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The NF-κB family member RelB Facilitates Apoptosis of Renal Epithelial Cells Caused by Cisplatin/TNFα Synergy by Suppressing an EMT-like Phenotypic Switch

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Running title: RelB in cisplatin/TNF-α-induced synergistic apoptosis

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ARF: acute renal failure; CDF curves: cumulative distance function curves; Cisplatin: *cis*-diamminedichloroplatinum(II); ECM: extracellular matrix; EMT: epithelial to mesenchymal transition; FA: focal adhesions; FAK: focal adhesion kinase; FDR: false discovery rate; I/R: ischemia/reperfusion; IFN-γ: mouse interferon-γ; IM-PTECs: immortalized proximal tubular epithelial cells; MLCK: myosin light chain kinase; NF-κB: nuclear factor κ B; PTECs: proximal tubular epithelial cells; ROCK: Ser/Thr kinase Rho-kinase; S100A4: S100 calcium binding protein A4shCtrl: control shRNA; shRelB: shRNARelB; TNF-α: tumor necrosis factor α; ZO-1: zona occludens 1; α-SMA: alpha smooth muscle actin

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Abstract

Cisplatin-induced renal proximal tubular apoptosis is known to be preceded by actin cytoskeleton reorganization, in conjunction with disruption of cell-matrix and cell-cell adhesion. In the present study we show that the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) aggravated these cisplatin-induced F-actin and cell adhesion changes, which was associated with enhanced cisplatin-induced apoptosis of immortalized proximal tubular epithelial cells (IM-PTECs). TNF-α induced RelB expression, and lenti-viral shRNA-mediated knock-down of RelB, but not other NF-κB members, abrogated the synergistic apoptosis observed with cisplatin/TNF-α treatment to the level of cisplatin-induced apoptosis. This protective effect was associated with increased stress fibre formation, cell-matrix and cell-cell adhesion in the shRelB cells during cisplatin/TNF-α treatment, mimicking an epithelial-to-mesenchymal phenotypic switch. Indeed, gene array analysis revealed that knock-down of RelB was associated with up-regulation of several actin regulatory genes, including snail2 and the Rho GTPase proteins Rhophilin and ARHGEF3. Pharmacological inhibition of Rho kinase signalling, re-established the synergistic apoptosis induced by combined cisplatin/TNF-α treatment of shRelB cells. In conclusion, our study shows for the first time that RelB is required for the cisplatin/TNF-α-induced cytoskeletal reorganization and apoptosis in renal cells by controlling a Rho kinase-dependent signalling network.

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Introduction

Acute renal failure (ARF) refers to a sudden deterioration of kidney function and is caused by either ischemia/reperfusion (I/R) or exposure to nephrotoxic xenobiotics like cisplatin (Thadhani et al., 1996). The proximal tubular epithelial cells (PTECs) are the primary target. After ischemic or nephrotoxic injury, morphological alterations occur in these PTECs. Disruption of the actin cytoskeleton of the PTECs is an early event, leading to loss of adhesion of cells from the extracellular matrix (ECM) as well as neighbouring cells, ultimately resulting in loss of cell function, pro-apoptotic signalling and cell death (Cordes, 2006; Gailit et al., 1993; Kruidering et al., 1998; Pabla and Dong, 2008; Qin et al., 2011; Van de Water et al., 1994; Zuk et al., 1998). Given the central role of the actin cytoskeletal network in cell adhesion maintenance and PTEC injury, it is important to better understand the molecular signaling networks that regulate the F-actin cytoskeleton organization in PTECs and, thereby, cell adhesion and cell survival during renal cell injury stress responses.

The actin cytoskeleton reorganization that takes place prior to and during PTEC injury, is directly associated with changes in cell-matrix and cell-cell adhesion. We have previously shown that exposure to nephrotoxic compounds, including cisplatin, affects the actin cytoskeleton network accompanied by disruption of focal adhesions (FA) followed by subsequent cell detachment and cell death in renal epithelial cells (Kruidering et al., 1998; van de Water et al., 2001; Van de Water et al., 1994; van de Water et al., 1999; van de Water et al., 2000). In addition, cisplatin treatment induces a loss of the tight junction and adherens junction proteins β-catenin and zona-occludens 1 (ZO-1) at the cell membrane in mouse proximal tubular cells and preservation of the cell-cell junctions protects against cisplatin-induced apoptosis (Imamdi et al., 2004; Qin et al., 2012). Understanding which PTEC signalling programs can control the actin signalling network, may lead to new therapeutic avenues to protect against cell detachment and cell death during renal injury. Here we focus on the regulation of actin organization during cisplatin-induced nephrotoxicity.

The pro-inflammatory cytokine tumor necrosis factor α (TNF-α) is involved in cisplatin-induced nephrotoxicity *in vivo* (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003). Recently, we

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showed that cisplatin-induced cell death of PTECs is aggravated by pre-exposure to TNF-α (Benedetti et al., 2012). This enhancement was associated with inhibition of TNF-α-induced nuclear factor κ B (NF-κB) activation by cisplatin. Interestingly, TNF-α mediated apoptosis has been associated with activation of the actin regulatory protein family of Rho GTPases in several cell lines (Mathew et al., 2009). This Rho GTPase family, including amongst others, Cdc42, Rac, RhoA, and regulating the Rho kinase (ROCK) (Leung et al., 1995)and myosin light chain kinase (MLCK) (Amano et al., 1996), is the most important family of proteins responsible for the actin cytoskeletal network changes that occur during PTEC injury (Kroshian et al., 1994; Raman and Atkinson, 1999). TNF-α has specifically been shown to induce the activation of the GTPases Cdc42, Rac and Rho (Mathew et al., 2009; Puls et al., 1999).

Here we investigated the involvement of TNF-α and downstream NF-κB signalling in the regulation of cisplatin toxicity through the control of actin cytoskeletal changes that occur during cisplatin cytotoxicity. We show that TNF-α aggravates cisplatin-induced disruption of actin stress fibres in association with reduction of focal adhesions and loss of cell-cell contacts, ultimately resulting in enhanced apoptosis. Intriguingly, knock-down of the NF-κB subunit RelB, but not other NF-kB family members, protected the cells against this synergistic apoptosis. This was associated with inhibition of the morphological changes induced by TNF-α. Gene array analysis revealed that knock-down of RelB was associated with an epithelial-to-mesenchymal (EMT)-like transition via up-regulation of snail2. Moreover, during cisplatin/TNF-α treatment, RhoA-pathway related gene expression, including Rhophilin and ARHGEF3, was increased. Inhibition of RhoA/ROCK signalling re-established the synergistic apoptosis with the cisplatin/TNF-α treatment in shRelB cells, indicating that TNF-α aggravated cisplatin-induced PTEC apoptosis via RelB-mediated RhoA/ROCK inactivation.

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Material and methods

Reagents and antibodies

Mouse recombinant TNF-α was acquired from R&D Systems (Abingdon, UK). The selective ROCK inhibitor Y27632 was from Bio-Connect (Huissen, The Netherlands). The MLCK inhibitor ML-7 was from Enzo Life Sciences (Antwerpen, Belgium). *cis*-diamminedichloroplatinum(II) (cisplatin) was from Sigma-Aldrich (Zwijndrecht, The Netherlands). AnnexinV-Alexa488 was made as described(Puigvert et al., 2010). The antibodies against β-actin, RelA, NF-κB1 and RelB were from Santa Cruz (Tebu-Bio, Heerhugowaard, The Netherlands) and the phospho-specific JNK antibody (Thr183/Tyr185) was from New England Biolabs (Leusden, The Netherlands). The antibody against NF-κB2 was from Cell Signaling (Bioké, Leiden, The Netherlands). The antibody against β-catenin was from BD Biosciences (San Jose, CA, USA), the antibody against P-paxillin Y118 was from Biosource (Bleiswijk, The Netherlands) and rhodamine phalloidin was from Molecular probes (Breda, The Netherlands).

Cell culture

Immortalized proximal tubular cells (IM-PTECs) described previously (Stokman et al., 2011) were cultured at 33°C in HK2 medium (DMEM/F12 medium (Invitrogen, Breda, The Netherlands) with 10% fetal bovine serum (Hyclone, Etten-Leur, The Netherlands), 5 µg/ml insulin and transferrin, 5 ng/ml sodium selenite (Roche), 20 ng/ml triiodo-thyrionine (Sigma-Aldrich), 50 ng/ml hydrocortisone (Sigma-Aldrich), and 5 ng/ml prostaglandin E1 (Sigma- Aldrich) with L-glutamine and antibiotics (both from Invitrogen) and mouse interferon-γ (IFN-γ) (1 ng/ml; R&D Systems)) in 5% CO2 and 95% air between passage 3 and 20. Prior to each experiment, the cells were differentiated into proximal tubular cells by culturing them for 4 days under restrictive conditions (at 37°C in the absence of IFN-γ). The cells were then plated in the appropriate assay plates and cultured for 2 more days. In total,

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IM-PTECs were cultured under restrictive conditions for 6 days, allowing the disappearance of SV40 activity and completion of differentiation (Stokman et al., 2011).

Exposures

IM-PTECs were exposed to 20 μM cisplatin or vehicle in the presence or absence of TNF-α (8 ng/ml). Exposure to TNF-α was performed 30 minutes prior to cisplatin as previously described (Benedetti et al., 2012). Cells were pre-incubated with Y27632 and ML-7 inhibitors 30 minutes prior TNF-α.

Lentiviral knock-downs

Stable IM-PTECs with knock-down of each NF-κB member were generated using lenti-viral shRNA vectors (Sigma-Aldrich, in collaboration with Rob Hoeben, Leiden University Medical Centre, The Netherlands) and selection with puromycin (1 μg/ml). The plasmid encoding non-target control SHC002 was used as a control, the plasmids encoding mouse RelA number TRCN55343 (CCGGGCGAATCCAGACCAACAATAACTCGAGT TATTGTTGGTC TGGATTCGCTTTTTG) and TRC55346 (CCGGCTGTCCTCTCACATCCGATTTCTCGAGAAATCGGATGTGAGAGGACAGTTTTTG), mouse RelB number TRC095314 (CCGGCGGTTCTCTTTGAGCCCATTTCTCGAGAAA TGGGCTCAAAGAGAACCGTTTTTG) and TRC095318 (CCGGCGTGACTGTCAATGTGTTCTTCTCGAGAAGAACACATTGACAGTCACGTTTTTG), mouse c-Rel number TRC042549 (CCGGCCATTGTTTCTAACCCAATTTCTCGAGAAATTGGGTTAGAAACAATGGTTTTTG) and TRC042550 (CCGGGCGACTTGAGTGCATCTAATTCTCGAGAATTAGATGCACTCAAGTCGCTTTTTG), mouse NF-κB1 number TRC009514 (CCGGCACTGCTTTGACTCACTCAATCTCGAGATTGAGTGAGTCAAAGCAGTGTTTTT), mouse NF-κB2TRC012343 (CCGGCCTGTCTAATCGAAATCTTATCTCGAGATAAGATTTCGATTAGACAGGTTTTT) and TRC012346 (CCGGCGTCATTTATCACGCTCAGTACTCGAGTACTGAGCGTGATAAATGACGTTTTT) were used.

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Live apoptosis

Real time induction of apoptosis was quantified using a live cell apoptosis assay previously described (Puigvert et al., 2010). Briefly, binding of annexin V-Alexa488 conjugate to phosphatidyl serine present on the membranes of apoptotic cells was followed in time by imaging the cells every hour after drug exposure with a BD Pathway 855 imager (Becton Dickinson, Erembodegem, Belgium). The total area of annexin V-Alexa488 fluorescence per image was quantified using Image Pro (Media Cybernetics, Bethesda, MD).

Western Blot

Cells were harvested as described (de Graauw et al., 2005). The samples were subjected to protein separation and blotted on Immobilon-P membranes (Millipore, Amsterdam, The Netherlands).The membranes were blocked overnight at 4°C in milk powder 5% (w/v) in Tris-buffered saline/Tween 20 (TBST-T). Primary antibody incubation was performed for one hour at room temperature followed by incubation with horseradish peroxidase-conjugated or Cy5-labeled secondary antibodies (Jackson Immunoresearch, Newmarket, UK) in TBS-T for 1 h at room temperature. Protein signals were detected with ECL (GE Healthcare) followed by film detection for the NF-κB members or by visualization on the Typhoon 9400 imager (GE Healthcare, Diegem, Belgium) for β-actin.

Immunofluorescence

Cultured cells were immuno-stained for β-catenin, P-paxillin-Y118 and F-actin (Rhodamine/Phalloidin) followed by secondary antibody labelling (goat anti-mouse Alexa488-labeled, goat anti-rabbit Alexa488-labeled (Molecular Probes, Breda, The Netherlands) and goat anti-mouse Cy3-labeled (Jackson Immunoresearch, Newmarket, UK)). Hoechst 33258 (2 µg/ml) was used

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to visualize the nuclei. Cells were imaged using a Nikon TiE2000 confocal microscope (Nikon, Amstelveen, Netherlands). All antibodies were used with a 1:1000 dilution.

Quantitative analysis of cell-cell junctions and focal adhesions

β-catenin segmentation and quantification was performed using Image J 1.44i software, as previously described (Qin et al., 2012), by calculating the average area occupied by β-catenin fluorescence signal in the cell-cell junction region of each cell and expressing it as β-catenin junction size per nucleus. Focal adhesion quantification was performed using Image J 1.43h software. Segmentation of the two signals P-paxillin-Y118 and Hoescht was followed by artificial cell region border creation. With the segmented P-paxillin-Y118 and nucleus masks together with the reconstructed cell region border, several phenotypic measurements were derived including focal adhesion size, dispersion, orientation and number. A minimum of 3400 focal adhesions and 100 cells per conditions was used for the quantification. The FA size distribution was represented as empirical cumulative distribution function (CDF) curves using the cdfplot function of the MATLAB® software (MathWorks®, Eindhoven, The Netherlands). The empirical cdf F(x) is defined as the proportion of X values less than or equal to x.

Gene array analysis

IM-PTECs wt, control shRNA (shCtrl) cells and shRNA RelB (shRelB) cells were exposed to vehicle, cisplatin (20μM), TNF-α (8ng/ml) or cisplatin in combination with TNF-α for 8 hours. Three replicates for each treatment were used. Total RNA was isolated from cells using an RNeasy® Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm (Nanodrop Technologies, Montchanin, DE, USA), and the integrity of the RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The synthesis of labelled cRNA and hybridization steps were performed by Service XS (Leiden, The Netherlands). Briefly, 100 ng of RNA was used to synthesize Biotin-labeled cRNA

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with the Affymetrix 3´ IVT Express Labeling Kit. The resulting labelled cRNA was quantified by absorbance at 260 nm (Nanodrop) and 6 μg was hybridized on an Affymetrix 24 all-in HT MG 430 PM Array plate following the procedures recommended by the manufacturer. After hybridization, the microarray slide was washed, dried, and scanned using the Hybridization, Wash and Stain Kit #901530 compatible with Affymetrix protocols. The Affymetrix Command Console (v3.0) and Expression Console software (v1.1) were used to analyse the performance of the washing, staining and scanning of the chips.

Subsequently, the raw intensity data were normalized with BRB array Tools software (developed by Dr. Richard Simon and BRB-ArrayTools Development Team; http://linus.nci.nih.gov/BRB-ArrayTools.html) using the Robust Multichip Average (RMA) method. Significantly differentially expressed genes (false discovery rate FDR≤0. 1) between the various experimental conditions were identified with a one-way blocked ANOVA test. A randomized blocked design was chosen to control for the different days that the replicate experiments were run. The ANOVA test was followed by calculation of the FDRs according to Benjamin and Hochberg (Benjamini, 1995). Since only one differentially expressed gene was found between wt and shCtrl cells, they were pooled to increase the statistical power of the analysis. Only probes with expression levels of 1.5-fold change or more relative to wt+shCtrl cells were selected for further analysis.

Commonly deregulated genes between the different groups were identified by Venn diagrams. Pathway analysis of the selected genes was performed using the GeneGoMetacore™ pathway analysis software (GeneGo, MI, USA). Heatmap representations were performed using the MultiArray Viewer software. The complete data set will be submitted to ArrayExpress (EMBL-European Bioinformatics Institue, Hinxton, England; http://wwww.ebi.ac.uk/arrayexpress) and will be available for public download shortly. Accession number referencing this data set will be available on the GEO database website (http://www.ncbi.nlm.nih.gov/geo/).

Statistical procedures

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All data are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was determined by GraphPad Prism using an unpaired two-tailed t-test. Data from focal adhesion quantification were analysed using the non-parametric Kolmogorov-Smirnow (KS) test via the MATLAB® software (MathWorks®, Eindhoven, The Netherlands) which computes test statistics that are derived from the empirical cdf.. The CDF curves are represented and the level of confidence is indicated by P-values in the figures.

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Results

TNF-α aggravates cisplatin-induced disruption of stress fibres, cell-ECM and cell-cell adhesion

In a recent study we showed that TNF-α enhanced cisplatin-induced cell death in immortalized proximal tubular epithelial cells (IM-PTECs) (Benedetti et al., 2012). As cisplatin alone is known to induce actin reorganization, and loss of cell-cell and cell-ECM adhesion prior to apoptosis of renal tubular cells (Imamdi et al., 2004; Kruidering et al., 1998; Qin et al., 2012; van de Water et al., 2000), we determined whether TNF-α could promote these events. IM-PTECs were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml) for 8 hours and thereafter immuno-stained to visualize the actin cytoskeleton, the focal adhesions and cell-cell adhesions. Treatment of the cells with TNF-α alone enhanced actin fibre formation in particular at cell-cell junctions (Figure 1A), but diminished the average size of focal adhesions (Figure 1B and D) and reduced the accumulation of β-catenin molecules at the cell membrane (Figure 1C and E). This effect of TNF-α aggravated the reorganization and disruption of the stress fibres (Figure 1A), reduced the size of the focal adhesions (Figure 1B and Di) and mildly aggravated the loss of β-catenin at the cell–cell contacts (Figure 1C and E) caused by cisplatin treatment alone. TNF-α only mildly affected the reduction in the number of focal adhesions induced by cisplatin alone (Figure 1Dii). These data show that prior to the onset of apoptosis, TNF-α aggravates the cytoskeletal and cell adhesion reorganization induced by cisplatin alone.

RelB knock-down suppresses cisplatin/TNF-α synergistic apoptosis

TNF-α activates NF-κB signalling. Furthermore, NF-κB signalling can affect the actin cytoskeletal organization (Nakamichi et al., 2007). The NF-κB transcription factor comprises five different members: RelA, RelB, NF-κB1, NF-κB2 and c-Rel (Hoffmann and Baltimore, 2006). Our next goal was therefore to determine which NF-κB subunit could contribute to the TNF-α mediated aggravation of both cisplatin-induced cytoskeletal reorganization and cell death. We therefore first systematically

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generated knock-down IM-PTEC cell lines using five individual lenti-viral shRNA targeting each individual NF-κB family member. Knock-down was confirmed using Western blotting (Supplemental Figure 1A). For NF-κB1 only one, instead of two out of 5 shRNA lentiviral vectors resulted in a sufficient and reliable protein knock-down. Due to its very weak expression in our IM-PTECs known from previous Affymetrix array data (Benedetti et al., 2012), c-Rel was not considered for further investigation.

Next, we investigated the effect of the knock down of each NF-κB member on cisplatin- and cisplatin/TNF-α-induced apoptosis. All cell lines were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml) and cell death was measured using live apoptosis imaging. RelA and NF-κB1 knock-down increased cisplatin- and cisplatin/TNF-α-induced apoptosis (Figure 2A); suggesting a role for the RelA/p50 NF-κB dimer to act in survival signalling. Interestingly, for both NF-κB2 knock-down cell lines, an increased cisplatin-induced apoptosis was observed, while cisplatin/TNF-α-induced apoptosis was unaffected (Figure 2A). This observation indicates that in our IM-PTECs, NF-κB2-based NF-κB signalling protects the cells from cisplatin-induced apoptosis. Importantly, knock-down of RelB using two independent shRNAs, suppressed the cisplatin/TNF-α-induced synergistic apoptosis without affecting cisplatin-induced apoptosis (Figure 2A). This suggests that RelB is the only NF-κB family member that is key in the regulation of the TNF-α-mediated enhancement of cisplatin-induced apoptosis. Of critical relevance, TNF-α itself increased expression of RelB both at the gene as well as protein expression level, which was not affected by cisplatin (Figure 2B and C). Together, these data indicate that the NF-κB family member RelB is directly involved in the pro-apoptotic activity of TNF-α and, thereby, enhances cisplatin-induced apoptotic cell death.

RelB knock-down inhibits cisplatin/TNF-α-induced cell adhesion and actin cytoskeleton changes

Next, we determined whether depletion of RelB also affected the TNFα- and cisplatin-mediated changes in actin cytoskeletal network, cell-matrix adhesions and cell-cell contacts. Cisplatin caused loss of F-actin organization and cell matrix and cell-cell adhesions in shCtrl cells as expected (Figure

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3A-E), which was further enhanced by TNFα. Knock-down of RelB resulted in some morphological changes compared to both wt and shCtrl cells. shRelB cells were more spread with a reorganization of stress fibres around the nucleus, larger focal adhesions and a reduced cortical-actin network. In addition, a loss in cell-cell contact formation was observed as indicated by the disappearance of β-catenin at the cell-cell border (Figure 3C and E). Although we expected these changes to influence cisplatin-induced renal cell death, no significant changes were observed between DMSO and cisplatin-treated cells. However, in sharp contrast to shCtrl cells, treatment of shRelB cells with cisplatin/TNF-α did not result in drastic morphological changes: cisplatin/TNF-α treated shRelB cells maintained their actin organization and did not show significant changes in focal adhesion size and number (Figure 3A, B and D and Supplemental Figure 2A) coinciding with the protection of the cells. Furthermore, although slightly diminished, β-catenin containing cell-cell adhesions remained largely intact during cisplatin/TNF-α treatment of shRelB cells compared to untreated cells (Figure 3C and E). Together, these data indicate that RelB-mediated signalling is essential in the nephrotoxic cytoskeletal reorganization in association with the onset of cell death during cisplatin/TNF-α treatment.

RelB knock-down leads to EMT-like driven cytoskeletal reorganization

To obtain detailed insight into the pathways involved in the actin cytoskeleton changes and the suppression of the synergistic apoptosis observed in shRelB cells, transcriptomic analysis was performed. Wt, shCtrl and shRelB cells were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml) for 8 hours, a time point at which cell death did not yet occur (Figure 2). Since wt cells and shCtrl cells only had one statistically different gene (Neurog3) with a FDR≤ 0.1, we pooled the datasets to increase the statistical power of our gene array analysis. We first analysed the effect of RelB knock-down on gene expression changes under control conditions. Gene expression changes with ≥ 1.5 fold-change and FDR ≤ 0.1for shRelB #1 and #2 cells compared to wt+ shCtrl cells were considered for further analysis. A total of 800 genes for shRelB #1 and 698 genes for shRelB #2 were identified with

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our selection criteria and 388 genes were common for both shRelB cell lines (Figure 4A).A pathway analysis was then performed on these common genes under control conditions using the GeneGoMetacore™ pathway analysis software. The top 7 significantly different pathways were all related to cytoskeletal and cell adhesion changes as well as induction of an EMT (Figure 4B). The most prominent cytoskeleton and cell adhesion-related genes that were up-regulated by RelB knock-down included actin (Actb), myosin IIB (Myh10), the gene encoding the tight junction-related protein cingulin (Cgn), the cell adhesion-related genes collagen type IV alpha 1 (Col4a1) and serpine 2 as well as the genes encoding the Cdc42 regulating proteins Cdc42 small effector 1 (Cdc42se1) and Par6 (Pard6g) (Figure 4C). In contrast, Vcan, encoding the cell adhesion protein versican was down-regulated in shRelB cells (Figure 4C). In addition, one of the key EMT inducing genes, Snai2, encoding the protein Snail2, was more than 2 fold up-regulated in both shRelB cell lines (Figure 4C), suggesting that the cytoskeletal changes described above could well be a consequence of a Snail2-induced EMT-like switch of the PTEC cells. Other EMT-related transcription factors, including Twist, were not affected.

RelB knock-down leads to Rho kinase pathway activation during cisplatin/TNF-α treatment

We next evaluated which pathways were affected by RelB knock-down under TNF-α, cisplatin or TNF-α/cisplatin conditions. Gene expression changes with $\geq$ 1.5 fold-change and FDR $\leq$ 0.1for shRelB #1 and shRelB#2 cells compared to wt+ shCtrl cells were considered for further analysis. Comparing differentially expressed genes between treatments resulted in identification of 21 and 17 uniquely changing genes for cisplatin and TNF-α treatments respectively compared to 118 and 70 uniquely changing genes for vehicle and cisplatin/TNF-α treatments respectively (Supplemental Figure 3 and Supplemental Table 1).

To identify pathways by which RelB controlled the synergistic apoptosis induced by cisplatin/TNF-a treatment, pathway analysis was performed on the common genes in cells treated with the combined cisplatin/TNF-α treatment using the GeneGoMetacore™ pathway analysis

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software. A total of 782 genes for shRelB #1 and 462 genes for shRelB #2 were identified under these treatment conditions and 278 genes were common for both shRelB cell lines (Figure 5A) comprising the 70 unique genes (Supplemental Figure 3 and Supplemental Table 1). Pathway analysis showed that also under cisplatin/TNF-α conditions, the top significantly different pathways were all related to cytoskeletal and cell adhesion changes as well as induction of an EMT (Figure 5B). The main genes involved in cell adhesion included the Ras-related protein 1a (Rap1a), the Eph receptor B3 (Ephb3) and the gap junction protein β2 (Gjb2) (Figure 5C) of which expression of Rap1a and Gjb2 was specifically changed under cisplatin/TNF-α conditions. Interestingly, the EMT gene snai2 was slightly induced in shCtrl cells; yet, depletion of RelB allowed an even further induction of snai2 expression after cisplatin treatment, which was approximately four times higher than under shCtrl conditions and independent of TNFα (Figure 5C). In addition, RhoGTPase pathway activation was observed in shRelB IM-PTECs (Figure 5B), including up-regulation of Rho guanine nucleotide exchange factor 3 (Arhgef3) and rhophilin 1 (Rhpn1) (Figure 5C). Together these data suggest a key role for RelB to suppress a stress-induced cytoskeletal reorganization response, which is likely part of an EMT-like program.

RelB knock-down protects against cisplatin/TNF-α treatment in a Rho kinase dependent manner

The Rho GTPase family is the most important family of proteins responsible for the regulation of the actin cytoskeletal network in cells and it is known to be involved in PTEC injury (Kroshian et al., 1994; Raman and Atkinson, 1999). Given the changes observed in the expression of some Rho-GTPase family members in shRelB cells, we determined whether RelB knock-down protects the IM-PTECs against cisplatin/TNF-α induced synergistic cell death via activation of the Rho GTPase pathway. Therefore we inhibited one of the key downstream components, Rho kinase using the Y27632 inhibitor. Cells were pre-exposed for 30 minutes with the ROCK inhibitor and thereafter treated with cisplatin (20 μM) and/or TNF-α (8 ng/ml). Y27632 caused disruption of stress fibres

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formation in both shCtrl and shRelB cells (Figure 6A). This disruption of the stress fibres was accompanied by an enhancement of apoptosis in shCtrl cells after both cisplatin and cisplatin/TNF-α treatment (Figure 6B). In shRelB cells, the apoptosis was only increased with cisplatin/TNF-α treatment and not with cisplatin treatment alone (Figure 6B). These data are indicative for mechanism whereby RelB knock down confers protection against cisplatin/TNF-α treatment in a Rho kinase dependent manner.

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Discussion

In the present study we demonstrate that TNF-α aggravated the cisplatin-induced changes in the actin cytoskeleton and cell adhesions that occur prior to the onset of apoptosis. Knock-down of the NF-κB family member RelB abrogated both the synergistic cisplatin/TNF-α-induced apoptosis as well as the changes in actin cytoskeleton and cell adhesions. RelB knock down resulted in an enhancement of the expression of Rho GTPase-related actin cytoskeletal regulators in association with a snail2-related EMT-like program. Likewise, a Rho kinase inhibitor, Y27632, reversed the cytoprotective effect of the RelB depletion.

Although the transcription factor NF-κB has been associated with morphological changes that occur prior to apoptosis (Nakamichi et al., 2007), the role of the family member RelB in Rho kinase regulation in relation to cell death has never been described before. Our study showed that up-regulation of RelB is responsible for the synergistic apoptosis induced by cisplatin/TNF-α treatment. This observation is contradictory to studies using thymocytes and cancer cells, in which RelB is involved in anti-apoptotic signalling (Guerin et al., 2002; Mineva et al., 2007; Wang et al., 2007); these effects were independent of TNF-α signalling. Moreover, different transcriptional programs may be activated by RelB in immune and cancer cells, compared to renal cells. Jacque et al. showed in mouse embryonic fibroblasts that although RelB entered the nucleus in response to TNF-α, it could not bind to DNA due to repression by RelA (Jacque et al., 2005). In our IM-PTECs TNF-α-induced RelA translocation was inhibited by cisplatin/TNF-α treatment (Benedetti et al., 2012). Furthermore, RelB expression was up-regulated during TNF-α treatment (Figure 2B-C). It could well be that the increase in apoptosis in our cells is due to the combination of RelB up-regulation and RelA translocation inhibition, thereby resulting in a different transcriptional program than the one occurring in immune and cancer cells. Additionally, other kinases regulating the cytoskeletal organization such as Src could be involved. v-Src has been shown to regulate the activation of RelB via accelerating IκB proteolysis in fibroblasts (Shain et al., 1999) and TNF-α increased Src activity in intestinal epithelial

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cells (Kawai et al., 2002). It could well be that in our cells, the increased RelB activity observed with TNF-α and cisplatin/TNF-α treatments is due to increased Src activation.

Transcriptomic analysis revealed activation of the RhoA/Rho kinase pathway in shRelB cells during treatment of the cells with cisplatin/TNF-α via increased gene expression of ARHGEF3 and rhophillin 1. ARHGEF3 has been shown to activate RhoA and RhoB specifically leading to assembly of stress fibres and thereby enlargement of focal adhesions in a Rho kinase-dependent manner (Arthur et al., 2002). Rhophilin 1 interacts strongly with GTP-bound RhoA, but its exact role in cytoskeletal organization remains elusive (Peck et al., 2002; Watanabe et al., 1996). Their role in apoptosis is unknown. Using the Rho kinase inhibitor we showed that RhoA pathway activation was crucial for cisplatin/TNF-α-induced synergistic apoptosis. It is well known that depending on the cell type and stimulus used, activation of the RhoA/ROCK pathway can either lead to apoptosis or cell survival. For example, ROCK inhibition enhanced cisplatin-induced cytotoxicity of lung carcinoma cells through focal adhesion kinase (FAK) suppression-independent mechanism (Igishi et al., 2003), while it attenuated I/R-induced renal injury in an *in vivo* model (Song and Gao, 2011). In addition, several other studies indicated a protective role of ROCK in several cell types including epithelial, cancer, and endothelial cells as well as *in vivo (*Amano et al., 2010*)*. Yet, to the best of our knowledge, the role of ROCK signalling in cisplatin-induced apoptosis in renal cells remained thus far unknown.

In addition to RhoA and ROCK pathway activation, our transcriptomic analysis demonstrated up-regulation of the EMT-regulatory gene snail2 in shRelB cells, which corresponded with the EMT-like switch observed in these cells. Since TNF-α strongly induces RelB expression (Figure 2C), this may suppress a cytoprotective snai2-based EMT-like switch initiated by cisplatin. RhoA pathway activation was shown to be a key step in renal EMT, leading to trans-differentiation of renal tubular cells to fibroblasts (Liu, 2004; Liu, 2010). It has been shown that renal regeneration during and after toxicant- or I/R-induced renal failure occurred by transformation of epithelial cells into fibroblasts via EMT. During an EMT, cells may acquire new mesenchymal markers such as vimentin, alpha smooth muscle actin (α-SMA), S100 calcium binding protein A4 (S100A4), the transcription factor Snail and N-cadherin (Liu, 2010). Although Snail was up-regulated over 2-fold, no changes occurred in gene

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expression of the mesenchymal markers vimentin, desmin, collagen I, fibronectin and N-cadherin.α-SMA gene expression was only increased in the shRelB #2 cells showing the highest knock-down but not in the shRelB #1 cells, while the S100A4 gene expression was down-regulated in both shRelB cell lines. Therefore, RelB knock-down did not induce a complete traditional EMT in IM-PTECs, but this partial EMT accompanied with RhoA/Rho kinase activation was sufficient to confer protection against the synergistic apoptosis induced by cisplatin/TNF-α treatment. In contrast to our data, Wang et al. showed that RelB expression was required to maintain the mesenchymal phenotype of breast cancer cells (Wang et al., 2007).The link between RhoA/ROCK pathway activation and RelB in this process is not yet clear.

Lastly, our transcriptomic analysis revealed specific and significant changes in the expression of the cell-cell proteins Rap1a and Gjb2 in cisplatin/TNF-α-treated shRelB cells. We have previously shown that Rap1a preserved cell-cell junctions and protected against cisplatin-induced apoptosis in proximal tubular cells (Qin et al., 2012). Gjb2 is part of the connexins that could be involved in the proximal tubular cells in the formation of hemi-channels regulating renal sodium, potassium, and water handling (Hanner et al., 2010). In mice, knock-out of GJB2 has been shown to responsible for cochlear cell apoptosis, so it could be that this connexin has also a protective role in renal cells (Zhang et al., 2012).

In conclusion, our observations suggest that TNF-α-induced up-regulation of RelB expression is responsible for the cisplatin/TNF-α-induced synergistic apoptosis and show, for the first time, the role of RelB in renal EMT-induction and RhoA/ROCK-mediated cytoskeletal regulation as a protective mechanism.

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Authorship contributions

Participated in research design: G. Benedetti, L. Fredriksson, M. de Graauw, B. van de Water

Conducted experiments: G. Benedetti

Contributed new reagents or analytic tools: K. Yan,B. Herpers

Performed data analysis: G. Benedetti, M. Fokkelman, J. Meerman, M. de Graauw

Wrote or contributed to the writing of the manuscript: G. Benedetti, M. de Graauw, B. van de Water

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Footnotes

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Figure legends

Figure 1. TNF-α aggravates cisplatin-induced disruption of stress fibres, cell-ECM adhesion and cell-cell contacts

IM-PTECs were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml) for 8 hours and immuno-stained to visualize the actin cytoskeleton, the focal adhesions and cell-cell adhesions. Cells were imaged using a Nikon TiE2000 confocal microscope. The effect of treatment on the actin cytoskeleton was visualized by staining of F-actin with rhodamine phalloidin (red) (A). The effect of treatment on focal adhesions (FA) was assessed by staining of P-paxillin-Y118 (green) (B). Quantification of P-paxillin was performed. The CDF curve indicates the direction of the changes in focal adhesion size with the different treatments. A KS test was performed relative to control treatment and indicates the differences observed in P-paxillin size between cisplatin and/or TNF-α treated cells and vehicle-treated cells. The p-values obtained are indicated in red. The number of focal adhesion was also assessed by quantifying P-paxillin fluorescence per cell (D). Cell-cell contacts disturbances after treatment were assessed by staining the adherens junction protein β-catenin (green) (C), which was quantified by assessing the average area occupied by β-catenin per cell (E). For all images, the nuclei were stained with Hoechst (blue) (A, B and C). The scale is indicated in the images in the bottom left corner. The data are representative of three independent experiments and expressed as mean ± S.E.M. * $P \leq 0.05$ and # $P \leq 0.05$, # # $P \leq 0.01$ compared to vehicle-treated cells.

Figure 2.RelB knock-down suppresses cisplatin/TNF-α synergistic apoptosis

IM-PTEC cell lines with knock-down for each NF-κB family member (NF-κB1, NF-κB2, RelA and RelB) were generated using lenti-viral shRNA (see materials and methods). Knock-down cells were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml). Cell death was followed over time using Annexin V-Alexa488 staining and automated imaging. Both apoptosis overtime and endpoints are

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represented. The dashed orange and red lines indicate the amount apoptosis observed with shCtrl cells after cisplatin or cisplatin/TNF-α treatment respectively (A). After cisplatin (5 μM) and/or TNF-α (8 ng/ml) treatment for 8 hours, the gene expression (B) and protein (C) levels of RelB were assessed. Gene expression values were obtained from previous Affymetrix gene array data (Benedetti et al., 2012) (B) and protein levels were assessed by Western blotting (C). B-actin was used as a loading control and the quantification indicated is the mean of three independent experiments (C). The data are representative of three independent experiments and expressed as mean± S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$ and # $P \leq 0.05$, # # $P \leq 0.01$ compared to vehicle-treated cells.

Figure 3.RelB knock-down inhibits cisplatin/TNF-α-induced cell adhesion and actin cytoskeleton changes

shCtrl and shRelB cells were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml) for 8 hours and immuno-stained to visualize the actin cytoskeleton, focal adhesions and cell-cell adhesions. Cells were imaged using a Nikon TiE2000 confocal microscope. The effect of RelB knock-down on actin cytoskeleton was visualized by staining of F-actin with rhodamine phalloidin (red) (A). The effect of RelB knock-down on focal adhesions was assessed by staining of P-paxillin-Y118 (green) (B). Cell-cell contacts disturbances after treatment were assessed by staining the adherens junction protein β-catenin (green) (C). Quantification of P-paxillin (D) and β-catenin (E) was performed as described under Figure 1 and materials and methods. For all images, the nuclei were stained with Hoechst (blue) (A, B and C). Images of shCtrl cells and shRelB cells have the same scale as indicated in the images in the bottom left corner. cispt=cisplatin, cispt+TNF-α and cispt+T=cisplatin+TNF-α. The data are representative of three independent experiments and expressed as mean ± S.E.M. * $P \leq 0.05$ and # $P \leq 0.05$, # # $P \leq 0.01$ compared to vehicle-treated cells.

Figure 4.RelB knock-down leads to EMT-like driven cytoskeletal rearrangements in vehicle-treated cells

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shCtrl and shRelB cells were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml) for 8 hours. Following hybridization and RMA normalization, the genes with ≥ 1.5 fold-change and FDR ≤ 0.1for shRelB #1 and #2 cell lines compared to wt+ shCtrl cells in control conditions were further selected. A Venn diagram was made comparing both shRelB cell lines in control conditions (A). Using the Metacore™ software, pathway analysis was performed on the genes commonly regulated in both shRelB cell lines in control conditions. The top pathways are represented in a heatmap (B). The gene expression changes of genes involved in cytoskeleton organization and EMT are represented for wt and shCtrl as well as both shRelB cell lines in control conditions. The data are representative of three independent experiments and expressed as mean ± S.E.M. * P ≤ 0.05 (C).

Figure 5.RelB knock-down is associated with up-regulation of RhoA pathway related genes during cisplatin/TNF-α treatment

shCtrl and shRelB cells were exposed to cisplatin (20 μM) and/or TNF-α (8 ng/ml) for 8 hours. Following hybridization and RMA normalization, the genes with ≥ 1.5 fold-change and FDR ≤ 0.1 for shRelB #1 and #2 cell lines compared to wt+ shCtrl cells after cisplatin/TNF-α treatment were further selected. A Venn diagram was made comparing both shRelB cell lines (A). Using the Metacore™ software, pathway analysis was performed on the genes commonly regulated in both shRelB cell lines with cisplatin/TNF-α treatment. The top pathways are represented in a heatmap (B). The gene expression changes of genes involved in cell adhesion, EMT and the RhoA pathway are represented for wt-shCtrl cells combined and both shRelB cell lines combined in cisplatin/TNF-α treated conditions The statistical significance is indicated only for the conditions where for both shRelB #1 and #2 the fold-change was ≥1.5. The data are representative of three independent experiments and expressed as mean ± S.E.M. * P ≤ 0.05 and ** P ≤ 0.05 . (C).

Figure 6.RelB knock-down protects against cisplatin/TNF-α treatment in a Rho kinase dependent manner

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shCtrl and shRelB cells were pre-exposed to the ROCK inhibitor Y27632 for 30 minutes prior to cisplatin (20 μM) and/or TNF-α (8 ng/ml). After 8 hours of exposures to cisplatin and/or TNF-α in combination with the ROCK inhibitor, actin was visualized by staining of F-actin with rhodamine phalloidin (red). The nuclei were visualized with Hoechst staining (blue). Images of both shCtrl cells and shRelB cells have the same scale as indicated in the images in the bottom left corner (A). Cell death after ROCK inhibition was followed over time using Annexin V-Alexa488 staining and automated imaging (B). ctl=control, cispt=cisplatin and cispt+T=cisplatin+TNF-α. The data are representative of three independent experiments and expressed as mean ± S.E.M. * $P \leq 0.05$ and # $P \leq 0.05$, # # $P \leq 0.01$ compared to vehicle-treated cells.

Figure 1

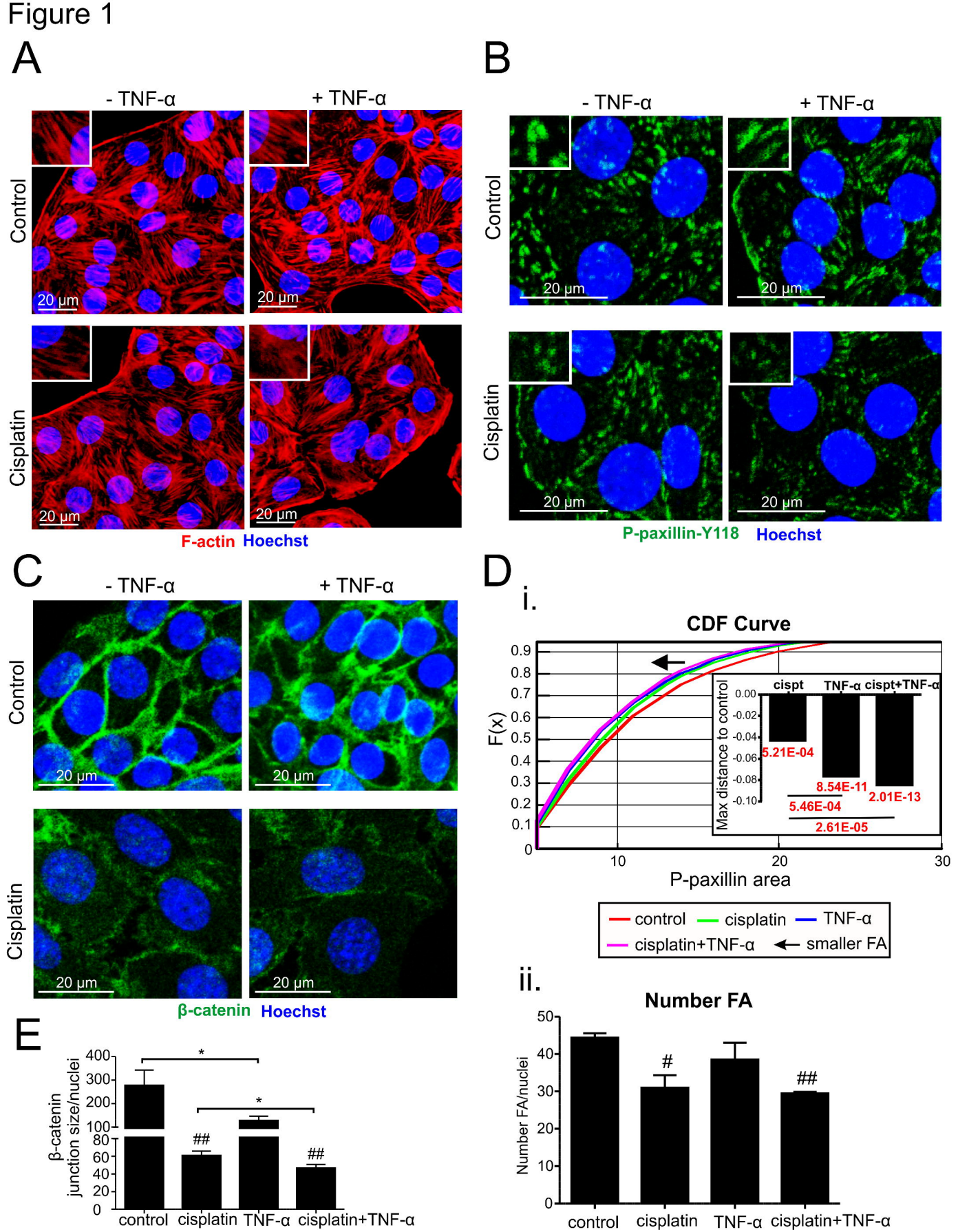
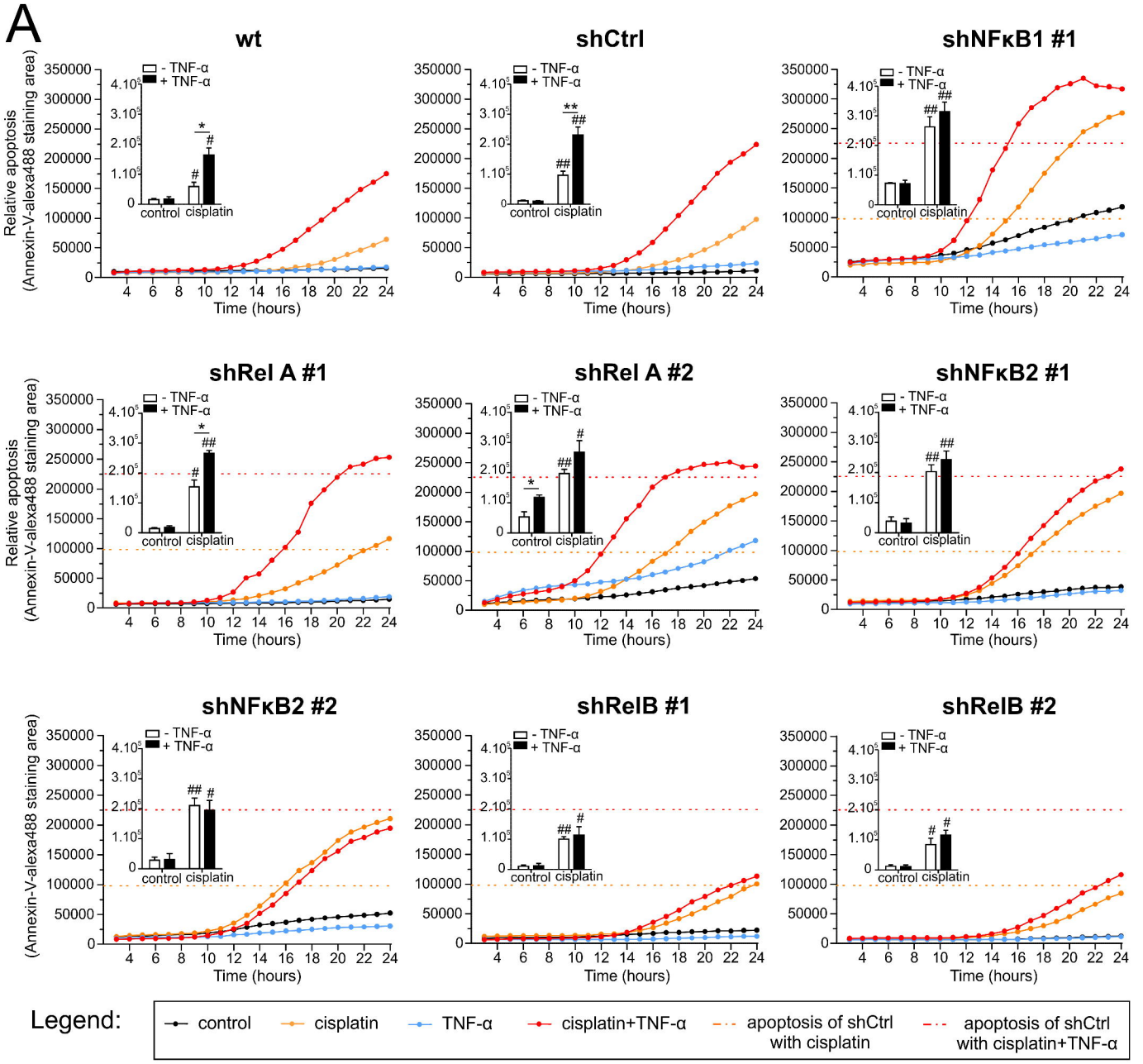
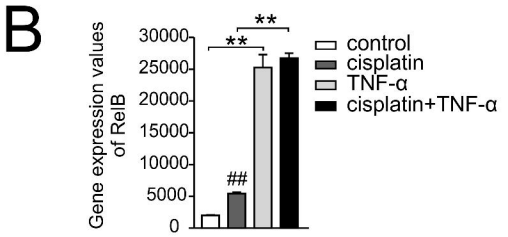

A
- TNF-α
+ TNF-α
Control
Cisplatin
20 μm
F-actin Hoechst
B
- TNF-α
+ TNF-α
Control
Cisplatin
20 μm
P-paxillin-Y118 Hoechst
C
- TNF-α
+ TNF-α
Control
Cisplatin
20 μm
β-catenin Hoechst
D
i.
CDF Curve
F(x)
P-paxillin area
cispt
TNF-α
cispt+TNF-α
Max distance to control
5.21E-04
8.54E-11
2.01E-13
5.46E-04
2.61E-05
control
cisplatin
TNF-α
cisplatin+TNF-α
smaller FA
ii.
Number FA
Number FA/nuclei
control
cisplatin
TNF-α
cisplatin+TNF-α
E
β-catenin junction size/nuclei
control
cisplatin
TNF-α
cisplatin+TNF-α

Figure 2


A
wt
shCtrl
shNFκB1 #1
shRel A #1
shRel A #2
shNFκB2 #1
shNFκB2 #2
shRelB #1
shRelB #2
Relative apoptosis (Annexin-V-alexa488 staining area)
Time (hours)
- TNF-α
+ TNF-α
control
cisplatin
Legend:
control
cisplatin
TNF-α
cisplatin+TNF-α
apoptosis of shCtrl with cisplatin
apoptosis of shCtrl with cisplatin+TNF-α


B
Gene expression values of RelB
control
cisplatin
TNF-α
cisplatin+TNF-α

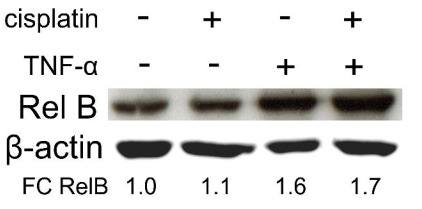
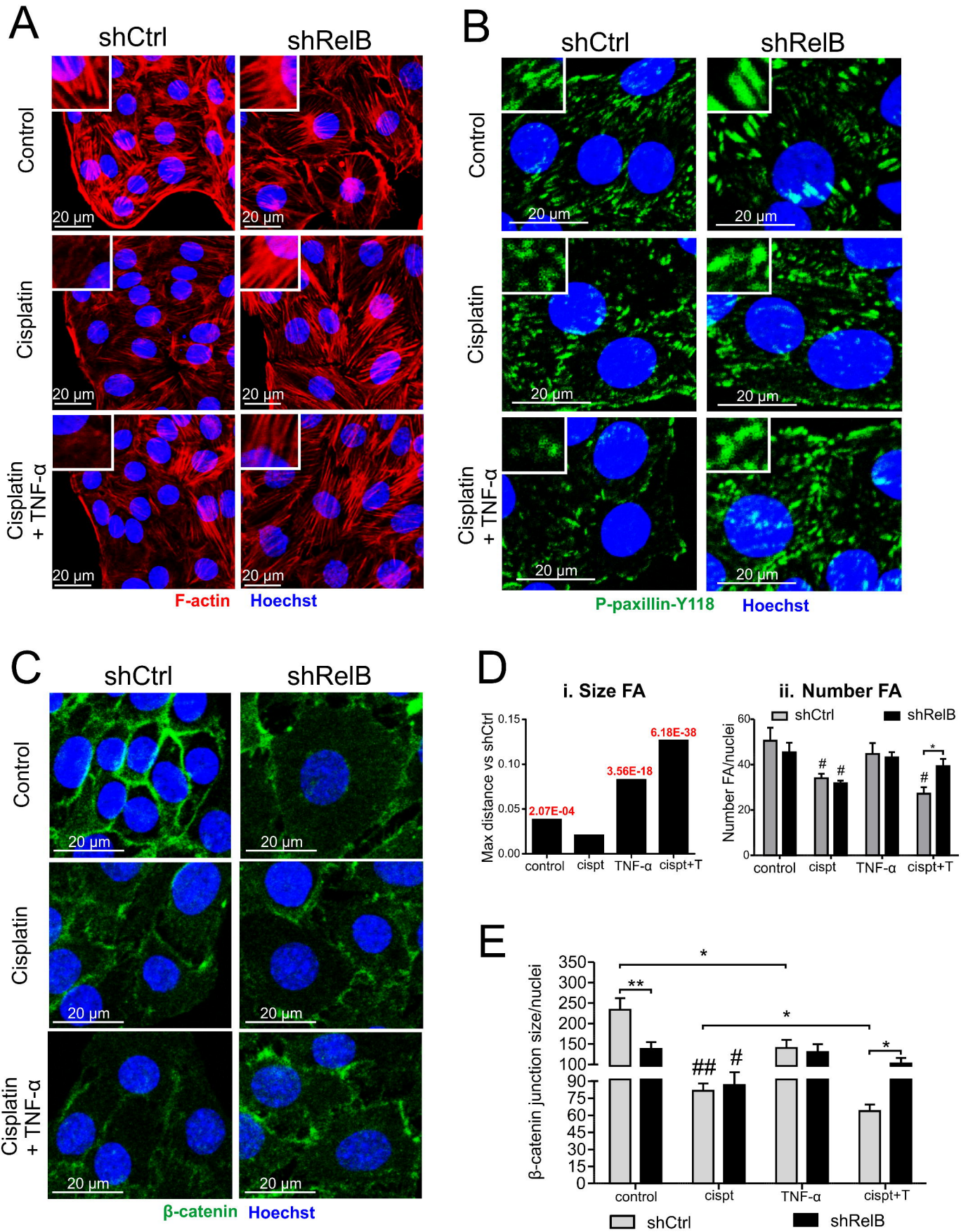

C
cisplatin
TNF-α
Rel B
β-actin
FC RelB 1.0 1.1 1.6 1.7

Figure 3


A
shCtrl
shRelB
Control
Cisplatin
Cisplatin + TNF-α
20 μm
F-actin
Hoechst
B
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Control
Cisplatin
Cisplatin + TNF-α
20 μm
P-paxillin-Y118
Hoechst
C
shCtrl
shRelB
Control
Cisplatin
Cisplatin + TNF-α
20 μm
β-catenin
Hoechst
D
i. Size FA
Max distance vs shCtrl
0.15
0.10
0.05
0.00
2.07E-04
3.56E-18
6.18E-38
control
cispt
TNF-α
cispt+T
ii. Number FA
shCtrl
shRelB
Number FA/nuclei
60
40
20
0
#
*
control
cispt
TNF-α
cispt+T
E
β-catenin junction size/nuclei
350
300
250
200
150
100
90
75
60
45
30
15
0
*
**
##
#
control
cispt
TNF-α
cispt+T
shCtrl
shRelB

Figure 4

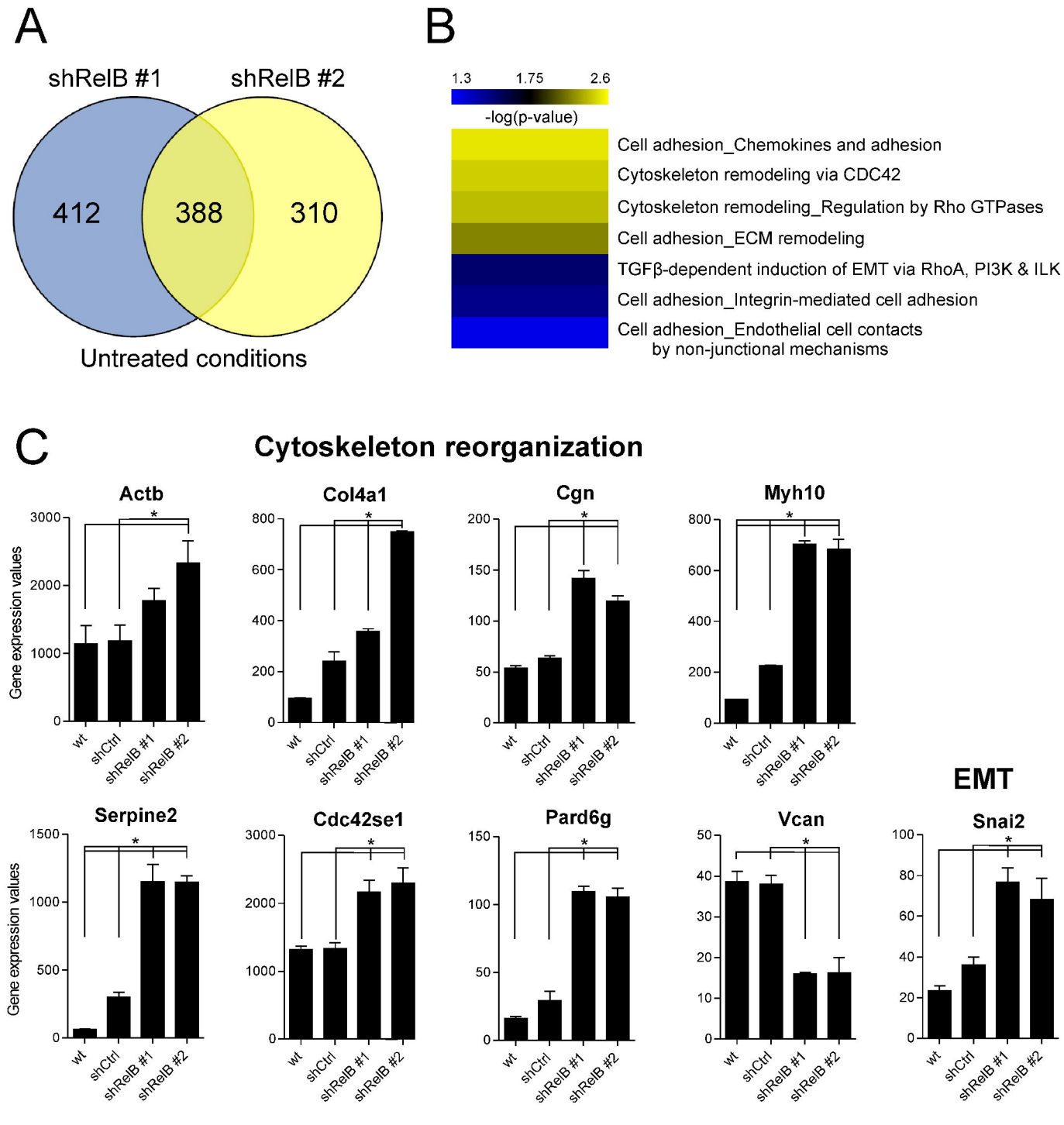

A
shRelB #1
shRelB #2
412
388
310
Untreated conditions
B
1.3
1.75
2.6
-log(p-value)
Cell adhesion_Chemokines and adhesion
Cytoskeleton remodeling via CDC42
Cytoskeleton remodeling_Regulation by Rho GTPases
Cell adhesion_ECM remodeling
TGFβ-dependent induction of EMT via RhoA, PI3K & ILK
Cell adhesion_Integrin-mediated cell adhesion
Cell adhesion_Endothelial cell contacts by non-junctional mechanisms
C
Cytoskeleton reorganization
Actb
Col4a1
Cgn
Myh10
Gene expression values
wt
shCtrl
shRelB #1
shRelB #2
Serpine2
Cdc42se1
Pard6g
Vcan
EMT
Snai2

Figure 5

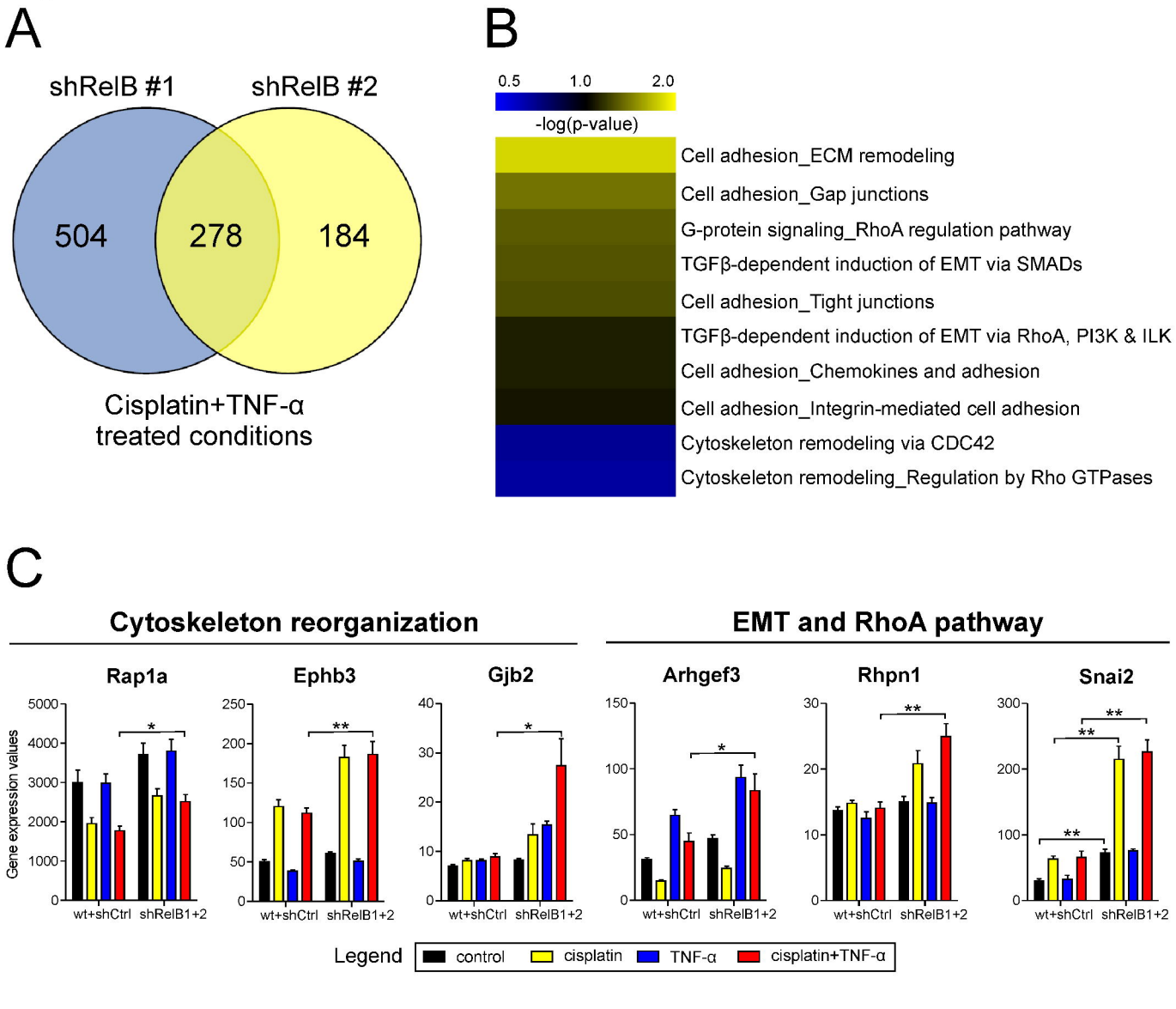
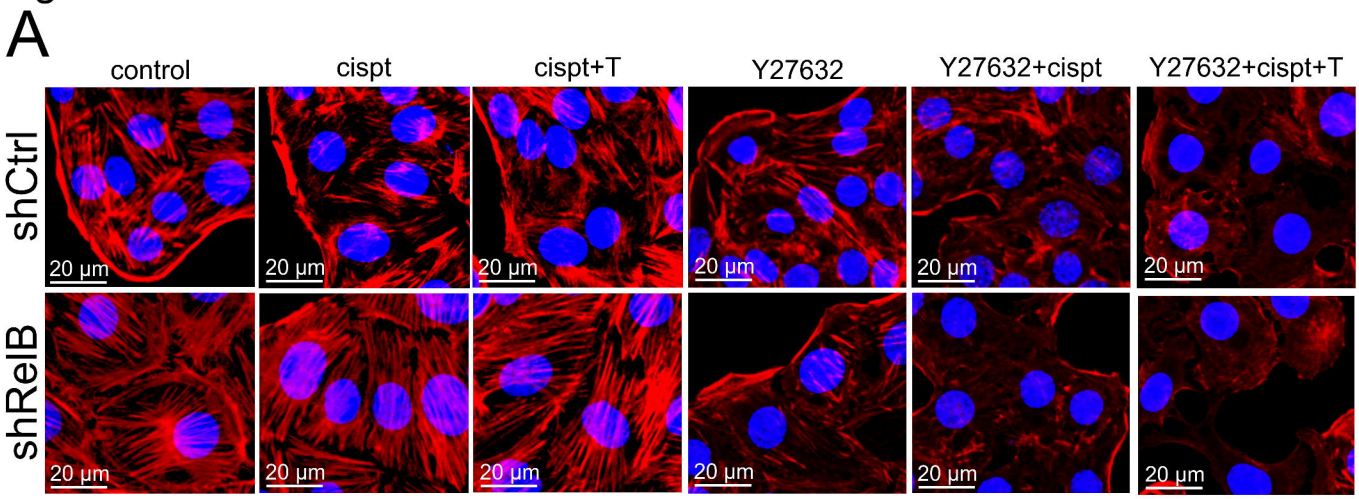
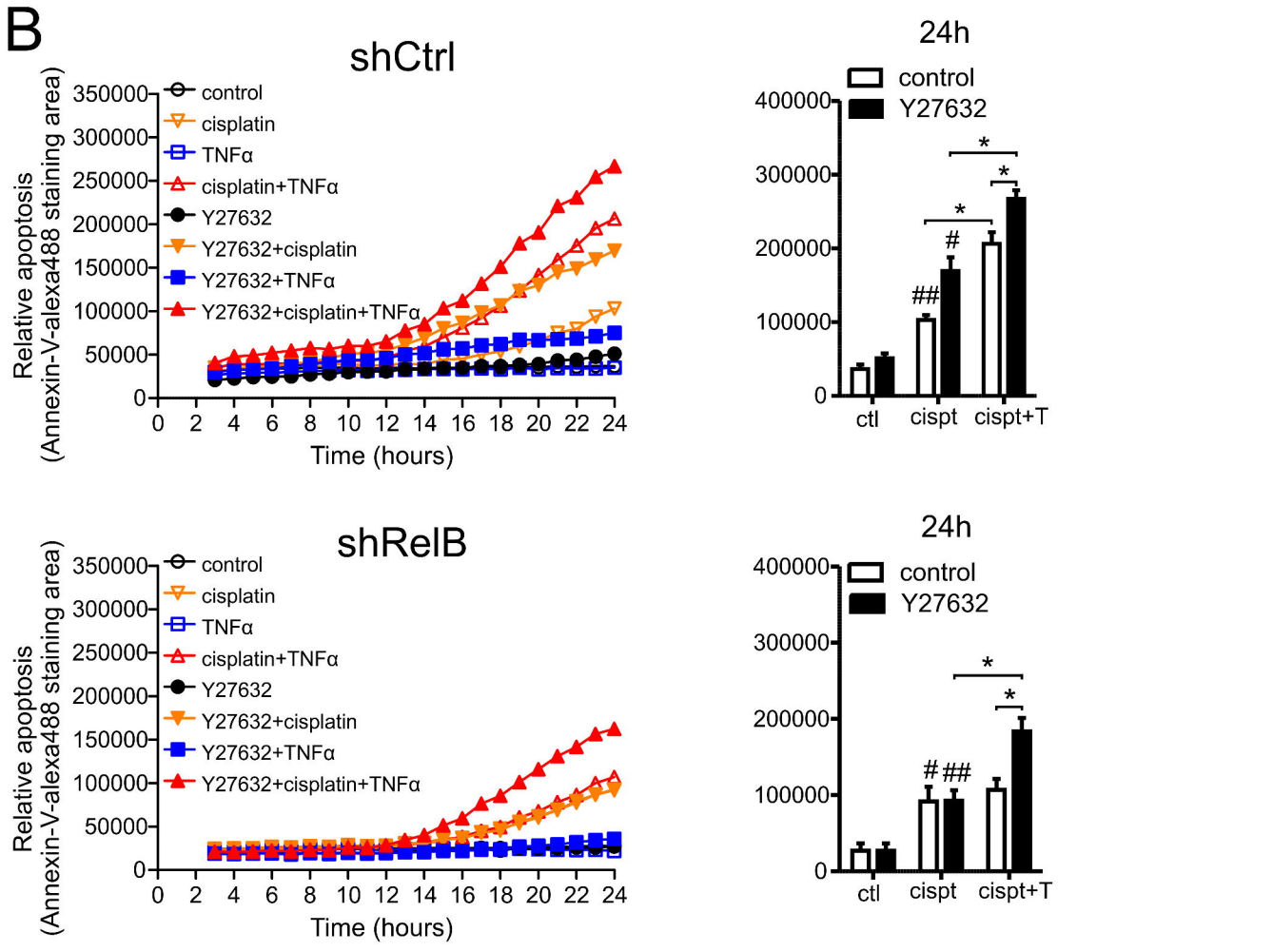

A
shRelB #1
shRelB #2
504
278
184
Cisplatin+TNF-α
treated conditions
B
0.5
1.0
2.0
-log(p-value)
Cell adhesion_ECM remodeling
Cell adhesion_Gap junctions
G-protein signaling_RhoA regulation pathway
TGFβ-dependent induction of EMT via SMADs
Cell adhesion_Tight junctions
TGFβ-dependent induction of EMT via RhoA, PI3K & ILK
Cell adhesion_Chemokines and adhesion
Cell adhesion_Integrin-mediated cell adhesion
Cytoskeleton remodeling via CDC42
Cytoskeleton remodeling_Regulation by Rho GTPases
C
Cytoskeleton reorganization
EMT and RhoA pathway
Rap1a
Ephb3
Gjb2
Arhgef3
Rhpn1
Snai2
Gene expression values
wt+shCtrl
shRelB1+2
Legend
control
cisplatin
TNF-α
cisplatin+TNF-α

Figure 6


A
control
cispt
cispt+T
Y27632
Y27632+cispt
Y27632+cispt+T
shCtrl
shRelB
20 μm


B
shCtrl
Relative apoptosis
(Annexin-V-alexa488 staining area)
control
cisplatin
TNFα
cisplatin+TNFα
Y27632
Y27632+cisplatin
Y27632+TNFα
Y27632+cisplatin+TNFα
Time (hours)
24h
control
Y27632
ctl
cispt
cispt+T
shRelB