Stress- and Rho-activated ZO-1–associated nucleic acid binding protein binding to p21 mRNA mediates stabilization, translation, and cell survival

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A central component of the cellular stress response is p21$^{WAF1/CIP1}$, which regulates cell proliferation, survival, and differentiation. Inflammation and cell stress often up-regulate p21 posttranscriptionally by regulatory mechanisms that are poorly understood. ZO-1–associated nucleic acid binding protein (ZONAB)/DbpA is a Y-box transcription factor that is regulated by components of intercellular junctions that are affected by cytokines and tissue damage. We therefore asked whether ZONAB activation is part of the cellular stress response. Here, we demonstrate that ZONAB promotes cell survival in response to proinflammatory, hyperosmotic, and cytotoxic stress and that stress-induced ZONAB activation involves the Rho regulator GEF-H1. Unexpectedly, stress-induced ZONAB activation does not stimulate ZONAB's activity as a transcription factor but leads to the posttranscriptional up-regulation of p21 protein and mRNA. Up-regulation is mediated by ZONAB binding to specific sites in the 3′-untranslated region of the p21 mRNA, resulting in mRNA stabilization and enhanced translation. Binding of ZONAB to mRNA is activated by GEF-H1 via Rho stimulation and also mediates Ras-induced p21 expression. We thus identify a unique type of stress and Rho signaling activated pathway that drives mRNA stabilization and translation and links the cellular stress response to p21 expression and cell survival.

epithelia | RhoGTPases | tight junction | necrosis | apoptosis

Cells developed regulatory pathways that are activated in response to proinflammatory challenges, adverse extracellular conditions, and cytotoxic stress to ensure cell survival and tissue integrity. These pathways regulate expression of genes encoding factors required to cope with stress conditions and involve transcription factors that stimulate the synthesis of mRNAs encoding proteins required for specific responses. However, expression of many crucial stress-induced genes is regulated posttranscriptionally by mechanisms that are not well understood.

A central regulator of cell proliferation and survival is p21$^{WAF1/CIP1}$, a protein up-regulated in response to inflammation, stress, and cell cycle entry (1). Although originally identified as a cell cycle inhibitor, p21 has multiple activities that link it to the regulation of proliferation, transcription, DNA repair, and cell death (1, 2). Control of p21 mRNA stability and translation is important for the cellular stress response, but the regulatory pathways that control these processes are not well understood (3).

Rho GTPases are central components of multiple subcellular signaling pathways and are known to regulate gene expression at the transcriptional level (4–6). Rho signaling also inhibits transcription of p21 by suppressing its promoter (7, 8); however, p21 protein and mRNA are often up-regulated in response to stimuli that activate Rho, such as TNFα, growth factor-induced cell cycle entry, and, depending on the cellular context, Ras activation (3, 4, 9). Hence, mechanisms must exist that can counteract Rho-stimulated inhibition of transcription. Although crucial for the understanding of p21 regulation and cell physiology, the underlying molecular mechanisms to explain these contradictory observations are not known.

A transcription factor activated by Rho signaling is ZO-1–associated nucleic acid binding protein (ZONAB)/DbpA, a member of the Y-box family of multifunctional nucleic acid binding proteins (10–13). ZONAB is activated in different types of cancers (14–20); hence, it would be important to know whether and how ZONAB promotes cell survival in response to cytotoxic stress and proinflammatory signals because targeting such survival pathways offers therapeutic benefits.

In epithelial cells, ZONAB associates with tight junctions by binding to ZO-1, a negative regulator, and its transcriptional activity is stimulated by GEF-H1, a RhoA exchange factor (10, 11). The transcriptional activity of ZONAB is also suppressed by binding to activated RalA in the cytoplasm downstream of Ras activation (21). GEF-H1 is activated by different stimuli including TNFα, EGF receptor and Erk signaling, and mechanical and environmental stress (11, 22–24). Although Y-box factors are multifunctional proteins that can bind RNA in vitro and have been proposed to function as general translational repressors (12, 13, 25), physiological relevance and targets of this mechanism are unclear. Whether and how GEF-H1, RhoA, and ZONAB regulate translation and mRNA stability is also not known.

Here, we identify a unique type of stress-activated pathway that consists of Rho-activated, sequence-specific ZONAB binding to p21 mRNA, resulting in mRNA stabilization and enhanced translational efficiency. This pathway is required for cell survival during stress and represents a unique type of Rho-activated mechanism.

Results

Cell Survival During Proinflammatory Signaling and Stress Is Regulated by ZONAB and GEF-H1. TNFα and cellular stress such as external mechanical forces and infections activate GEF-H1, a guanine nucleotide exchange factor that mediates RhoA activation, and that, in turn, stimulates the transcription factor ZONAB (11, 22–24, 26–28). Therefore, we asked whether ZONAB is activated by GEF-H1 during the cellular stress response and is thus required for cell survival during stress. As a model system, we first used renal epithelial Madin–Darby canine kidney (MDCK) cell lines that allow the selective shRNA-mediated depletion of ZONAB and GEF-H1 expression (29, 30) and then assessed cell death by LDH release as a measure of necrosis, caspase 3/7 activation as an indicator of apoptosis, and redox activity to monitor metabolic activity.

Fig. 1*A* shows that both proteins were effectively depleted in cell lines expressing respective shRNAs. Necrosis and apoptosis

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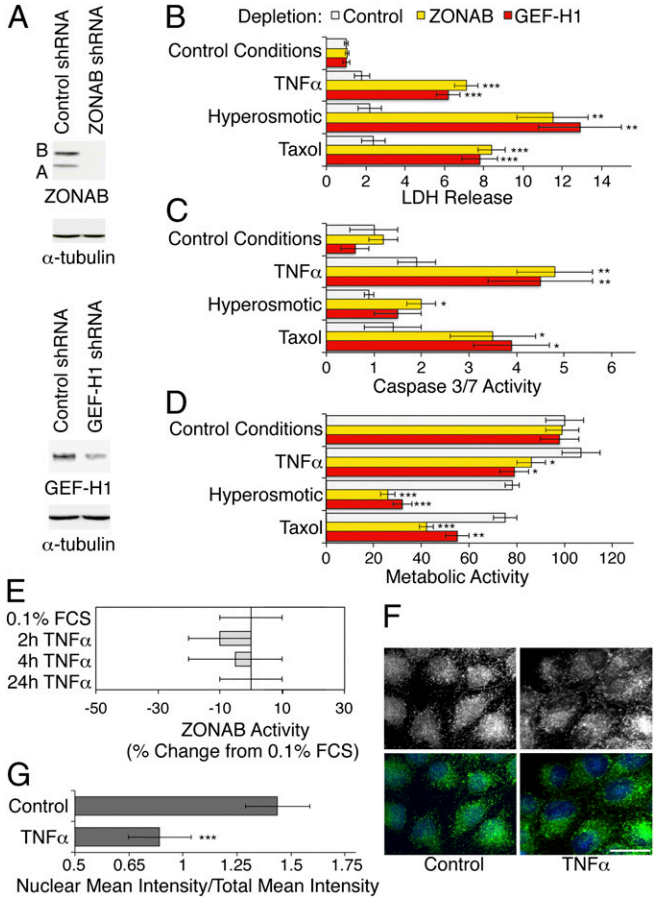

Fig. 1. Regulation of cell survival by ZONAB. (*A*) Depletion of ZONAB and GEF-H1 in MDCK cell lines expressing control or specific shRNAs under the control of a tetracycline-regulated promoter. (*B–D*) MDCK cells were treated as in *A* and were then incubated for 20 h with either normal medium or medium supplemented with 10 ng/mL TNFα, 150 mM NaCl (i.e., 600 mOsm), or 1 μM Taxol. Cells were then analyzed for LDH release (*B*), Caspase 3/7 activation (*C*), or metabolic activity (*D*). All values are expressed relative to control MDCK cells kept in normal medium and are means ± 1 SD, $n = 4$. (*E*) Confluent MDCK cells were transfected with a *firefly* luciferase reporter construct containing a ZONAB-responsive promoter and a control encoding *renilla* luciferase driven by a promoter lacking a ZONAB binding site but that is otherwise identical. Cells were then incubated as indicated before measuring expression of the luciferases. ZONAB activity was normalized to control cells. Shown are means ± 1 SD, $n = 4$. (*F*) Control and TNFα-treated cells fixed and processed for immunofluorescence using antibodies against ZONAB. Nuclei were stained with Hoechst 33258. Shown are epifluorescence images. (Scale bar: 20 μm.) (*G*) Images such as those in *F* were quantified by measuring the mean intensities over the nucleus and the total area of the cells and calculating the ratios. Shown are means ± 1 SD, $n = 7$ (seven fields were quantified with at least 12 cells per field). Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

assays revealed that the proinflammatory cytokine TNFα, hyperosmotic, and taxol-induced cytotoxic stress selectively induced cell death in ZONAB and GEF-H1 depleted cells along with a decrease in metabolic activity of the cultures (Fig. 1 *B–D*). Survival of nonstressed cells was not affected by depletion of either protein as reported (31). Experiments using transient transfection of siRNAs, targeting different sequences as an alternative strategy for ZONAB depletion, also enhanced cell death in response to stress (Fig. S1 *A–C*). Transfection reagents are well-known to induce cell stress and death (32); consequently, transfection itself already led to an increase in cell death upon ZONAB depletion. ZONAB expression is thus required for survival of MDCK cells during stress.

We next analyzed the role of ZONAB in two human epithelial cell lines to determine whether the survival function of ZONAB extends beyond kidney cells, which need to be well adapted to cope with conditions causing hyperosmotic stress. Fig. S1 *D–F* demonstrates that survival of human corneal epithelial (HCE) cells and the intestinal epithelial adenocarcinoma cell line Caco-2 cells was similarly comprised by ZONAB depletion, indicating that ZONAB mediates survival of cells form different epithelial tissues.

We next used GEF-H1 targeting siRNAs to test the role of ZONAB downstream of GEF-H1. In control cells, depletion of GEF-H1 using siRNAs also led to increased cell death upon TNFα treatment (Fig. S2 *A* and *B*). In cells overexpressing ZONAB, however, no significant increase was observed upon GEF-H1 depletion, suggesting that overexpression can counteract depletion of GEF-H1 (Fig. S2*C*).

TNFα and Stress Do Not Stimulate the Transcriptional Activity of ZONAB. ZONAB is a Y-box transcription factor that is active in proliferating cells and stimulates expression of genes that promote proliferation such as cyclin D1 and PCNA (10, 29, 31). Activation of GEF-H1 in proliferating cells leads to the dissociation of ZONAB/GEF-H1 complexes (11). TNFα and hyperosmotic shock also reduced coimmunoprecipitation of GEF-H1 with ZONAB (Fig. S3). Hence, we tested whether stress induces the transcriptional activity of ZONAB by using a dual luciferase reporter assay, in which firefly luciferase expression is driven by a promoter with a ZONAB binding site, and the control renilla luciferase by a promoter that lacks such a site but is otherwise identical (21). To increase the sensitivity of the assay, cells were first serum-starved to reduce ZONAB activity and then stimulated with TNFα.

Fig. 1*E* shows that stimulation of cells with the cytokine for different periods of time did not lead to differences in luciferase expression, indicating that the transcriptional activity of ZONAB was not increased. When proliferating cells in which ZONAB is active were stressed, the transcriptional activity of ZONAB was attenuated (Fig. S4*A*). Similarly, transcription from a full-length cyclin D1 promoter, which is stimulated by ZONAB, was attenuated (Fig. S4*B*). Stress thus leads to a reduction in ZONAB's transcriptional activity.

ZONAB is regulated by its subcellular localization. In proliferating cells, ZONAB is enriched in the nucleus and transcriptionally active and increased RalA activation or ZO-1 expression leads to cytoplasmic sequestration (21, 31). Indeed, TNFα also induced a redistribution of ZONAB from the nucleus to the cytoplasm (Fig. 1 *F* and *G*). The junctional staining for ZONAB was also reduced, and the staining was enriched in the perinuclear cytoplasm (Fig. S5).

These results thus indicate that the cell survival function of ZONAB does not rely on its transcription factor function but involves a cytosolic activity of the protein.

Induction of p21 Expression and Cell Survival. A protein crucial for cell survival that is up-regulated during stress and proinflammatory signaling is p21, because it inhibits apoptosis by attenuating the activation of caspase-3 and blocks Ask-1 (1, 33, 34). p21 expression is regulated at different levels including post-transcriptonal mechanisms (3). Therefore, we tested whether induction of p21 by stress depends on ZONAB and GEF-H1. Fig. 2*A* shows that stimulation with TNFα led to a ZONAB and GEF-H1–dependent up-regulation of p21 protein. Similar observations were made when cells were stressed or stimulated with EGF, hyperosmotic shock, or taxol (Fig. 2*B*). Thus, p21 expression in response to different types of stress, as well as cytokine and growth factors, is regulated by ZONAB and GEF-H1.

If p21 is a functionally important target, depletion of p21 should affect cell survival. We hence depleted p21 by transfecting specific siRNAs (Fig. 2*C*) and then analyzed induction of necrosis and apoptosis (Fig. 2 *D* and *E*). If p21 was depleted in MDCK, HCE, and Caco-2 cells, stress induced cell death (Fig. 2 *D* and *E*).

Thus, p21 is a functionally important component of the cellular stress response in epithelial cells that is up-regulated in a ZONAB and GEF-H1–dependent manner. Because the

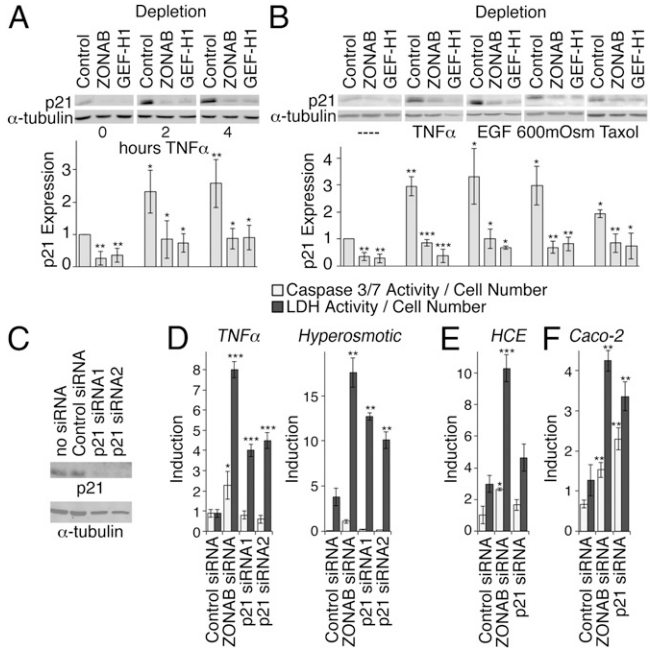

Fig. 2. Regulation of p21 expression and cell survival. (*A*) Control, ZONAB, or GEF-H1 depleted cells were serum starved for 20 h and were then stimulated by TNFα as indicated. Expression of p21 and α-tubulin was determined by immunoblotting total cell extracts. The bar graph shows a quantification of immunoblots ($n = 3$; all values were normalized to control cells that had not been treated with TNFα) (*B*) Cells were treated and processed as in *A*, but incubated for 24 h as indicated. For the quantification, all values were normalized to control cells that had not been stressed ($n = 3$). (*C* and *D*) MDCK cells were transfected as indicated. After 3 d, the cells were analyzed by immunoblotting (*C*) or assayed for cell numbers, LDH secretion, and Caspase 3/7 activation after TNFα stimulation or hyperosmotic shock (*D*) ($n = 4$). LDH and Caspase values were divided by cell numbers. (*E* and *F*) Caco-2 and HCE cells were analyzed as MDCK cells in *D* after a hyperosmotic shock ($n = 4$). All graphs show means ± 1 SD. Statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

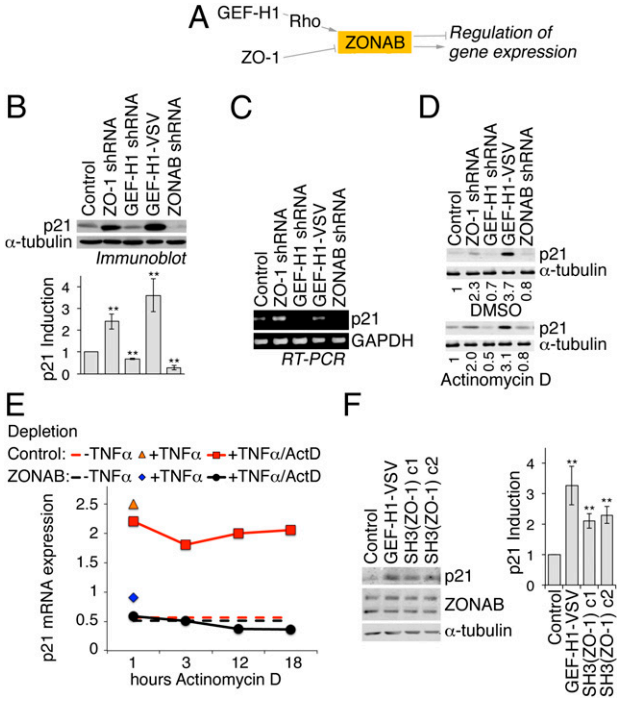

Fig. 3. Posttranscriptional regulation of p21 expression. (*A*) Scheme showing the role of GEF-H1 and ZO-1 in the regulation of ZONAB and gene expression. (*B–D*) The indicated MDCK cell lines were analyzed by immunoblotting (*B* and *D*) or RT-PCR (*C*). In *D*, the solvent control DMSO and actinomycin D (ActD) were added as indicated. In *B*, three experiments were quantified, normalizing all values to control cells. In *D*, averages of two experiments are indicated underneath the respective lanes. (*E*) Control and ZONAB-depleted cells were incubated with or without actinomycin D. and, if indicated, TNFα for 1 h before the addition of actinomycin D. Expression of p21 mRNA was then analyzed by reverse transcription and real-time PCR. Indicated values represent averages of duplicate cultures. The dashed lines represent the baselines obtained from samples not treated with TNFα ($n = 8$). (*F*) Expression of p21 was analyzed by immunoblotting in cells expressing VSV-tagged GEF-H1 or two clones expressing the SH3 domain of ZO-1, an inhibitor of the transcriptional activity of ZONAB. For the quantification, all values were normalized to control cells ($n = 3$). Graphs show means ± 1 SD; statistical significance: $*P < 0.05$; $**P < 0.01$.

relative extents of necrosis and apoptosis differed between ZONAB and p21 depletion and between different types of stress and cell lines, however, p21 is unlikely to be the only stress-induced ZONAB target.

Posttranscriptional Regulation of p21 Expression. We next analyzed the molecular mechanism by which ZONAB induces the expression of p21 by determining the effect of ZONAB and its regulators on p21 protein and mRNA expression. We used previously established conditional MDCK cell lines that allow the down-regulation of ZO-1, a junction-associated inhibitor of ZONAB; the overexpression or down-regulation of GEF-H1, an activator of ZONAB; or depletion of ZONAB (Fig. 3*A*) (29, 30).

ZONAB activation by either depletion of its inhibitor ZO-1 or expression of its activator GEF-H1 stimulated, whereas depletion of GEF-H1 or ZONAB itself reduced p21 protein and mRNA expression (Fig. 3 *B* and *C*). The relative expression levels remained the same even when transcription was attenuated with actinomycin D, suggesting that regulation of p21 expression by ZONAB is a posttranscriptional process (Fig. 3*D*). Expression was lost when translation was inhibited with cycloheximide, suggesting that the up-regulation is not due to an inhibition of degradation of p21 protein (Fig. S6*A*). Real-time PCR experiments further demonstrated that TNFα stimulation led to a ZONAB-dependent increase in p21 mRNA levels that remained stable even after prolonged inhibition of transcription, whereas ZONAB depletion resulted in a rapid drop in mRNA expression to levels observed in unstimulated cells (Fig. 3*E*).

Our data suggest that ZONAB mediates stabilization of p21 mRNA and posttranscriptonal up-regulation of p21 protein expression. These observations are in agreement with previous work suggesting that mRNA stabilization is important for the TNFα-induced p21 induction (9). Nevertheless, we also measured the effect of ZONAB on the p21 promoter itself by performing luciferase reporter assays with a construct containing 2.4 kB of the p21 promoter region (7). Fig. S6*B* shows that depletion of ZONAB did not affect the activity of the promoter, further supporting the conclusion that ZONAB does not regulate p21 on the transcriptional level. Finally, we made use of MDCK cells expressing the SH3 domain of ZO-1, a cytosolic construct that binds ZONAB, preventing its nuclear accumulation and, thereby, inhibits transcription (10, 31). Immunoblotting revealed that expression of the SH3 domain was sufficient to induce p21 (Fig. 3*F*), indicating that, in contrast to transcription, cytoplasmic retention of ZONAB does not inhibit p21 induction and ZONAB's role in the posttranscriptional regulation of gene expression.

Stimulation of p21 mRNA/ZONAB Complexes. Up-regulation of p21 mRNA requires ZONAB; hence, we tested whether the two molecules form complexes in response to ZONAB activation. We immunoprecipitated ZONAB complexes from extracts derived from MDCK cells with reduced or increased GEF-H1 and ZONAB expression. The presence of specific mRNAs in isolated immunocomplexes was then tested by reverse transcription PCR.

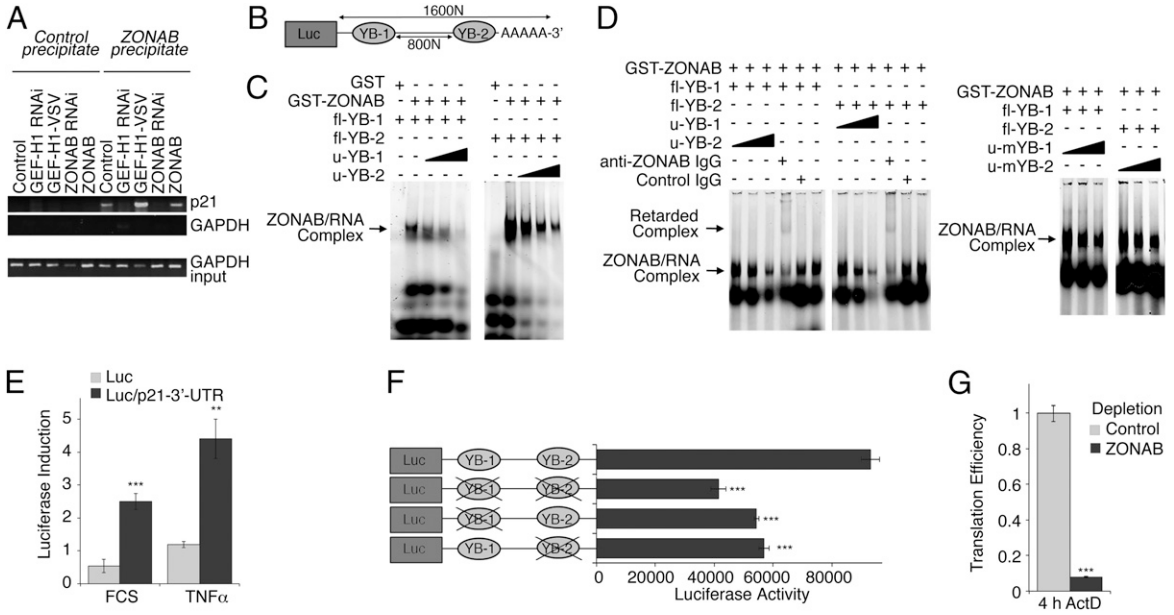

Fig. 4. Binding of ZONAB to p21 mRNA. (*A*) The indicated cell lines were incubated as for the experiments in Fig. 3*C*. The cells were then extracted and precipitated with control or anti-ZONAB IgG beads. Total mRNAs were extracted from precipitates and samples from the inputs. The amounts of p21 and GAPDH mRNAs were then determined by RT-PCR. (*B*) Scheme of the chimeric luciferase/p21 3′-UTR construct. Indicated are the two predicted Y-box factor binding sites, which are an approximate size of the 3′-UTR. Note that all known human p21 transcripts contain the two predicted binding sites. (*C* and *D*) RNA electrophoretic mobility shift assays were performed with fluorescent oligonucleotides representing the predicted Y-box binding sites (fl-YB-1 and fl-YB-2) and purified recombinant ZONAB (GST-ZONAB; GST was used as a control). In *C*, binding was quenched with the respective unlabeled oligonucleotides (u-YB-1 or u-YB-2). In *D*, quenching experiments were performed with unlabeled wild-type (u-YB-1 or u-YB-2) and mutant (u-mYB-1 or u-mYB-2) oligonucleotides. (*E*) Induction of luciferase from constructs without (Luc) or with the entire p21 3′-UTR (Luc/p21-3′-UTR) was determined after treating serum-starved cells with either serum or TNFα. (*F*) Expression of luciferase was measured after transfection of chimeric constructs containing either wild-type or the indicated mutant 3′-UTRs. (*G*) The translational efficiency of the chimeric Luc/p21-3′-UTR construct was determined in TNFα-treated cells after 4 h of actinomcyin D treatment. Expression of mRNA was determined by reverse transcription and real-time PCR, and luciferase protein was determined by measuring luciferase activity. All graphs show means ± 1 SD, $n = 4$; statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$.

Fig. 4*A* shows that p21 mRNA was detected in ZONAB immunoprecipitates from control, but not ZONAB or GEF-H1–depleted cells. If ZONAB was stimulated by overexpression of GEF-H1, the association of p21 mRNA with ZONAB was enhanced. A control mRNA encoding GAPDH was not detected in the ZONAB precipitates. Thus, activated ZONAB forms complexes with p21 mRNA.

Y-box factors can bind nucleic acids in vitro by targeting sequences with central C(N)CAUC motifs in RNA (35). Sequence analysis revealed that all known human p21 transcripts have two such potential binding sites in their 3′-UTR (Fig. 4*B*). In electrophoretic mobility shift assays with fluorescently labeled RNA oligonucleotides, probes representing these sites bound to GST-ZONAB, but not GST, and both probes could quench binding if added in nonlabeled form to either of the two fluorescent probes as long as the central binding motif was not mutated by substitution, indicating that binding was specific (Fig. 4 *C* and *D*). Addition of antibodies against ZONAB resulted in a supershift, confirming the presence of ZONAB in the reconstituted complexes (Fig. 4*D*). ZONAB can thus bind single-stranded RNAs corresponding to specific regions of the p21 mRNA 3′-UTR.

We next constructed a chimeric reporter to determine the functional importance of the 3′-UTR and the ZONAB binding sites suggested by the in vitro binding studies. Addition of the entire 3′-UTR of human p21 mRNA to a luciferase cDNA resulted in an induction of luciferase expression in response to growth factors or TNFα (Fig. 4*E*). Full activity of the 3′-UTR was only achieved if both ZONAB binding sites were present but not if they were substituted (Fig. 4*F*), indicating that the p21-3′-UTR mediates induction of protein. Quantifications of luciferase protein and mRNA expression after TNFα treatment further demonstrated that the presence of ZONAB resulted in increased translational efficiency of the chimeric mRNA (Fig. 4*G*).

These experiments demonstrate that ZONAB activation leads to the formation of p21 mRNA/ZONAB complexes via specific binding sites in the 3′-UTR, leading to p21 mRNA stabilization and enhanced translation.

Stimulation of p21 Expression by Rho and Ras Signaling. p21 protein and mRNA are up-regulated in response to many stimuli that also activate Rho, such as TNFα, and, depending on the cellular context, Ras activation; however, Rho signaling inhibits transcription of p21 (7, 8). For example, stimulation of cell cycle entry by Ras activation leads to Rho activation and increased expression of p21, which is required for efficient cell cycle progression (4). The mechanism that drives p21 up-regulation under these conditions is unknown.

Because ZONAB is activated by the Rho activator GEF-H1 and GEF-H1 stimulates p21 expression, we first tested whether GEF-H1 mediates p21 up-regulation via Rho and ZONAB. Fig. 5*A* shows that increased expression of p21 in response to GEF-H1 transfection required ZONAB because it was strongly reduced in ZONAB-depleted cells. Inhibition of Rho by C3 transferase suppressed p21 expression in control and GEF-H1 overexpressing cells to similar levels, indicating that GEF-H1 function is Rho dependent (Fig. 5*B*). GEF-H1 thus stimulates p21 expression via a Rho- and ZONAB-dependent mechanism.

TNFα is a strong inducer of Rho signaling and activates GEF-H1 via EGF receptor and Erk (11, 23, 24). EGF release in response to TNFα is mediated by TACE, a process that depends on p38 activation (24, 36). Phosphorylation of GEF-H1 in response to TNFα is also observed in MDCK cells (Fig. S7).

To test the role of MAP kinases and Rho activation, we first asked whether the regulatory activity of the 3′-UTR is regulated by Rho using the chimeric luciferase 3′-UTR construct. Fig. 5*C* shows that transfection of a constitutively active RhoA mutant stimulated the reporter. Moreover, TNFα and growth factor-

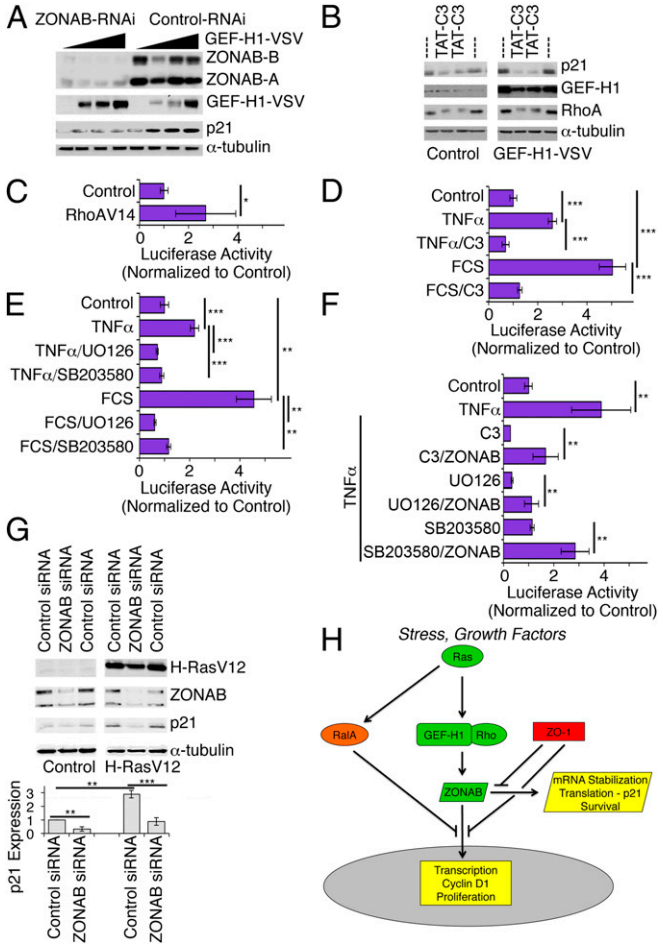

Fig. 5. Regulation of p21 expression by Rho and Ras signaling. (*A*) Control and ZONAB-depleted cells were transfected with increasing amounts of a plasmid encoding GEF-H1. Extracts were then immunoblotted as indicated. (*B*) Control or GEF-H1–overexpressing cells were incubated with TAT-C3 to inhibit Rho signaling for 16 h, and were then analyzed by immunoblotting. (*C–F*) MDCK cells transfected with the Luc/p21-3′-UTR construct were incubated as indicated before measuring expression of luciferase activity. Control refers to cells kept in low serum without stimulation (0.1%). In *F*, ZONAB was cotransfected with the reporter as indicated. Values were normalized to controls. All graphs show means ± 1 SD, $n \geq 4$. (*G*) Control or H-RasV12–expressing MDCK cells were transfected with control or ZONAB targeting siRNAs and analyzed by immunoblotting. Expression of p21 was quantified and normalized to control cells transfected with control siRNAs ($n = 3$). (*H*) Regulation of the ZONAB pathway during stress. Activation of Ras leads to stimulation of ZONAB via GEF-H1 and Rho activation, stimulating the transcriptional and posttranscriptional roles of ZONAB. Ras also activates RalA, which functions as a tuner of ZONAB's transcriptional activity by regulating its nuclear accumulation. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

induced luciferase expression were blocked by the Rho inhibitor C3 transferase (Fig. 5*D*). Inhibition of Erk with UO126 and, to a lesser extent, inhibition of p38 with SB203580 attenuated p21 protein expression in response to TNFα (Fig. S8 *A* and *B*); these inhibitors also blocked up-regulation of the chimeric luciferase mRNA by TNFα and growth factors (Fig. 5*E*). Induction of p21 expression by ZONAB overexpression was not affected by UO126, which is compatible with ZONAB functioning downstream of Erk (Fig. S8*C*). These data thus indicate that TNFα and growth factors regulate the activity of the 3′-UTR via Erk and Rho activation. Expression of constitutively active RhoA could counteract the effect of the MAP kinase inhibitors, suggesting that it functions downstream p38 and Erk (Fig. S9*A*). Moreover, overexpression of ZONAB stimulated luciferase expression in inhibitor treated cells, compatible with a role of the Y-box factor downstream of Erk and Rho activation (Fig. 5*F*). Blocking Erk activation also prevented complex formation by ZONAB and p21 mRNA, supporting the results with the reporter construct (Fig. S9*B*).

To test whether up-regulation of p21 by Ras is ZONAB dependent, we transfected MDCK cells with an active form of Ras, H-RasV12, and monitored the expression of p21. Expression of increasing amounts of active Ras led to increasing levels of p21 expression (Fig. S10*A*). However, if ZONAB was depleted, cells expressing H-RasV12 failed to up-regulate p21 (Fig. 5*G*). ZONAB is thus required for p21 induction stimulated by constitutively active Ras signaling. Unlike transcription, which is inhibited by Ras-stimulated Ral activation (21), the activity of the 3′-UTR reporter was not significantly affected by the expression of an inhibitor of Ral signaling (Fig. S10*B*). This observation suggests that, in contrast to transcription, the posttranscriptional activity of ZONAB is not inhibited by Ral signaling.

Discussion

We have identified a cytosolic stress response pathway that mediates cell survival by inducing p21 expression in a Rho-dependent manner by mRNA stabilization and increased translational efficiency. In response to Rho activation, the Y-box factor ZONAB complexes with p21 mRNA by binding to specific sites in the 3′-UTR (Fig. 5*H*). This mechanism thus links specific mRNA stabilization and regulation of translational efficiency to the Rho signaling network.

p21 represents a functionally important ZONAB target that has been shown to affect apoptosis by regulating the activation of caspase-3 (33). It is intriguing that depletion of p21 can also lead to necrosis, suggesting that it does not only function as an inhibitor of apoptosis but plays a more general role in cell survival. However, the molecular mechanisms that control and mediate necrosis are still not well understood (37, 38). The extent of necrosis and apoptosis differed in different cell lines and in response to different types of stress, suggesting that the here-identified pathway cross-talks with other survival mechanisms and, as expected, is influenced by the metabolic state of the cells (37, 39). Despite the functional importance of p21, depletion of ZONAB induced necrosis and apoptosis more strongly than depletion of p21. It will thus be important to identify additional ZONAB targets to fully comprehend its role in cell survival.

Many of the stimulants that activate GEF-H1, such as TNFα, EGF, and mechanical stress, induce activation of Ras and Erk signaling (22–24, 26), indicating that the GEF-H1/ZONAB pathway can be activated by multiple external stimuli. GEF-H1 is also activated by various pathogens, junction dissociation, and tissue disruption (28, 30, 40, 41). Moreover, ZONAB is stimulated by reduced expression of ZO-1, a cellular inhibitor of the transcriptional and posttranscriptional function of ZONAB, linking it to the integrity of tight junctions (10). ZONAB thus represents a stress-activated signaling mechanism that can receive signals from a range of stimuli, including growth factors, cytokines, pathogens, environmental stress, and oncogene-induced transformation that leads to tissue damage and dissociation of cell-cell adhesion.

Ras signaling plays an important role in the regulation of ZONAB (Fig. 5*H*). Ras activation inhibits ZONAB's function as a transcription factor by inducing the formation of cytoplasmic complexes between activated RalA and ZONAB (21). RalA is activated by different types of stress and transmits cytokine-induced signals; hence, RalA activation seems to be a general mechanism to switch off the transcriptional activity of ZONAB (42–45). However, Ral signaling does not affect ZONAB's posttranscriptional activity. The second Ras-activated pathway that regulates ZONAB stimulates GEF-H1/RhoA signaling via Erk activation and can stimulate the transcriptional and the posttranscriptonal activity of ZONAB. The Erk/GEF-H1/RhoA axis thus reflects a general activation pathway, whereas RalA functions as a tuner that modulates the transcriptional function by cytoplasmic retention.

The here-identified mechanism of Rho-stimulated p21 expression also provides a molecular explanation for apparently contradictory observations. The p21 promoter is suppressed by Ras-activated Rho signaling, but p21 mRNA and protein are up-regulated by Ras signaling and cell cycle entry (4). Our data now show that ZONAB is activated by Ras signaling and promotes p21 expression posttranscriptionally even when transcription driven by the p21 promoter is reduced. Activation of Rho signaling thus leads to two opposed mechanisms that regulate p21 mRNA and protein expression: a pathway that reduces the activity of the p21 promoter and, thus, inhibits transcription and a ZONAB-dependent, posttranscriptional mechanism the leads to p21 mRNA stabilization and enhanced translation. Nevertheless, expression of oncogenic Ras and cell transformation also stimulates junction dissociation; hence, oncogenic Ras signaling stimulates ZONAB not only via RhoA.

Ras signaling is an important driver of tumorigenesis (4). The ZONAB-dependent survival pathway thus provides a possible therapeutic opportunity to target ZONAB to induce cell death in cancer cells. This survival pathway seems up-regulated in different types of carcinomas because ZONAB has been reported to be up-regulated in cancers form different tissues (14–20). Therapeutic targeting of ZONAB might also improve the effectiveness of existing therapies, because depletion enhanced cell death in response to Taxol (Fig. 1).

From an evolutionary perspective, it is intriguing that mammalian Y-box factors, in which a cold shock domain mediates the interaction with nucleic acids, are linked to RNA binding in response to stress. Cold shock domain proteins have first been identified in bacteria as small proteins containing just the cold shock domain that bind RNAs in response to cold shock to prevent secondary structure formation (46). Hence, cold shock domain-containing proteins retained an evolutionarily conserved role in binding RNA during cellular stress to ensure RNA function and cell survival.

Materials and Methods

All cell lines were grown under standard conditions and transfected as described (11, 30, 31). Necrosis and apoptosis were analyzed by measuring LDH release and caspase 3/7 activity, respectively. A comprehensive description of all experimental procedures is included in *SI Materials and Methods*.

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