. This derepression of transcriptional activity by H-Ras (G12V) was inhibited by coexpression of the RBD of RalBP1 to sequester RalA-GTP and thereby prevent it from forming complexes with ZONAB. These results indicate that H-Ras (G12V) can overcome transcriptional repression mediated by ZONAB and that this effect is mediated through activation of RalA.

In Figure 5, we suggest a model to account for the effects of RalA on transcriptional repression mediated by ZONAB. In proliferating cells growing at low density, ZONAB is present in the nucleus where it represses transcription through bind-

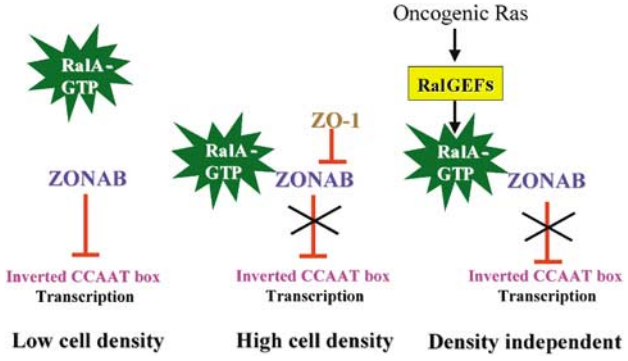


Figure 5 Proposed model for regulation of inverted CCAAT box transcription by the RalA/ZONAB complex. Under conditions of low cell density when RalA/ZONAB complex levels are low, ZONAB is present in the nucleus repressing transcription through binding to inverted CCAAT boxes. When cells become more dense, the RalA–ZONAB complex increases resulting in derepression of inverted CCAAT box-dependent transcription. Oncogenic Ras activates RalA-GTP and causes localisation to the plasma membrane leading to an increase in RalA–ZONAB complex and derepression. It is also known that ZONAB is complexed to ZO-1 under conditions of high cell density leading to derepression of inverted CCAAT box transcription. At present, it is not known how or if RalA and ZO-1 might interact to control transcriptional repression by ZONAB.

ing to inverted CCAAT boxes (Balda and Matter, 2000). Under these conditions, we show that little ZONAB is complexed with RalA. When cells become denser, ZONAB becomes complexed to ZO-1 and RalA, less ZONAB is found in the nucleus and transcriptional repression is relieved. Our data showing that blocking RalA function in dense cells leads to restoration of the repressive activity of ZONAB argue that the RalA–ZONAB interaction plays a major role in the regulation of ZONAB activity. The increase in RalA–ZONAB complexes as cells become denser shows that the amount of the complex is under regulation. On the basis of measurements of total RalA-GTP (Figure 2B), this change in the amount of the RalA–ZONAB complex does not appear to result from increased RalA-GTP but must reflect some other level of regulation. In dense cells, RalA is localised at the plasma membrane; so it is possible that the change in RalA localisation as cells become dense is responsible for the density dependence of complex formation with ZONAB. Consistent with this idea that the cellular localisation of RalA determines whether the RalA–ZONAB complex is formed, we find that when low-density cells are transfected with oncogenic Ras or activated RalA, RalA is found at the plasma membrane (Figures 3C and 4B) and there is relief of transcriptional repression by ZONAB. While it is clear from these studies that binding of RalA to ZONAB influences ZONAB function in transcriptional regulation, it remains to be determined by future studies whether RalA plays a role in other functions of ZONAB such as the binding of cell cycle regulators (Balda *et al*, 2003).

The experiments with an inhibitor of Ras signalling (Ad-CMV-Myc-Raf-1-RBD-Caax) show that the activation of RalA to the GTP-bound state does not appear to be a consequence of Ras signalling in untransformed MDCK cells (Figure 2C), suggesting that this activation is mediated either by RalGEFs that lack an RA domain such as RalGEF2 (de Bruyn *et al*, 2000) or a Ras-independent regulation of an RA-containing GEF (Hofer *et al*, 1998). However, expression of oncogenic Ras leads to an increase in RalA-GTP (Figure 2C), membrane localisation of RalA (Figure 3C) and a RalA-mediated relief of ZONAB-dependent transcriptional repression (Figure 4D), suggesting that oncogenic Ras can subvert the normal regulation of RalA activity and the RalA–ZONAB complex. By these means, oncogenic Ras signalling to RalA can overcome transcriptional repression by ZONAB. Interestingly, a recent study shows that Ras regulates the promoter of beta-galactoside alpha2,6-sialyltransferase (ST6Gal I) in a RalGEF-dependent manner (Dalziel *et al*, 2004). Examination of the promoter of this gene shows that it contains an inverted CCAAT box, which is a possible ZONAB-binding site.

A number of studies have shown that signalling through Ral is an important contributor to oncogenesis by Ras (Urano *et al*, 1996; Aguirre-Ghiso *et al*, 1999; Hamad *et al*, 2002; Rangarajan *et al*, 2004). However, the mechanism through which this occurs is not clear. Ras signalling to RalA leading to derepression of inverted CCAAT box-containing promoters regulated by ZONAB may provide at least in part such a mechanism.

Materials and methods

Cell culture

MDCK cells were maintained in DMEM cell culture medium (Life Technologies) with 10% fetal calf serum (FCS) (Life Technologies) and antibiotics at 37°C. For density experiments, MDCK cells were plated at different densities and harvested simultaneously at either 48 or 72 h later.

Plasmids

The rat pituitary cDNA library, pYes2 (Invitrogen) and the methionine expression plasmid, p425-Met25, were previously described (Hubsman *et al*, 2001). The different RalA constructs were subcloned into p425-Met25 by *Hin*dIII–*Xho*I. The different ZONAB constructs were subcloned into pYesRas by *Eco*RI–*Xho*I. The library represents over 4×10^6 independent cloning events. All RalA and ZONAB variants were expressed in mammalian cells using pRK5 or pCB6 and are as previously described (Balda and Matter, 2000; Rosario *et al*, 2001, #58). The sequence encoding the Ral-binding domain (RBD) of RalBP1/RLIP76 (amino acids 397–518) was inserted into pRK5-myc as previously described (Wolthuis *et al*, 1998). The Ras-binding domain (RBD) of Raf-1 with a K-Ras Caax motif (Ad-CMV-Myc-Raf-1-RBD-Caax) or the Raf-1-RBD harbouring the mutation (R89L) that inhibits binding to Ras-GTP (Ad-CMV-Myc-Raf-1(R89L)-RBD-Caax) was inserted into the pAd-CMV-Myc vector (Stratagene) using standard cloning techniques.

Adenoviral infection

Recombinant adenoviruses were generated using the Adeasy™ system (Stratagene) according to the manufacturer's instructions. MDCK cells grown to 100% confluence were exposed to adenovirus (2000 PFU/ml) for 90 min followed by replenishment with 10% DMEM. Cells were harvested 24 h after infection and analysed for RalA-GTP levels as described (see pull-down assays) and phosphorylated ERK (ppERK) levels using a phosphospecific antibody (Sigma).

Preparation of bacterially expressed recombinant proteins.

For S-His-RalA, the ORF of simian RalA (G23V) was PCR cloned into the vector pTriEx4 (Novagen). A 10 ng portion of pTriEx4RalAv23 was used to transform *E. coli* strain C41(DE3). The transformed cells were inoculated into 1 l 2 × YT medium and grown at 37°C to an OD_{600} of 0.4. Cells were cooled to 20°C before inducing RalA expression with 100 μM IPTG. RalA was expressed for 16 h before cells were pelleted and stored at −20°C. Cells were lysed for 1 h in 25 ml of buffer A (25 mM Tris–HCl, 500 mM NaCl, 10 mM imidazole, 2% (v/v) Triton X-100, 10 mg lysozyme, 250 U Benzonase (Novagen), pH 8.0). The cell lysate was clarified by centrifugation at 10 000 *g* for 1 h at 4°C. The supernatant was applied to a 5 ml HiTrap chelating column (nickel loaded) on an Akta Explorer 100 (Amersham Biosciences). Specifically bound protein was eluted with buffer B (25 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0). The S-His-RalA (G23V) protein was concentrated to 30 mg/ml in a Vivascience spin concentrator (10 000 MWCO) and injected onto a Superdex 75 (26/60) (Amersham Biosciences) gel filtration column in buffer C (25 mM Tris–HCl, 150 mM NaCl, 1 mM TCEP, 1 mM $MgCl_2$). S-His-RalA (G23V) was collected as the major protein peak and stored at 4°C. GST-ZONAB-A was prepared as previously described (Balda and Matter, 2000).

Immunoprecipitations

Immunoprecipitations were performed at 4°C. Wild-type MDCK cells were harvested and lysed with extraction buffer (PBS containing 1% Triton X-100, 40 μg/ml phenylmethylsulphonyl fluoride (PMSF), 10 mM sodium fluoride, 10 mM pyrophosphate and 1 mM sodium vanadate). Since protein content of MDCK cells changes with increasing cell density, inputs were normalised to equal cell numbers rather than cell protein. After centrifugation, the supernatants were transferred to tubes containing a rabbit polyclonal anti-ZONAB antibody covalently coupled to Sepharose beads (Balda and Matter, 2000). After incubating for 2 h, the beads were washed twice with extraction buffer and once with PBS. Bound proteins were eluted by boiling in SDS–PAGE sample buffer lacking dithiothreitol (DTT) to avoid partial elution of the antibody. The eluates were transferred to fresh tubes, 0.1 M DTT was added and the samples were analysed by SDS–PAGE and immunoblotting. Western blots were quantitated using a Cy5-labelled antibody and capturing the signal with a Phosphorimager.

Pull-down assays

For analysis of RalA-GTP levels, lysates from equal numbers of cells were incubated for 1 h at 4°C with glutathione–Sepharose beads

that had been precoupled to recombinant GST-RalBP1-RBD as previously described (Wolthuis *et al*, 1998). The beads were washed three times in large volumes of NP-40 buffer, and bound endogenous RalA-GTP was analysed by Western blotting with an anti-RalA antibody (BD-Transduction Laboratories). Western blots were quantitated using a Cy5-labelled antibody and capturing the signal with a Phosphorimager. For the *in vitro* binding studies, 100 nM of S-His-RalA (G23V) was loaded with 10 mM of either GTP or GDP (Sigma) in nucleotide loading buffer containing Tris–HCl (pH 7.5, 100 mM), EDTA (15 mM), BSA (1 mg/ml) and TCEP (2 mM) at 37°C followed by addition of 50 mM $MgCl_2$ and incubation in binding buffer containing Tris–HCl (pH 7.5, 50 mM), KCl (150 mM), TCEP (1 mM), Triton X-100 (1%), BSA (2 mg/ml) and $MgCl_2$ (5 mM) at 4°C for 45 min with glutathione–Sepharose beads (Amersham Biosciences) that had been precoupled to either recombinant GST-ZONAB-A or the control GST-Pak (N-term regulatory domain). The beads were washed three times in wash buffer containing Tris–HCl (pH 7.5, 50 mM), KCl (150 mM), TCEP (1 mM) and Triton X-100 (1%), and bound S-His-RalA was analysed by Western blotting with an anti-RalA antibody (BD-Transduction Laboratories).

Antibodies and immunofluorescence

Mouse monoclonal anti-RalA and anti-Ras were from BD Biosciences, ERK2 and phosphospecific ERK were from Sigma and rabbit polyclonal ZO-1 was from Zymed. Anti-ZONAB N-terminus antibody has been described previously (Anderson *et al*, 1988; Balda and Matter, 2000, #79). Fluorescein (FITC) goat anti-mouse, Texas red goat anti-rabbit, Cy5 donkey anti-rat and Cy5 donkey anti-mouse were from Jackson ImmunoResearch. Cells were fixed and permeabilised with methanol at −20°C without pre-extraction, incubated with antibodies and mounted as previously described (Balda and Matter, 2000). Images were acquired using a Biorad MRC1024 confocal microscope.

Reporter gene assays

A short version of the ErbB-2 promoter containing a functional ZONAB-binding site was used to drive firefly luciferase expression (ErbB-2: S; Balda and Matter, 2000). A mutant promoter of the same length but in which the ZONAB-binding site had been inactivated by substituting the inverted CCAAT box (ErbB-2: S-A; Balda and Matter, 2000) was used to replace the CMV promoter in pRL-CMV (Promega). This construct was then used as a control promoter driving the expression of *Renilla* luciferase. For reporter assays, MDCK cells were plated at low or high densities, and the reporter plasmids were then cotransfected the next day by calcium phosphate precipitation (Balda and Matter, 2000). The expression vectors to manipulate RalA activation were cotransfected as indicated, and the total DNA concentrations of all samples were equalised with empty expression vector. The ZONAB and control RNAi plasmids were described previously (Balda *et al*, 2003). The incubations of the cells with the precipitates were performed overnight, and expression of the luciferases was assayed 30 h after removing the precipitates using a double luciferase reporter assay system that allows the sequential determination of firefly and *Renilla* luciferase activities from the same sample (Promega).

Acknowledgements

We thank Steven Hooper for constructing the adenoviruses, and Georgia Mavria, Ana Gonzalez-Garcia, Joana Barros and Simon Wilkinson for helpful discussions. PF and CJM are supported by Cancer Research UK. CJM is a Gibb Life Fellow of Cancer Research UK. MB and KM are supported by the Wellcome Trust. AA is supported by The Israel Science Foundation (grant no. 534/01-1).

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