Up-regulation of the immediate early gene product RhoB by the cytotoxic necrotizing factor 1 from *Escherichia coli*

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Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation “Up-regulation of the immediate early gene product RhoB by the cytotoxic necrotizing factor 1 from *Escherichia coli*” selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 28.05.08

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Stefanie Dreger

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Vorträge

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Mainz (Germany), 13th – 15th March 2007, 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
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Posterpräsentationen

Mainz (Germany), 11th – 13th March 2008, 49th Spring Meeting of the German Society of Pharmacology and Toxicology.
B. Hirsch**, S.C. Dreger**, J. Huelsenbeck, I. Just, G. Schmidt, H. Genth.
Effect of the cytotoxic necrotizing factors from *Escherichia coli* and *Yersinia pseudotuberculosis* on cell division and cell viability. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 377: p. 29, 109, Suppl. 1 MAR 2008

Mainz (Germany), 11th – 13th March 2008, 49th Spring Meeting of the German Society of Pharmacology and Toxicology.
I. Bock**, S.C. Dreger**, G. Schmidt, H. Genth.
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Maribor (Slovenia), 6th – 9th June 2007, 2nd International Clostridium difficile Symposium.
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Maribor (Slovenia), 6th – 9th June 2007, 2nd International Clostridium difficile Symposium.
J. Huelsenbeck, **S. Dreger**, R. Gerhard, J. Just, H. Genth.
Active RhoB is Required for the Cytotoxic Effect of Clostridium difficile Toxin B.

Mainz (Germany), 13th – 15th March 2007, 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
H. J. Meyer, **S. Dreger**, G. Fritz, K. Aktories, G. Schmidt, H. Genth.
Up-regulation of RhoB by the Cytotoxic Necrotizing Factor 1 from *Escherichia coli*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 38, 151, Suppl. 1 MAR 2007

Nottingham (United Kingdom), 21th – 25th June 2006, 5th International Meeting on the Molecular Biology and Pathogenesis of the Clostridia.
H. Genth, **S. Dreger**, J. Huelsenbeck, I. Just.
Distinct dynamics of the cytopathic and cytotoxic effect induced by *Clostridium difficile* toxin B.

Mainz (Germany), 4th – 6th April 2006, 47th Spring Meeting of the German Society of Pharmacology and Toxicology.
S. Dreger, J. Huelsenbeck, I. Just, H. Genth.
The Cytopathic Activity of Clostridium difficile Toxin B Includes Up-Regulation of the Immediate Early-Gene RhoB and Caspase-3-Activation. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 186, Suppl. 1 MAR 2006

Berlin (Germany), 13th – 15th October 2005, 9th International Dahlem Symosium on Cellular Signal Recognition and Transduction.
H. Genth, J. Huelsenbeck, **S. Dreger**, F. Hofmann, R. Gerhard.
Clostridial factors associated to *Clostridium difficile* toxin A and toxin B responsible for their cytotoxic activity.

Mainz (Germany), 15th – 17th March 2005, 46th Spring Meeting of the German Society of Pharmacology and Toxicology.
J. Huelsenbeck, **S. Dreger**, B. Hartmann, R. Gerhard, H. Genth.
Up-regulation of RhoB protein by glucosylating toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R118-R118 493 Suppl. 1 FEB 2005

Zusammenfassung

Der zytotoxisch nekrotisierende Faktor 1 (CNF1) aus uropathogenen *Escherichia coli* Stämmen (UPEC) aktiviert die niedermolekularen GTP-bindenden Proteine RhoA, Rac1 und Cdc42. Dies hat eine Reorganisation des Aktinzytoskeletts und Polyploidie zur Folge. Diese Arbeit basiert auf der Hypothese, dass die Aktivierung von Rac1 durch CNF1 eine Hochregulation des „immediate early gene“ Produkts RhoB bewirkt. RhoB unterdrückt möglicherweise die CNF1-induzierte Polyploidie.

Die ektopische Expression von konstitutiv aktivem Rac1 sowie die Aktivierung von endogenem Rac1 durch CNF1 aktivierten den *rhoB* Promotor. Die Aktivierung des *rhoB* Promotors durch CNF1 hatte eine Hochregulation des *rhoB* mRNA- und RhoB Proteinspiegels zur Folge. Mit Hilfe des Rac1 Inhibitors NSC23766 konnte bestätigt werden, dass die CNF1-induzierte RhoB Hochregulation auf der Aktivierung von Rac1 beruhte. c-Myc und die p38 MAP Kinase werden von Rac1 reguliert. Beide waren in die CNF1-induzierte RhoB Hochregulation eingeschaltet. c-Myc verstärkte die RhoB Hochregulation, während p38 eine supprimierende Wirkung auf die CNF1-induzierte RhoB Antwort hatte. In CNF1-behandelten Zellen stieg der RhoB Spiegel für mindestens 48 h an, obwohl RhoB eine kurze Halbwertzeit (< 1 h) besaß. Anscheinend wurde die RhoB Expression in CNF1-behandelten Zellen langanhaltend stimuliert. Die RhoB Hochregulation ging mit dessen Aktivierung einher.

Die Behandlung von NIH3T3, ME Fibroblasten und humanen Kolonozyten (HCT 116) mit CNF1 führte zur Polyploidie. Die CNF1-induzierte Polyploidie könnte mit folgendem Modell erklärt werden: Der Abschluss der Zytokinese erfordert die Inaktivierung von RhoA durch Mgc GAP. Die Deamidierung verhindert die Mgc-vermittelte RhoA Inaktivierung, was den Abschluss der Zytokinese verhindert. Dies führt zur Polyploidie.

Es wurde vorgeschlagen, dass p53 einen Zellzyklusarrest in tetraploiden Zellen auslöst (“Tetraploidie Kontrollpunkt”). p53 verhinderte die CNF1-vermittelte Polyploidie nicht, da diese in p53 WT und p53 -/- HCT 116 Zellen mit vergleichbarer Kinetik ablief. Dieses Ergebnis steht im Widerspruch zu der Existenz eines durch p53 regulierten Tetraploidie Kontrollpunktes. RhoB unterdrückte die CNF1-induzierte Polyploidie, da diese in RhoB -/- ME Fibroblasten gegenüber RhoB +/- und NIH3T3 Fibroblasten verstärkt war. CNF1 wird häufig als anti-apoptotischer Faktor beschrieben. CNF1-Behandlung führte in RhoB -/- Fibroblasten zum apoptotischen Zelltod, jedoch nicht in RhoB +/- oder NIH3T3 Fibroblasten. Dieser Befund legte den Schluss nahe, dass RhoB für das Überleben der CNF1-behandelten Zielzelle benötigt wird.

Die Inhibition der Zytokinese durch CNF1 verlangsamt möglicherweise die Erneuerung von Epithelzellen des Ureters, was die Kolonisierung durch CNF1-produzierenden *E. coli* begünstigt.

Schlagworte: zytotoxisch nekrotisierender Faktor; RhoB; Polyploidie

Abstract

The cytotoxic necrotizing factor 1 (CNF1) from uropathogenic *Escherichia coli* (UPEC) activates the low molecular weight GTP-binding proteins RhoA, Rac1, and Cdc42. Thereby, CNF1 causes actin re-organisation and polyploidy. This study is based on the hypothesis that Rac1 activation by CNF1 causes up-regulation of the immediate early gene product RhoB. RhoB may suppress CNF1-incuced polyploidy.

Ectopic expression of constitutively active Rac1 and activation of endogenous Rac1 by CNF1 caused activation of the *rhoB* promoter. The *rhoB* promoter activation induced by CNF1 was reflected by an increase of the *rhoB* mRNA and RhoB protein level. Inhibition of Rac1 by NSC23766 confirmed that CNF1-induced RhoB up-regulation was based on Rac1 activation. c-Myc and p38 MAP kinase are downstream effectors of Rac1. Both were involved in CNF1-induced RhoB up-regulation. c-Myc positively, while p38 negatively regulated the RhoB response. In CNF1-treated cells, the level of RhoB was elevated for at least 48 h, even though RhoB exhibited a short half-life period (< 1 h). Thus, RhoB up-regulation was permanently triggered by CNF1 for at least 48 h. Up-regulated RhoB was active in CNF1-treated cells, as determined by effector pulldown assay.

Treatment with CNF1 caused polyploidy in NIH3T3 and ME fibroblasts, as well as in the human colon carcinoma cell line HCT 116. We suggest the following model for CNF1-induced polyploidy: Completion of cytokinesis requires the inactivation of RhoA by Mgc GAP. Deamidation blocks the Mgc-induced inactivation of RhoA, leading to blocked cytokinesis, which results in polyploidy.

p53 has been suggested to induce cell cycle arrest of tetraploid cells (“tetraploidy checkpoint”). Polyploidy was not suppressed by p53, as the kinetics of CNF1-induced polyploidy were comparable in p53 WT and p53 -/- HCT 116 cells. Our data challenge the existence of a p53-dependent tetraploidy checkpoint. RhoB was a suppressor of polyploidy, as CNF1-induced polyploidy was enhanced in RhoB -/- compared to either MEF RhoB +/- or NIH3T3 fibroblasts. CNF1 is often characterised as anti-apoptotic. In MEF RhoB -/- but not in MEF RhoB +/- or NIH3T3 fibroblasts, CNF1 induced apoptotic cell death. Thus, RhoB is required for the survival of the CNF1-treated mammalian target cells.

Inhibition of cytokinesis by CNF1 may inhibit cell renewal, resulting in prolonged colonisation of adherent uropathogenic bacteria in the epithel.

Keywords: cytotoxic necrotizing factor; RhoB; polyploidy

Content

1 Introduction

1.1 Low molecular weight GTP-binding proteins

The Ras (**Ra**t **s**arcoma) superfamily of low molecular weight GTP-binding proteins consists of more than 150 members (molecular weight: 18-26 kDa). Based on structural, sequential, and functional similarities, the Ras superfamily has been divided into 5 subfamilies: Ras, Rho, Rab, Ran, and Arf (Table I) (Wennerberg et al., 2005). GTP-binding proteins are molecular switches that cycle between an inactive, GDP-bound, and an active, GTP-bound, conformation (Hall and Nobes, 2000). The GTPase cycle of low molecular weight GTP-binding proteins (shown for Rho) is regulated by two groups of proteins (Fig. I) (Jaffe and Hall, 2005):

1. Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP against GTP, thereby activating GTP-binding proteins.
2. GTPase activating proteins (GAPs) potentiate the intrinsic GTPase activity. GTP hydrolysis causes inactivation of the Ras proteins.

Rho and Rab proteins are further regulated by guanine nucleotide dissociation inhibitors (GDIs). GDIs stabilise the inactive, GDP-bound, conformation of Rho and Rab proteins in the cytosol (Olofsson, 1999).

The exchange of GDP against GTP causes conformational changes in the switch I region (residues 32–40 in RhoA) and switch II region (residues 62-78 in RhoA) (Wittinghofer and Pai, 1991; Fujisawa et al., 1998). The switch I is critical for effector-binding and the switch II for GTP hydrolysis. The conformational changes allow the interaction of Ras proteins with their effectors. Effectors of GTP-binding proteins include serine/threonine/tyrosine kinases, lipid kinases, lipases, oxidases, and scaffold proteins (Jaffe and Hall, 2005).

An important biochemical feature of the majority of low molecular weight GTP-binding proteins is their post-translational modification (Wennerberg et al., 2005). Most Ras proteins are isoprenylated at the C-terminal CAAX (C=Cys, A=aliphatic, X=any amino acic) motif (Cox and Der, 2002). Isoprenylation at the cysteine residue within the CAAX-motif by either geranylgeranyl-transferases (GGT) or farnesyl-transferases

(FT) facilitates membrane targeting and is thus required for subcellular localisation of the Ras proteins (Wennerberg et al., 2005). GDIs mask the isoprenyl moiety of Rho and Rab proteins to prevent membrane targeting (Wennerberg et al., 2005).

Table I: Functions of the 5 subfamilies of the Ras superfamily of low molecular weight proteins

subfamily	regulation of
Ras	gene expression, cell differentiation, cell proliferation
Rho	actin cytoskeleton, cell cycle progression, gene expression
Rab	transport of proteins and vesicles
Arf	transport of proteins and vesicles
Ran	nucleocytoplasmic transport of RNA and proteins

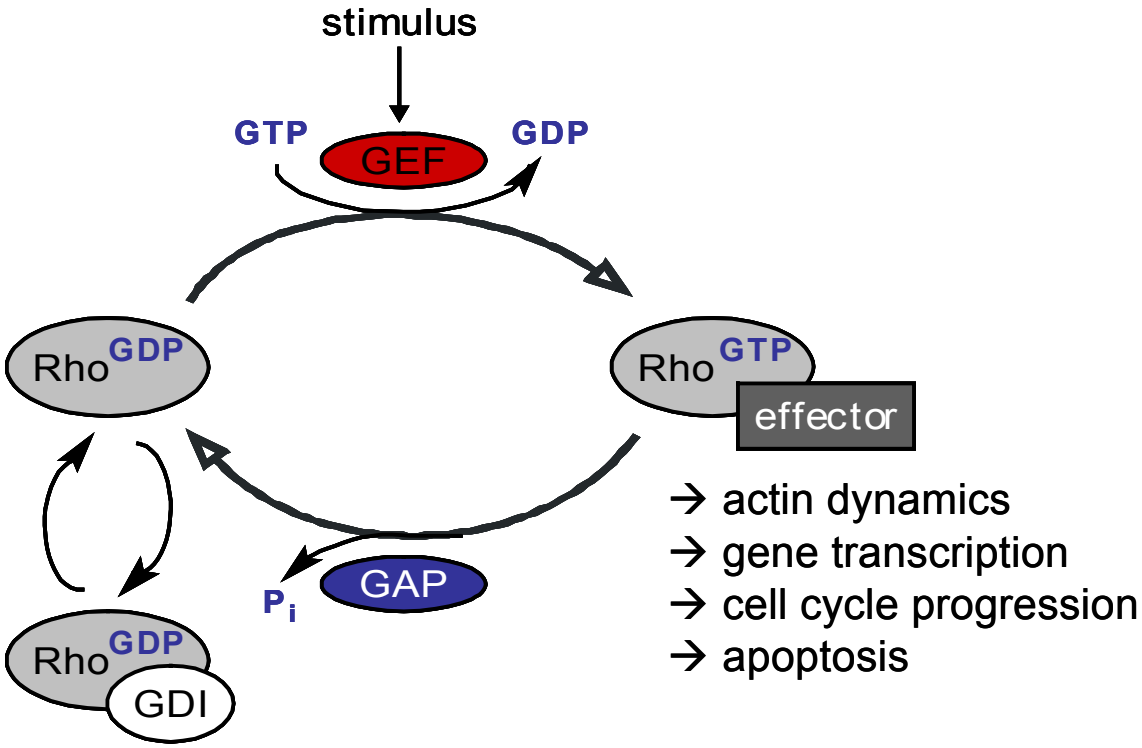

Fig. I: The GTPase cycle of Rho proteins

Rho proteins cycle between an inactive, GDP-bound, and an active, GTP-bound, conformation. In the GTP-bound conformation, they interact with their effector proteins to regulate signal transduction. The GTPase cycle is regulated by guanine nucleotide exchange factors (GEF, activating), GTPase activating proteins (GAP, inactivating), and guanine nucleotide dissociation inhibitors (GDI, inactivating) (adapted from Jaffe and Hall, 2005).

1.2 Rho proteins

The Rho (**R**as **ho**mologous) subfamily consists of 23 members: Rho(A,B,C), Rac(1,2,3), RhoG/AhrG, Cdc42, TC10, TCL, Wrch-1, Wrch-2/Chp, RhoH/TTF, Rnd1, Rnd2, RhoE/Rnd3, RhoD/AhrD, RhoF/Rif, as well as the atypical Rho proteins: RhoBTB(1,2,3), and Miro-1 and 2 (Bustelo et al., 2007).

Rho family proteins regulate the actin cytoskeleton, cell-cycle progression, gene transcription, and vesicle trafficking (Jaffe and Hall, 2005). The best studied Rho family proteins are RhoA, Rac1, and Cdc42. They are described as key regulating proteins of the actin cytoskeleton (Wennerberg and Der, 2004). RhoA activation induced by lysophosphatidic acid (LPA) causes the formation of stress fibres in fibroblasts (Hall, 1998). At the plasma membrane, stress fibres end in focal adhesions, which connect the actin cytoskeleton with the extracellular matrix (Ridley and Hall, 1994). Moreover, RhoA-induced stress fibres are required for the formation of the contractile ring during cytokinesis (Wadsworth, 2005). Activation of Rac1 by platelet-derived growth factor (PDGF) or insulin leads to the assembly of actin filaments at the cell periphery, namely lamellipodia and membrane ruffles (Hall, 1998). Active Cdc42 is responsible for the formation of actin-based surface protrusions called filopodia (Nobes and Hall, 1995). Consequently, RhoA, Rac1, and Cdc42 regulate cell polarity, cell shape, cell movement, and cell-cell-interactions (Ridley, 2001).

1.3 RhoB

RhoA and RhoB are 86 % identical at the amino acid level. Both are involved in the regulation of the actin cytoskeleton. They interact with the same GEF and effector proteins (Wennerberg and Der, 2004). RhoB, however, displays several properties distinct from RhoA. While RhoA is constitutively expressed, RhoB is transcriptionally regulated. The RhoB expression is low in quiescent cells due to RhoA- and (H/K/N)Ras-dependent suppression of the *rhoB* promoter activity (Jiang et al., 2004). RhoB up-regulation occurs physiologically during G1- and S-phase (Zalcman et al., 1995). Furthermore, RhoB is up-regulated in response to genotoxic stress and

growth factors and is thus classified as an immediate early gene product. (Fritz and Kaina, 2001; Jähner and Hunter, 1991). Inactivation of RhoA or (H/K/N)Ras by either farnesyl-transferase/geranylgeranyl-transferase inhibitors (Stamatakis et al., 2002) or Rho/Ras-inactivating bacterial protein toxins (Gerhard et al., 2005; Huelsenbeck et al., 2007) causes “de-suppression” of the *rhoB* promoter, resulting in RhoB up-regulation. RhoB can be either farnesylated (RhoB-F) or geranylgeranylated (RhoB-GG), and is additionally modified by palmitic acid (Adamson et al., 1992a). Opposing functions of RhoB have been suggested to be based on the nature of RhoB prenylation (Mazieres et al., 2005). RhoB-F is associated with the plasma membrane, whereas RhoB-GG localises to endomembrane vesicles (endosomes and lysosomes) (Adamson et al., 1992b) (Michaelson et al., 2001). The distinct subcellular localisation may be responsible for the distinct biological activities of RhoB-F and RhoB-GG (Mazieres et al., 2005). RhoB regulates the transport of endosomes (Rojas et al., 2004) and causes G2-arrest after UV-light exposure (Milia et al., 2005). Furthermore, RhoB contributes to the initiation of apoptosis by alkylating mutagens and toxin B from *Clostridium difficile* (Fritz and Kaina, 2000; Huelsenbeck et al., 2007). Several studies, however, show a protective role of RhoB against cell death (Ader et al., 2002; Canguilhem et al., 2005). RhoB-F has been shown to suppress multinucleation and centrosome overduplication in cells exposed to UV-light to prevent mitotic cell death (Milia et al., 2005). Accordingly, RhoB has been proposed to participate in the decision of a damaged cell to undergo cell death or to initiate repair mechanisms rather than to regulate cell death itself (Fritz and Kaina, 2000).

1.4 Rho-modifying bacterial pathogenicity factors

A variety of bacterial pathogenicity factors targets Rho proteins to manipulate host-cell functions. These factors can be divided into two subgroups: Rho-inactivating and Rho-activating pathogenicity factors.

1.5 Rho-inactivating bacterial pathogenicity factors

C3 exoenzyme from *Clostridium botulinum* (C3bot) was the first pathogenicity factor identified as Rho-inactivating. C3bot specifically ADP-ribosylates Rho(A,B,C) at asparagine 41 using the co-substrate NAD^+ (Sekine et al., 1989). This modification renders Rho(A,B,C) inactive, as it blocks the GEF-mediated nucleotide exchange (Sehr et al., 1998) and promotes binding of the GTP-binding proteins to their GDI proteins (Genth et al., 2003). The clostridial glucosylating toxins (CGT), e.g., toxin A (TcdA) and toxin B (TcdB) from *Clostridium difficile* and lethal toxin (TcsL) from *Clostridium sordellii*, are further inactivating toxins. They catalyse the transfer of a glucose moiety from their co-substrate UDP-glucose onto a pivotal threonine residue (Thr37 in RhoA, Thr35 in Rac1) within the switch I domain of Rho/Ras proteins (Just et al., 1995a; Just et al., 1995b; Just et al., 1996; Popoff et al., 1996). The glucosylation blocks the interaction of Rho/Ras proteins with their effector proteins, thereby causing their functional inactivation (Sehr et al., 1998; Genth et al., 1999; Hermann et al., 1998).

1.6 Rho-activating bacterial pathogenicity factors

Rho-activating protein toxins have been isolated exclusively from gram-negative bacteria (Lemonnier et al., 2007): the cytotoxic necrotizing factors (CNFs) CNF1, CNF2, and CNF3 from *Escherichia coli*, CNF_Y from *Yersinia pseudotuberculosis*, and the dermonecrotic toxin (DNT) from *Bordetella* species. The first identified toxin was CNF1, which was isolated from enteritis-affected children (Caprioli et al., 1983). The toxin is cytotoxic for HeLa cells and exhibits a necrotizing effect on skin when injected subcutaneously in rabbits, mice, or guinea pigs. Based on these effects, it was called cytotoxic necrotizing factor (Caprioli et al., 1983). About 60 % of uropathogenic *E. coli* strains (UPECs) carry the *cnf1* gene. Mutation of this gene attenuates the virulence of UPECs (Rippere-Lampe et al., 2001). These observations indicate that CNF1 plays a role in UPEC-mediated urinary tract infection. CNF_Y is one of many pathogenicity factors of *Yersinia pseudotuberculosis* (Lockman et al., 2002). Its role in pathogenicity is unclear.

1.7 Structure and up-take mechanism of CNF1 and CNF_Y

The chromosomally located *cnf1* and *cnf_Y* genes encode single-chained proteins with molecular masses of about 115 kDa (Falbo et al., 1993; Lockman et al., 2002). Both are 65 % identical at the nucleotide level (Lockman et al., 2002). CNF1 and CNF_Y are multi-domain protein toxins. They can be classified as A-B toxins with a C-terminal deamidase domain and an N-terminal delivery domain (Fig. II). The delivery domain harbours the receptor binding domain (amino acids (aa) 53-190) (Hoffmann and Schmidt, 2004). The cellular receptor of CNF1 has been suggested to be the laminin receptor precursor p37, a subunit of the mature non-integrin 67 kDa laminin receptor. In a yeast two-hybrid system, CNF1 has been shown to bind to the ubiquitously expressed p37 (Chung et al., 2003; Kim et al., 2005). CNF_Y binds to a different cellular receptor. Recent data, however, suggested overlapping regions of the receptors for CNF1 and CNF_Y (Blumenthal et al., 2007). Possible candidates, exhibiting overlapping structures, are heparan sulfate proteoglycans (HSPGs) (Blumenthal et al., 2007). Receptor binding of CNFs initiates receptor-mediated endocytosis (Fig. III) (Hoffmann and Schmidt, 2004). A further domain within the delivery domain is the central translocation domain, which contains two hydrophobic helices (aa 350-372 and aa 387-412) (Fig. II). This domain is most likely responsible for the translocation of CNF from the endosome into the cytosol. Acidification of the endosome induces re-folding of the toxin to allow the insertion of the translocation domain into the endosome membrane (Contamin et al., 2000; Pei et al., 2001). The C-terminal deamidase domain (aa 720-1014) is then released into the cytosol (Fig. II+III) (G. Schmidt, personal communication). The auto-catalytic cleavage of the deamidase domain is mediated by an inherent protease domain within the delivery domain (Fig. II) (G. Schmidt, personal communication). A similar mechanism has recently been described for the glucosyltransferase domain of CGT (Egerer et al., 2007).

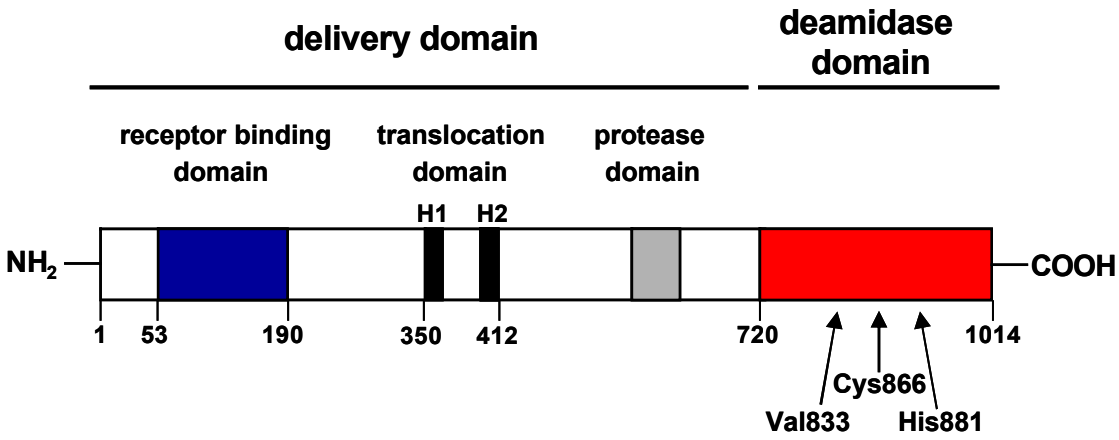

Fig. II: Schematic representation of the multi-domain structur of *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1)

The single-chained CNF1 is an A-B toxin, which consists of an N-terminal delivery domain and a C-terminal deamidase domain. The delivery domain harbours the receptor binding domain, the translocation domain, which contains two hydrophobic helices (H1, H2), and a protease domain. Val833, Cys866, and His881 are the crucial amino acids for the deamidase activity.

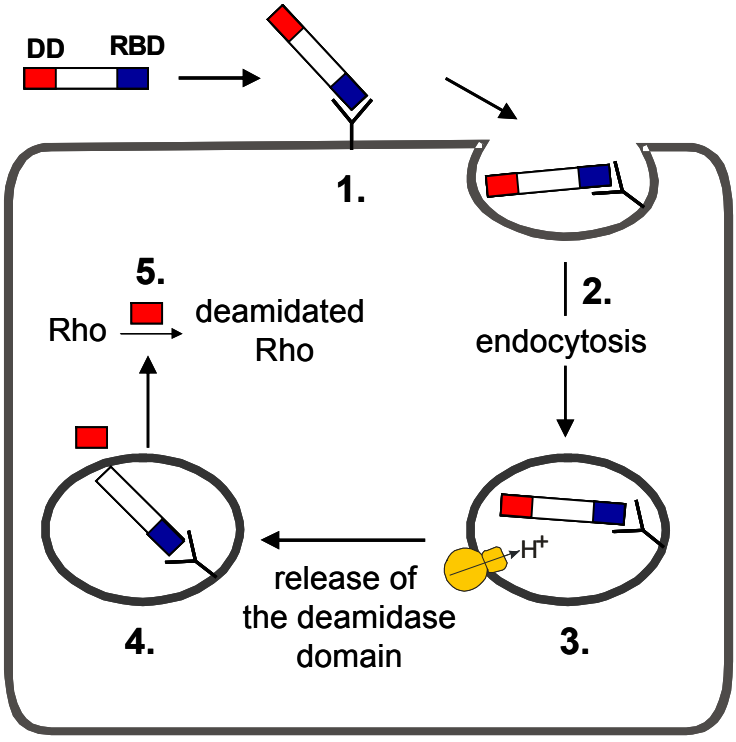

Fig. III: Schematic representation of the proposed up-take mechanism of cytotoxic necrotizing factors

The receptor binding domain (RBD) binds to the receptor (1.), by which means endocytosis is initiated (2.). Subsequently, the endosome is acidified by an H^+-ATPase (3.). Re-folding of the toxin allows the insertion of the translocation domain into the endosome membrane. The deamidase domain (DD) is proteolytically cleaved into the cytosol (4.), where it deamidates cellular Rho proteins (5.).

1.8 CNF-induced deamidation of Rho proteins

CNF1 and CNF_Y catalyse the deamidation of Rho family GTP-binding proteins. Valine 833, cysteine 866, and histidine 881 are the three crucial amino acids for this activity (Fig. II), as identified by mutation studies and crystal structure analysis (Buetow et al., 2001; Schmidt et al., 1998). CNF1 deamidates glutamine 63 of RhoA or glutamine 61 of Rac1 and Cdc42 using H_2O as co-substrate (Fig. IV) (Schmidt et al., 1997; Lerm et al., 1999). CNF_Y selectively deamidates RhoA (Fig. IV) (Hoffmann et al., 2004). Glutamine 63/61 is located within the switch II region of the Rho proteins and essential for GTP hydrolysis. Deamidation impairs the intrinsic as well as the GAP-stimulated GTPase activity (Flatau et al., 1997; Schmidt et al., 1997). Accordingly, CNF-induced deamidation results in constitutive activation of Rho proteins (Flatau et al., 1997; Schmidt et al., 1997). Deamidation of Rho proteins sensitises them for ubiquitinylation and subsequent degradation by the 26S proteasome (Boyer et al., 2006; Doye et al., 2002). This effect, however, is not general but cell-type-specific (Boyer et al., 2006).

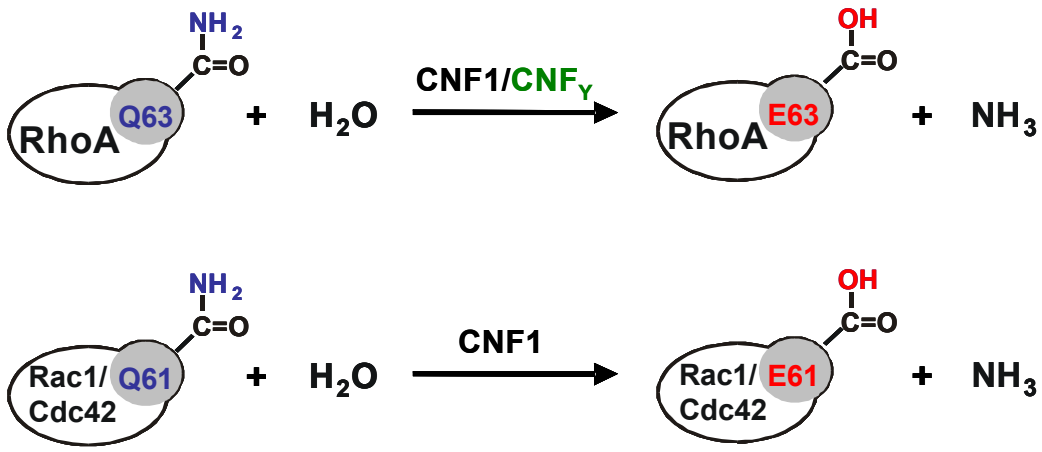

Fig. IV: Deamidation of Rho proteins by cytotoxic necrotizing factors 1 and $_Y$

CNF1 and CNF_Y deamidate RhoA at Gln63 using H_2O as co-substrate. CNF1 deamidates the further substrates Rac1 and Cdc42 at Gln61.

1.9 Cell biological consequences of Rho-protein deamidation

Deamidation, i.e., activation of RhoA, Rac1, and Cdc42 by CNF1 causes cell spreading, formation of actin stress fibers, membrane ruffles, and filopodia (Hoffmann and Schmidt, 2004). Membrane ruffles and lamellipodia are required for phagocytosis and endocytosis. CNF1-treated epithelial cells behave like phagocytes and develop macropinocytotic activity (Falzano et al., 1993; Fiorentini et al., 2001). Furthermore, CNF1 increases the permeability of epithelial cells, thereby it disturbs the barrier function (Gerhard et al., 1998). CNF1 induces the formation of multinucleated cells (Oswald et al., 1989), which has been suggested to be based on impaired cytokinesis with ongoing karyokinesis (Denko et al., 1997; Oswald et al., 2005). Rac1 activation may trigger multinucleation in CNF1-treated cells (Malorni and Fiorentini, 2006). Furthermore, CNF1 activates Rho-dependent signalling including the JNK, the p38 MAP kinase, and the NF-κB pathways (Boyer et al., 2004; Lerm et al., 2002; Munro et al., 2004). Activation of these pathways results in the production of pro-inflammatory mediators, e.g., TNF-α, IL-6, IL-8, and reactive oxygen species (ROS) (Falzano et al., 2003; Munro et al., 2004). When first identified, CNF1 was described as cytotoxic (Caprioli et al., 1983). Recent data, however, suggest a pro-survival activity of CNF1. CNF1 inhibits UV-light-induced apoptosis in an NF-κB-dependent manner (Miraglia et al., 2007).

CNF_Y, which selectively deamidates RhoA, induces the formation of actin stress fibres and multinucleated cells (Hoffmann et al., 2004).

2 Aims of this project

The cellular level of the immediate early gene product RhoB is low in most cell lines, as the *rhoB* promoter is suppressed by RhoA and (H/K/N)Ras. Inactivation of either RhoA or (H/K/N)Ras by Rho/Ras-inactivating bacterial toxins leads to “de-suppression” of the *rhoB* promoter, resulting in up-regulation of RhoB.
RhoB is also up-regulated by the cyctotoxic necrotizing factor 1 from *Escherichia coli* that activates RhoA, Rac1, and Cdc42. This finding is surprising, as one must expect that RhoA activation by CNF1 causes suppression rather than up-regulation of RhoB.

In this thesis, this apparent contradiction is solved. In the first part of this study, a new Rac1-dependent pathway is characterised that positively regulates RhoB.

p38 MAP kinase and c-Myc are downstream effector proteins of Rac1. Their involvement in the CNF1-induced RhoB up-regulation is analysed in this study.

CNF1 induces the formation of prominent actin cables, lamellipodia, and filopodia, indicating the activation of RhoA, Rac1, and Cdc42, respectively. CNF1 further causes formation of multinucleated cells and cell death. In the second part of this study, the involvement of RhoB in the latter effects is characterised.

3 Materials and Methods

3.1 Materials

The GST-C21 and the GST-PAK-Crib constructs were a gift of Dr. J. Collard (Amsterdam) (Sander et al., 1998).

Expression vectors encoding for the constitutively active Rho GTPases myc-RhoA-L63, myc-RhoB-L63, myc-Cdc42-L61, and myc-Rac1-L61 were a gift from P. Aspenström (Uppsala) (Aspenström et al., 2004).

The 3.5 kb *rhoB* promoter CAT construct was provided by G. Fritz (Mainz) (Fritz and Kaina, 1997).

Commercially obtained reagents: actinomycin D, MG132, c-Myc inhibitor ((Z,E)-5-(4-Ethylbenzylidine)-2-thioxothiazolidin-4-one), Rac1 inhibitor (NSC23766), SB 203580 (Calbiochem); cycloheximide, rhodamine-conjugated phalloidin (Sigma); DAPI (4',6-diamidino-2-phenylindole) (Serva); propidium iodide (Fluka); Annexin V-Alexa fluor 488 (Invitrogen); WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt), Complete-EDTA (Roche).

Antibodies: RhoA (26C4), RhoB (sc-8048) (SantaCruz); Rac1 (clone 23A8) (Upstate Technologies); beta-actin (AC-40) (Sigma); horseradish peroxidase conjugated secondary antibody, mouse (Rockland).

3.2 Toxin Preparation

In this study, recombinant toxins were applied. CNF1, CNF1-C866S, and CNF_Y were expressed as GST fusion proteins in *E. coli* and purified by affinity chromatography using glutathione-sepharose (Hoffmann et al., 2004). GST-CNF1, GST-CNF1-C866S, and GST-CNF_Y were eluted from the beads using glutathione.

3.3 Cell culture

In this study, fibroblast cell lines were applied to study the function of Rho proteins. For functional analysis of RhoB, mouse embryonic fibroblasts (MEFs) RhoB +/- and RhoB -/- were applied. The role of the p38 MAP kinase was studied using MEF $p38_\alpha$

WT and MEF $p38_{\alpha}$ -/-. The human colon carcinoma cell lines HCT 116 p53 wild type and HCT 116 p53 -/- were applied to investigate the function of p53. All cell lines are sensitive to the cytotoxic necrotizing factors.

Fibroblasts were cultivated in Dulbecco's modified essential medium (Biochrom, + 10 % FCS, 100 µg/ml penicillin, 100 U/ml streptomycin and 1 mM sodium pyruvate) at 37 °C and 5 % CO_2. HCT 116 cells were cultivated in McCoy's 5A medium (Biochrom, + 10 % FCS, 100 µg/ml penicillin, 100 U/ml streptomycin and 1 mM sodium pyruvate) at 37 °C and 5 % CO_2. Upon confluence, cells were passaged. The cells were washed with phosphate buffered saline (PBS) and trypsinised. Cells were then suspended in full growth medium and centrifuged at 800 rpm for 5 min. Pellets were re-suspended in 5-10 ml full growth medium and counted using a Neubauer-counting chamber. 10^5 cells were seeded per 3.5 cm^2 culture dish.

3.4 Treatment of cells

Subconfluent cells were treated with CNF1 or CNF_Y as indicated or CNF1-C866S (1 µg/ml). The following concentrations of the used agents were applied: MG132 (20 µM), a reversible and cell permeable inhibitor of the 26S proteasome; cycloheximide (1 mM), a protein synthesis inhibitor; actinomycin D (5 µM), a transcription inhibitor; NSC23766 (60 µM), a Rac1 inhibitor; (Z,E)-5-(4-Ethylbenzylidine)-2-thioxothiazolidin-4-one (100 µM) a c-Myc inhibitor; SB 203580 (10 µM), an inhibitor of the p38 MAP kinase.

3.5 Analysis of the *rhoB* promoter activity

The activity of the *rhoB* promoter was determined using a reporter gene assay. Chloramphenicol acetyltransferase (CAT) was used as reporter. Its expression in cells transfected with the 3.5 kb *rhoB* promoter CAT construct (*rhoB* subcloned into pCAT basic) reflects the activity of the *rhoB* promoter (Fritz and Kaina, 1997). Subconfluent NIH3T3 fibroblasts were either transiently transfected with 2 µg of the 3.5 kb *rhoB* promoter CAT construct for 14 h (Fritz and Kaina, 1997) and then treated with the toxins as indicated, or cells were co-transfected with 2 µg of the 3.5 kb *rhoB*

promoter CAT construct and 2 µg expression plasmids coding for the constitutively active forms of the small GTPases RhoA, RhoB, Cdc42, or Rac1. For transient transfection experiments, FuGENE 6 Transfection Reagent (Roche) was applied according to the manufacturer's instructions. Cells were harvested and the protein concentration of the lysates was normalised using Bradford test. The level of CAT expression was analysed using an enzyme-linked immunosorbent assay (CAT-Elisa kit, Roche) according to the manufacturer's instructions.

3.6 RNA purification and RT-reaction

Total RNA was purified from NIH3T3 fibroblasts using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, ethanol was added to cell lysates. Lysates were applied to silica membranes to extract RNA. After DNase digestion, total RNA was washed and eluted with RNase free water.

Reverse Transcription (RT)-reaction is a process, in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA). RT-reaction was performed using the Omniscript RT-Kit (Qiagen) according to the manufacturer's instructions. 2 µg of RNA were used as template for cDNA synthesis.

3.7 Semi-quantitative real-time PCR

Real-time polymerase chain reaction (PCR) is a method that allows exponential amplification of DNA sequences. In this study, we applied cDNA obtained from RT-reaction of purified total RNA to detect changes of mRNA levels. For detection of the synthesised PCR product, the fluorescent dye SYBR green, which specifically intercalates into double stranded DNA, was applied. The fluorescence intensity was measured after each cycle to monitor the synthesis of the DNA product. For quantification, a threshold value for the fluorescence intensity was set. The number of PCR cycles required to reach this threshold (crossing point) was used to compare treated samples with control samples. Control samples were set to 1.0. The real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler (Roche). The cDNA obtained from the RT-reaction was diluted (1:10-

1:100) to avoid overloading. Primers (3 µM) used were: β-actin: 5'– CCT GCT TGC TGA TCC ACA TC– 3' and 5'–GCA TTG CTG ACA GGA TGC AG–3', RhoB: 5'–CCG AGG TAA AGC ACT TCT GC–3' and 5'–CCG AGC ACT CGA GGT AGT CA–3'.

3.8 Cell lysis

After toxin treatment, cells were washed and scraped into Laemmli sample buffer. The obtained suspension was agitated for 10 min at 37 °C and subsequently sonified on ice. The lysate was then incubated for 10 min at 95 °C and submitted to SDS-PAGE.

3.9 Western blot analysis

Complete lysate proteins were separated using SDS-PAGE and subsequently transferred onto nitrocellulose membranes by a tank blot system. The membranes were blocked with 5 % (w/v) non-fat dried milk in TRIS buffered saline plus Tween-20 (1 % (w/v)) buffer for 60 minutes. Incubation with primary antibody was conducted over night at 4 °C, treatment with the secondary antibody for 1 h (22 °C). For the chemoluminescence reaction, ECL Femto (Pierce) was used. All signals were analysed densitometrically using the KODAK 1D software and normalised to beta-actin signals.

3.10 Rho GTPase pulldown assay

Rho proteins cycle between an inactive, GDP-bound, and an active, GTP-bound, conformation. In the active conformation, they interact with their effector proteins. This protein interaction is used in the effector pulldown assay to determine the activity state of Rho proteins.

The Rho binding domain C21, encoding the N-terminal 90 amino acids of Rhotekin (RhoA/B effector) or the CRIB domain (amino acids 56–272) of PAK (Rac1/Cdc42 effector), were expressed as GST fusion proteins in *E. coli* and purified by affinity chromatography using glutathione-sepharose. NIH3T3 fibroblasts were lysed in lysis

buffer (50 mM Tris pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1 % NP-40, 1 mM PMSF, 5 mM DTT, Complete-EDTA). Lysates were then added to either glutathione-bound GST-C21 (detection of GTP-RhoA/B) or GST-PAK-CRIB (detection of GTP-Rac1/Cdc42) for 1 h (4°C). Bound Rho GTPases were eluted by incubation with Laemmli sample buffer at 95 °C for 10 min. Samples were submitted to SDS-PAGE and Western blot analysis.

3.11 Fluorescence microscopy

NIH3T3 fibroblasts seeded on coverslips were washed with PBS and subsequently fixed in 4 % formaldehyde in phosphate buffered saline (PBS) (pH 7.4) at room temperature for 15 min. Cells were then washed and permeabilised with 0.3 % (w/v) Triton X-100 in PBS supplemented with 5 % BSA. F-actin staining was performed using rhodamine-conjugated phalloidin (30 µg/ml) for 30 min at room temperature. Then, a 0.1 µg/ml solution of DAPI in PBS supplemented with 0.1 % (w/v) Tween-20 was used for nuclei staining for 15 min at 37°C. Cells were analysed by fluorescence microscopy using a Zeiss Axiovert 200 M.

3.12 DNA content determination by flow cytometry

Flow Cytometry is a method that can be used to determine the DNA content of cells. The proportions of cells in different phases of the cell cycle can be analysed. Furthermore, cells with an abnormal DNA content can be detected.

Cells were washed in PBS and fixed in 80 % ethanol on ice for 30 min. The cells were then centrifuged at 1000 rpm for 5 min. RNA was removed by incubation with 30 µg/ml RNase at room temperature for 1 h. Subsequently, cells were stained with propidium iodide (50 µg/ml). The stained cells were analysed using a FACScan flow cytometer (Becton Dickinson) using CellQuest Pro software (Becton Dickinson). Quantitative analysis was performed using WinMDI 2.8.

3.13 Detection of apoptosis by flow cytometry

In viable cells, the membrane component phosphatidylserine is exclusively located to the cytosolic side of the plasma membrane due to membrane heterogeneity. Apoptosis is associated with loss of membrane heterogeneity. Therefore, phosphatidylserine becomes exposed to the outer leaflet of the plasma membrane during apoptosis. Annexin V specifically binds to phosphatidylserine, which becomes accessible only in apoptotic cells. Phosphatidylserine exposure can be detected using Annexin V labeled with Alexa Fluor 488.

A marker of necrosis and late apoptosis is the loss of membrane integrity, which can be detected in terms of propidiom iodide up-take.

Annexin V and propidium iodide staining was assessed by flow cytometry. Cells were washed in phosphate-buffered saline and trypsinised. The cells were then centrifuged at 1000 rpm for 5 min. Subsequently, they were stained with Annexin V-Alexa fluor 488 (2.5 µl) in 50 µl binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, 0.1 % BSA) for 15 minutes on ice. Subsequently, 430 µl of binding buffer were added, followed by addition of 10 µl propidium iodide (50 µg/ml). The stained cells were immediately analysed with a FACScalibur flow cytometer using CellQuest Pro software (Becton Dickinson). Quantitative analysis was performed using WinMDI 2.8.

3.14 WST-1 assay

The viability of cells was assessed using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Roche)) according to the manufacturer's instructions. This colorimetric assay is based on the reduction of the tetrazolium salt WST-1 to soluble formazan by cellular dehydrogenases, which can be detected by photometry. Cells were exposed to the toxins as indicated. WST-1 was added directly into the medium. The reduction of WST-1 to formazan by mitochondrial dehydrogenases was quantified by photometry using a scanning multiwell spectrophotometer at 450 nm.

4 Results

4.1 The *rhoB* promoter activity is stimulated by constitutively active Rac1 and CNF1

The immediate early gene *rhoB* has been suggested to be regulated by other Rho family GTP-binding proteins. While Rac1 positively regulates the activity of the *rhoB* promoter, RhoA does the opposite (Fritz and Kaina, 1997). To re-investigate the Rho-dependent regulation of the *rhoB* promoter, we co-transfected NIH3T3 fibroblasts with constitutively active forms of Rac1, Cdc42, RhoA, or RhoB and a 3.5 kb fragment of the *rhoB* promoter cloned into pCAT basic for 24 h (Fig. 1A) (Fritz and Kaina, 1997). The expression of the reporter chloramphenicol acetyltransferase (CAT) in transfected fibroblasts reflects the activity of the *rhoB* promoter. Rac1-Q61L increased the *rhoB* promoter-driven CAT expression by 1.5-fold (Fig. 1A), whereas RhoA-Q63L and RhoB-Q63L caused a reduction (Fig. 1A). Cdc42-Q61L did not affect the *rhoB* promoter activity (Fig. 1A). These results confirmed the former findings of Fritz and Kaina, showing that the *rhoB* promoter is in fact activated by Rac1. RhoA and RhoB suppressed its activity.

Cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* is a protein toxin that deamidates and thus activates RhoA, Rac1, and Cdc42 (Hoffmann and Schmidt, 2004). We hypothesised that Rac1 activation induced by CNF1 activates the *rhoB* promoter. CNF1 increased the *rhoB* promoter-driven CAT expression in a concentration-dependent manner (Fig. 1B). In contrast, the specific activation of RhoA by the cytotoxic necrotizing factor from *Yersinia pseudotuberculosis* CNF_Y hardly increased the *rhoB* promoter activity (Fig. 1B). These data provided further evidence that *rhoB* is positively regulated by Rac1.

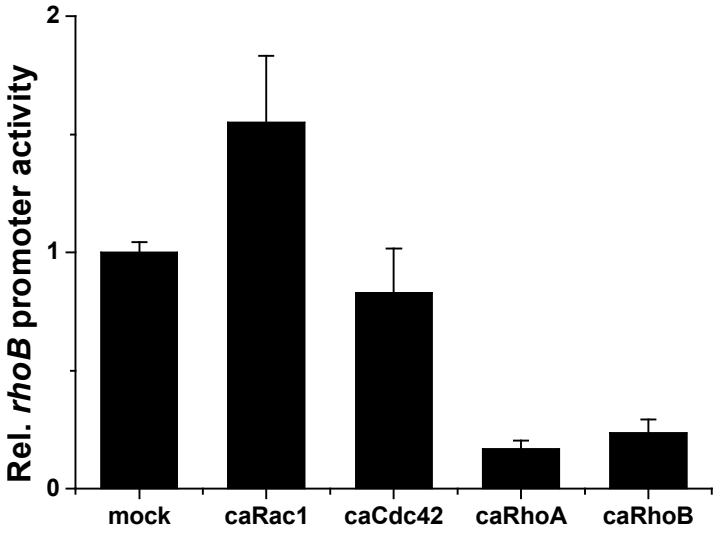

Fig. 1A: The *rhoB* promoter is regulated by constitutively active Rho proteins

2 µg of the 3.5 kb *rhoB* promoter-CAT construct were co-transfected or not (mock) with 2 µg of the expression plasmids coding for the constitutively active (ca) GTPases as indicated into NIH3T3 fibroblasts. 24 h after transfection, cells were harvested and the CAT protein level was quantified. The CAT level of control (mock) cells was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

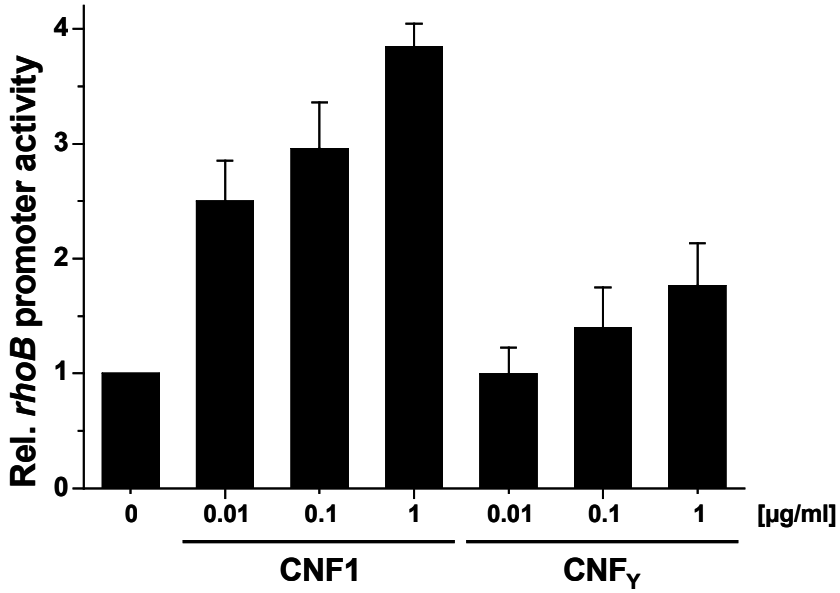

Fig. 1B: CNF1 activates the *rhoB* promoter

Fibroblasts were transfected with 2 µg of the 3.5 kb *rhoB* promoter CAT construct for 14 h. Cells were treated with increasing concentrations of CNF1 or CNF_Y or left untreated for 7 h. Cells were harvested and the CAT protein level was quantified. The CAT level of untreated cells was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

4.2 CNF1-induced RhoB up-regulation depends on mRNA and protein *de novo* synthesis and its deamidase activity

To check, whether the CNF1-induced activation of the *rhoB* promoter was reflected by an up-regulation of *rhoB* mRNA, semi-quantitative real-time RT-PCR was applied (Fig. 2A). CNF1 increased the *rhoB* mRNA level up to 7-fold (Fig. 2A). In contrast, CNF_Y failed to do so (< 3-fold). The CNF1-induced RhoB up-regulation was further analysed at the protein level (Fig. 2B). CNF1 increased the RhoB protein level in a concentration-dependent manner. Both actinomycin D, an inhibitor of mRNA synthesis, and cycloheximide, an inhibitor of protein synthesis, abolished this effect (Fig. 2B). Thus, CNF1-induced RhoB up-regulation was based on both mRNA and protein *de novo* synthesis.

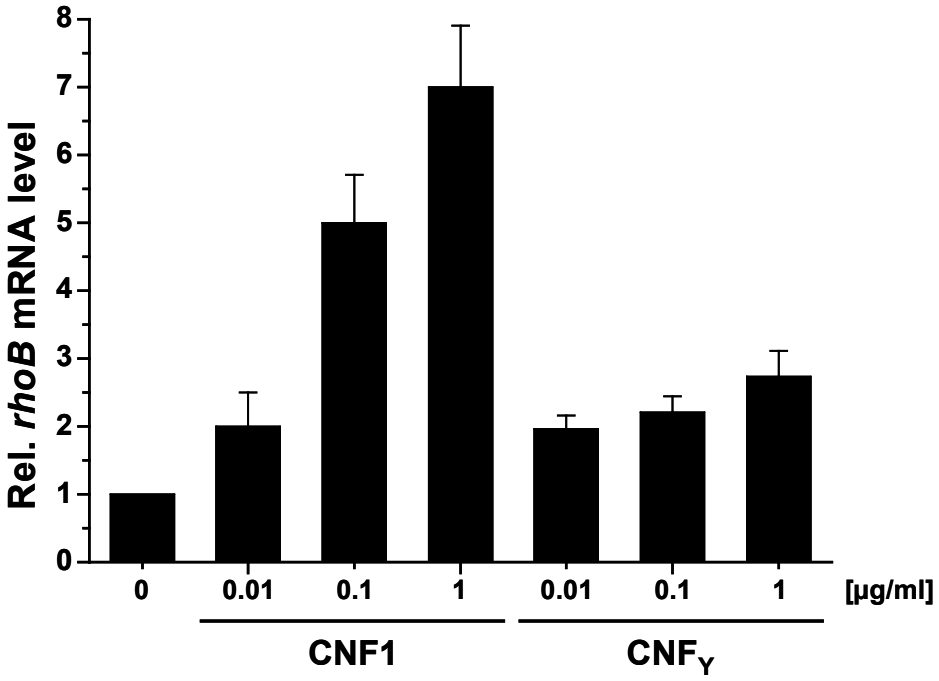

Fig. 2A: CNF1-induces up-regulation of *rhoB* mRNA

Fibroblasts were exposed to increasing concentrations of CNF1 or CNF_Y for 4 h. Total mRNA was prepared and submitted to real-time RT-PCR. *rhoB* signals were normalised to beta-actin signals. The control level was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

Lipopolysaccharide (LPS) has recently been shown to cause up-regulation of RhoB in dendritic cells (Kamon et al., 2006). LPS is a cell wall component of gram-negative bacteria, which is released after microbial cell death. CNF1 applied in this study was prepared from *E. coli* and purified as GST fusion protein. Thus, LPS may be a contamination of the CNF preparation. To exclude that LPS was the trigger of RhoB up-regulation, fibroblasts were treated with the deamidase-deficient mutant of CNF1, CNF1-C866S (Blumenthal et al., 2007). CNF1-C866S failed to cause a strong RhoB up-regulation (Fig. 2C). RhoB up-regulation was therefore based on the deamidation of Rho proteins. It was not due to an enzyme-independent activity of the toxin or based on LPS-contamination. Minor activation of the *rhoB* promoter and up-regulation of *rhoB* mRNA by CNF_Y may be based on LPS (Fig. 1B+2A).

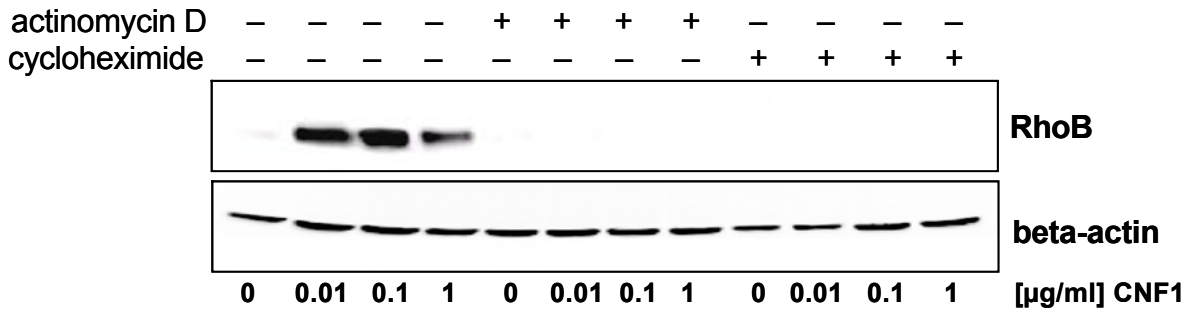

Fig. 2B: CNF1-induced RhoB up-regulation is inhibited by actinomycin D and cycloheximide
Fibroblasts were treated with either actinomycin D (5 µM) or cycloheximide (1 mM) or left untreated for 1 h. CNF1 was then added as indicated and incubation continued for 4 h. Cells were lysed and submitted to immunoblot against RhoB and beta-actin. Western blots from representative experiments are shown (n = 3).

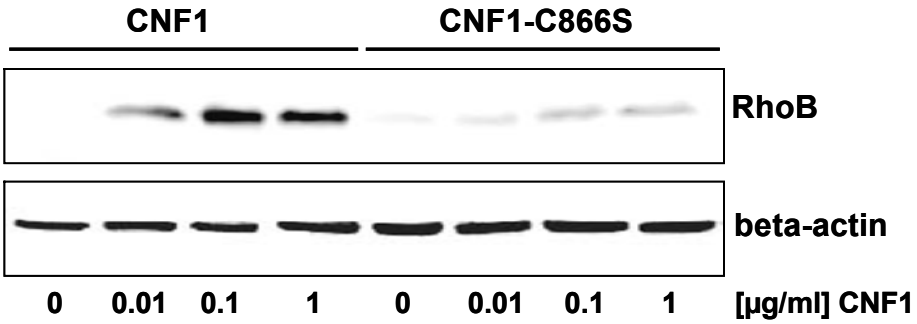

Fig. 2C: CNF1-induced RhoB up-regulation depends on its deamidase activity
Fibroblasts were treated with increasing concentrations of CNF1 or CNF1-C866S. After 4 h, cells were lysed and submitted to immunoblot against RhoB and beta-actin. Western blots from representative experiments are shown (n = 3).

4.3 CNF1 causes up-regulation of RhoB via Rac1 and c-Myc

CNF1 and CNF_Y-induced deamidation of Rho proteins results in their activation. Thus, we assessed the state of activity of RhoA, RhoB, Rac1, and Cdc42 in fibroblasts treated with increasing concentrations of CNF1 or CNF_Y for 4 h. Effector pulldown assays using the GTPase binding domain of Rhotekin or the CRIB domain of PAK to precipitate active RhoA/RhoB or Rac1/Cdc42, respectively, were performed (Fig. 3A). CNF1 induced RhoB up-regulation as well as RhoB activation (Fig. 3A). In contrast, CNF_Y hardly increased the RhoB protein level. The activity of RhoB, however, increased in CNF_Y-treated fibroblasts (Fig. 3A). This finding suggests that RhoB was deamidated by CNF_Y. RhoA was activated by both CNF1 and CNF_Y in fibroblasts, with CNF_Y being more efficient (Fig. 3A). The total level of RhoA was not affected by the toxins. Furthermore, CNF1 but not CNF_Y activated Rac1 and Cdc42, indicating their deamidation. The total levels of Rac1 and Cdc42 slightly decreased in a toxin concentration-dependent manner (Fig. 3A). Thus, the activity of Rac1 correlated with the up-regulation of RhoB in CNF1-treated fibroblasts (Fig. 3A).

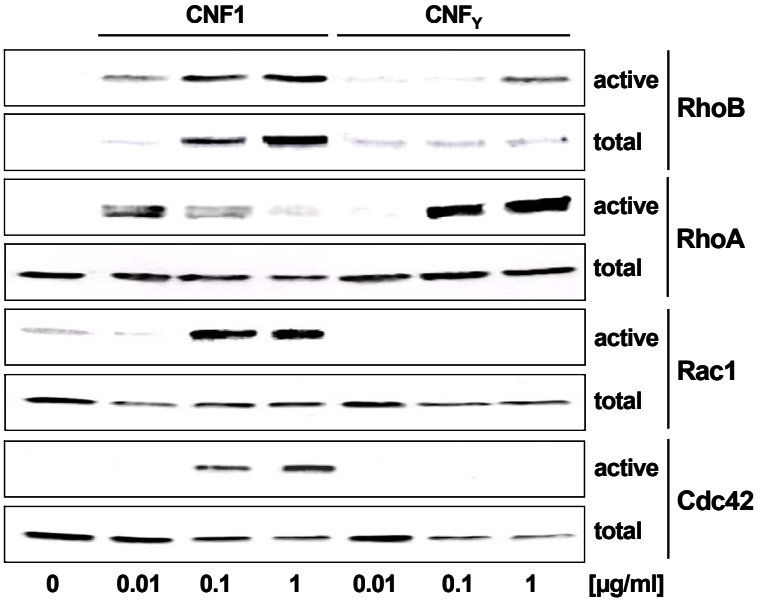

Fig. 3A: Activation of Rho proteins by CNF1 and CNF_Y

NIH3T3 fibroblasts were treated with increasing concentrations of CNF1 for 4 h. Cell lysates were submitted to either Rhotekin (RhoA, RhoB) or PAK (Rac1, Cdc42) pulldown assay to assess the activity. Total and precipitated Rho proteins were analysed by Western blot analysis with the indicated antibodies. Western blots from representative experiments are shown (n = 3).

We investigated next, whether active Rac1 was indeed the trigger of the CNF1-induced up-regulation of RhoB. To this end, we applied NSC23766, an inhibitor of Rac1. NSC23766 inhibits Rac1 by blocking its binding to the GEF proteins Tiam and Trio (Gao et al., 2004). In cells treated with NSC23766, the CNF1-induced activation of Rac1 was clearly reduced (Fig. 3B). The reduced Rac1 activation was reflected by reduced RhoB up-regulation (Fig. 3B). Correspondingly, NSC23766 reduced the CNF1-induced activation of the *rhoB* promoter (Fig. 3C), confirming that RhoB up-regulation was in fact based on Rac1 activation.

NSC23766 inhibits the interaction of Rac1 with GEF proteins, which in turn are activated by growth factors. The CNF1-induced Rac1 activation and RhoB up-regulation may thus depend on the presence of growth factors in the serum. In serum-starved fibroblasts, both Rac1 activation and RhoB up-regulation were reduced compared to serum-cultured fibroblasts (Fig. 3B). Accordingly, the CNF1-induced increase of the *rhoB* mRNA was reduced in the absence of serum (Fig. 3D). CNF1-induced Rac1 activation and subsequent up-regulation of RhoB was thus enhanced in the presence of serum. These results suggested that the presence of growth factors facilitated Rac1 activation and subsequent RhoB up-regulation.

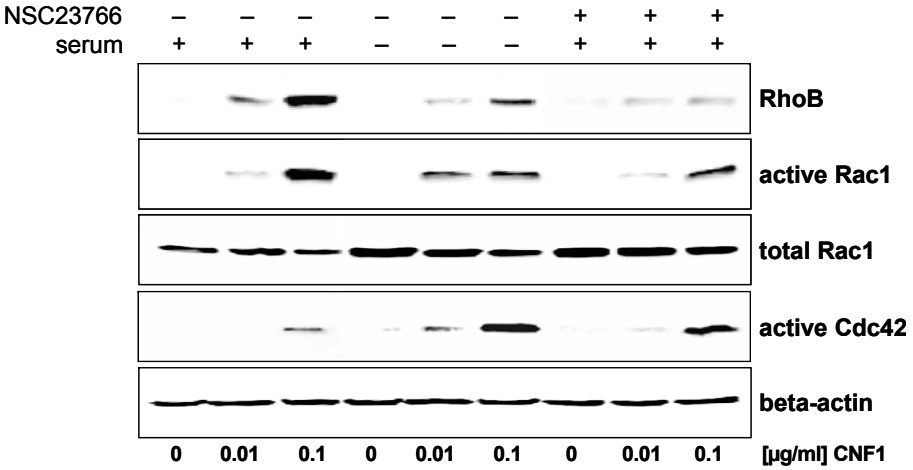

Fig. 3B: CNF1-induced RhoB up-regulation is Rac1-dependent and reduced in serum-starved fibroblasts

Fibroblasts were either serum-starved for 24 h, or pre-treated with NSC23766 (60 µM) for 1 h, or left untreated. Cells were exposed to increasing concentrations of CNF1 for 4 h. Cell lysates were submitted to PAK pulldown assay to assess the activity of Rac1 and Cdc42. Total and precipitated Rho proteins were analysed by Western blot analysis with the indicated antibodies. Western blots from representative experiments are shown (n = 3).

CNF1-induced activation of Cdc42 was enhanced in the presence of NSC23766 and also in serum-starved compared to serum-cultured fibroblasts (Fig. 3B). This finding showed that NSC23766 blocked Rac1 but not Cdc42 activation.

Rac1 positively regulates the transcription factor c-Myc (Boureux et al., 2005). c-Myc is up-regulated in response to PDGF in a Rac1-dependent manner in fibroblasts. Hence, CNF1 induces the expression of c-Myc (Chiariello et al., 2001). We hypothesised that c-Myc played a role in the CNF1-mediated RhoB up-regulation. Therefore, we treated fibroblasts with the c-Myc inhibitor (Z,E)-5-(4-Ethylbenzylidine)-2-thioxothiazolidin-4-one (Fig. 2F+H). This inhibitor specifically blocks the c-Myc-Max interaction, thereby preventing the transactivation of c-Myc target gene expression (Yin et al., 2003). The c-Myc inhibitor reduced both the CNF1-induced activation of the *rhoB* promoter and RhoB up-regulation (Fig. 3C+E). These findings showed that RhoB was up-regulated in a c-Myc-dependent manner.

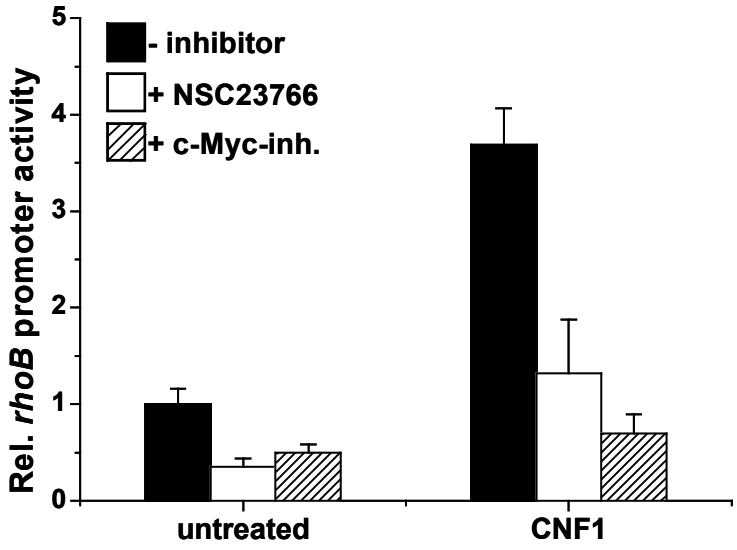

Fig. 3C: CNF1-induced activation of the *rhoB* promoter is responsive to inhibition of Rac1 and c-Myc

Fibroblasts were transfected with 2 µg of the 3.5 kb *rhoB* promoter CAT construct for 14 h. Cells were treated with either NSC23766 (60 µM, empty bars) or (Z,E)-5-(4-Ethylbenzylidine)-2-thioxothiazolidin-4-one (c-Myc inhibitor, 100 µM, striped bars) or left untreated (filled bars) for 1 h. CNF1 (0.1 µg/ml) was then added and incubation continued for 7 h. Cells were harvested and the CAT protein level was quantified. The CAT level of untreated cells was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

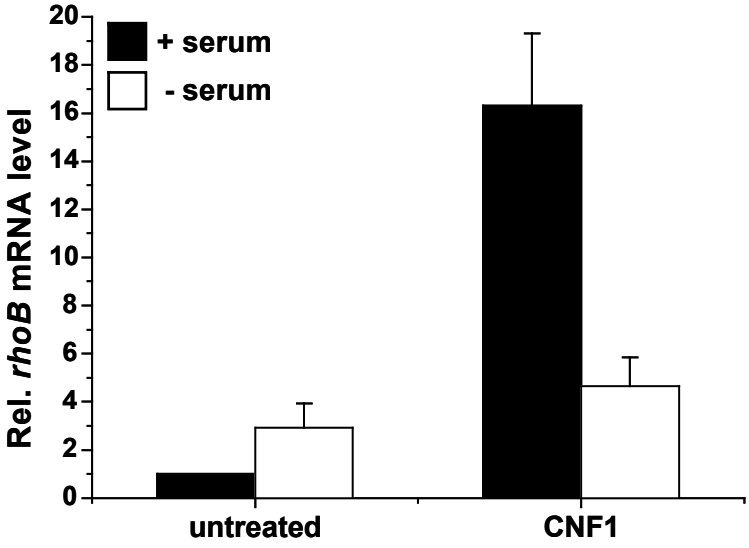

Fig. 3D: CNF1-induced up-regulation of *rhoB* mRNA is reduced in serum-starved fibroblasts
Fibroblasts were either serum-starved (empty bars) or not (filled bars) for 24 h. Cells were then exposed to CNF1 (1 µg/ml) for 4 h. Total mRNA was prepared and submitted to real-time RT-PCR. *rhoB* signals were normalised to beta-actin signals. The control level was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

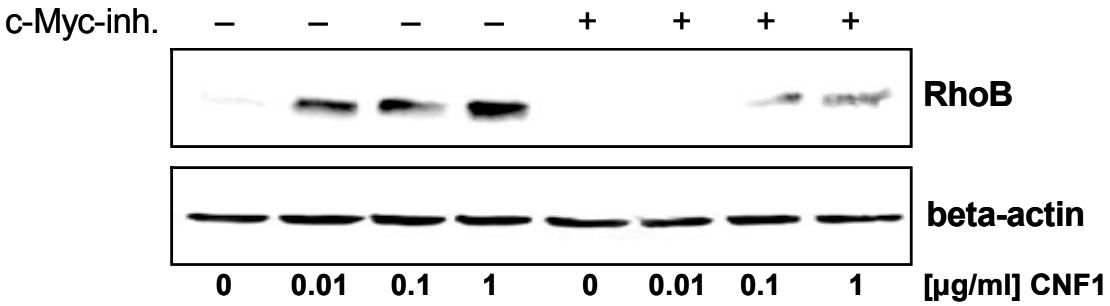

Fig. 3E: CNF1-induced RhoB up-regulation is c-Myc-dependent
Fibroblasts, either 1 h pre-treated or not with (Z,E)-5-(4-Ethylbenzylidine)-2-thioxothiazolidin-4-one (c-Myc inhibitor, 100 µM), were exposed to CNF1 as indicated. After 4 h, cells were lysed and submitted to immunoblot against RhoB and beta-actin. Western blots from representative experiments are shown (n = 3).

4.4. The p38 MAP kinase negatively regulates the CNF1-induced RhoB up-regulation

The p38 mitogen-activated protein (MAP) kinase is activated in CNF1-treated cells (Brest et al., 2004). We checked, whether p38 played a role in the CNF1-induced RhoB up-regulation. RhoB up-regulation was analysed in NIH3T3 fibroblasts pre-treated with the pharmacological inhibitor of p38 MAP kinase, SB 203580 (Fig. 4A). SB 203580 enhanced the CNF1-induced RhoB up-regulation (Fig. 4A). In this line, SB 203580 also enhanced the CNF1-induced up-regulation of the *rhoB* mRNA level and the activation of the *rhoB* promoter (Fig. 4B+C). These findings suggested a suppressive role of p38 in the transcriptional activation of *rhoB* in CNF1-treated cells.

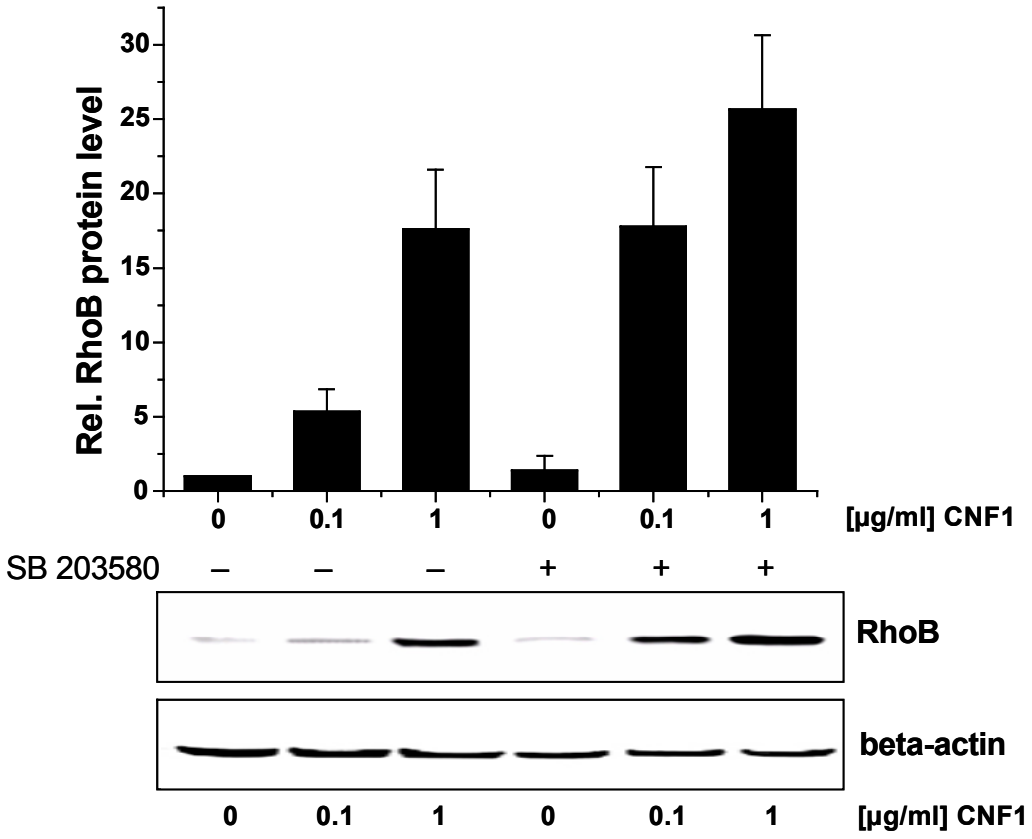

Fig. 4A: CNF1-induced RhoB up-regulation is enhanced by SB 203580

Fibroblasts were pre-treated with SB 203580 (10 µM) or left untreated for 1 h. CNF1 was then added as indicated and incubation continued for 4 h. Cells were lysed and submitted to immunoblot against RhoB and beta-actin. Signals were analysed densitometrically. RhoB signals were normalised to beta-actin signals. The control level was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

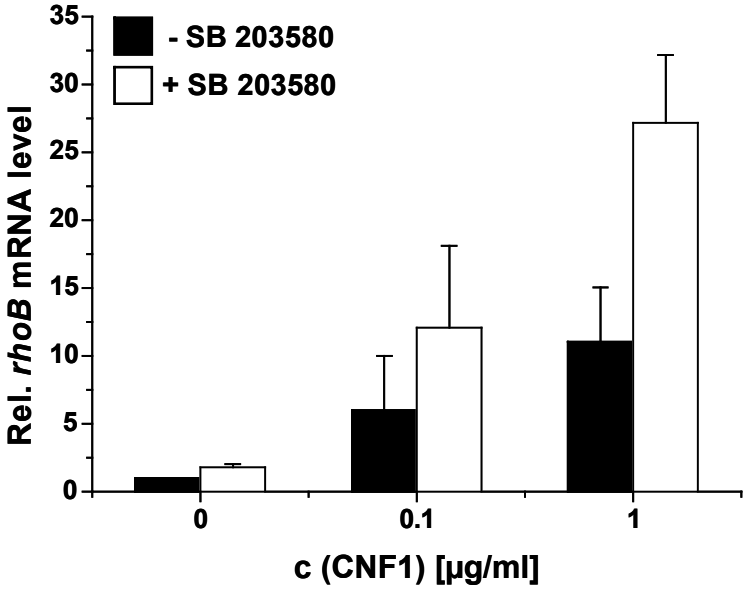

Fig. 4B: CNF1-induced up-regulation of *rhoB* mRNA is enhanced by SB 203580

Fibroblasts were pre-treated with SB 203580 (10 µM) or left untreated for 1 h. Cells were then exposed to increasing concentrations of CNF1 for 4 h. Total mRNA was prepared and submitted to real-time RT-PCR. *rhoB* signals were normalised to beta-actin signals. The control level was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

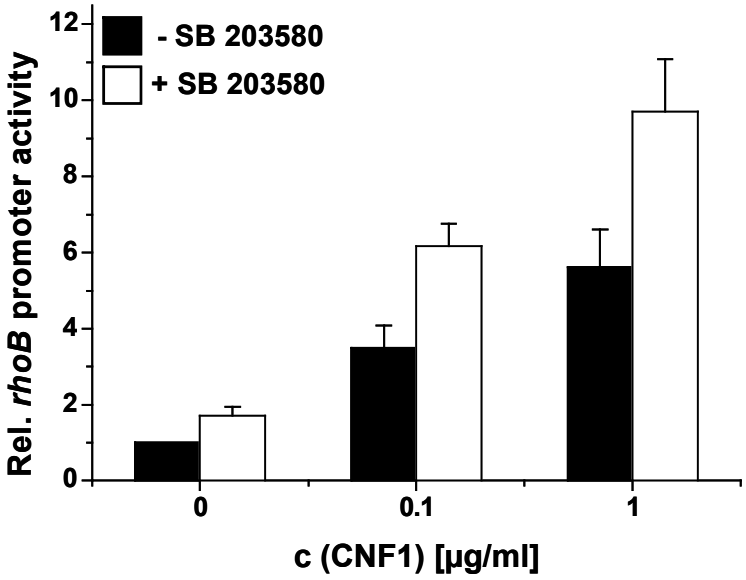

Fig. 4C: CNF1-induced activation of the *rhoB* promoter is enhanced by SB 203580

Fibroblasts were transfected with 2 µg of the 3.5 kb *rhoB* promoter CAT construct for 14 h. Cells were pre-treated with SB 203580 (10 µM) or left untreated for 1 h. CNF1 was then added as indicated and incubation continued for 7 h. Cells were harvested and the CAT protein level was quantified. The CAT level of untreated cells was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

This finding was confirmed using a $p38_{\alpha}$ MAP kinase -/- mouse embryonic fibroblast (MEF) cell line. $p38_{\alpha}$ is the most abundant of the four isoforms of p38 MAP kinase. The applied MEFs do not express the other isoforms β, γ and, δ. Thus, the $p38_{\alpha}$ -/- cells represent a cell line devoid of p38 MAP kinase signalling. In $p38_{\alpha}$ -/- MEFs, the CNF1-induced RhoB up-regulation was enhanced compared to wild type cells (Fig. 5A). Accordingly, up-regulation of *rhoB* mRNA and activation of the *rhoB* promoter were enhanced in $p38_{\alpha}$ -/- MEFs compared to wildtype cells (Fig. 5B+C). These data are consistent with the above results obtained applying the inhibitor in NIH3T3 fibroblasts. Wild type MEFs pre-treated with SB 203580 also exhibited enhanced RhoB up-regulation, comparable to that observed in $p38_{\alpha}$ -/- MEFs (Fig. 5D). The inhibitor was without effect in CNF1-treated $p38_{\alpha}$ -/- fibroblasts, confirming its specificity (Fig. 5D). In conclusion, the p38 MAP kinase pathway suppressed RhoB up-regulation in CNF1-treated fibroblasts.

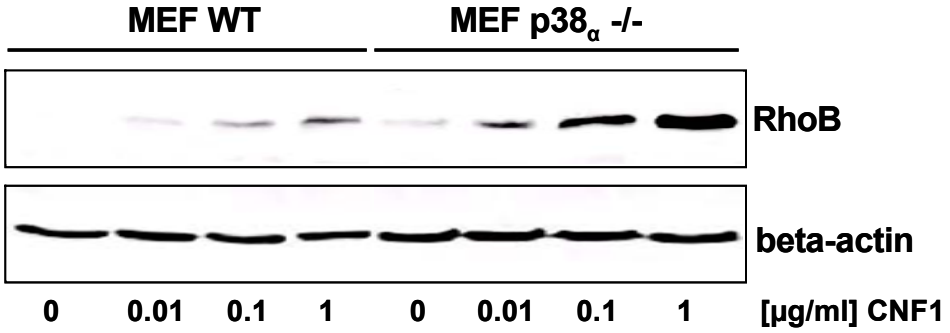

Fig. 5A: CNF1-induced up-regulation of RhoB protein is enhanced in $p38_{\alpha}$ -/- ME fibroblasts
WT or $p38_{\alpha}$ -/- ME fibroblasts were exposed to the indicated concentrations of CNF1 for 4 h. Cells were lysed and submitted to immunoblot against RhoB and beta-actin. Western blots from representative experiments are shown (n = 3).

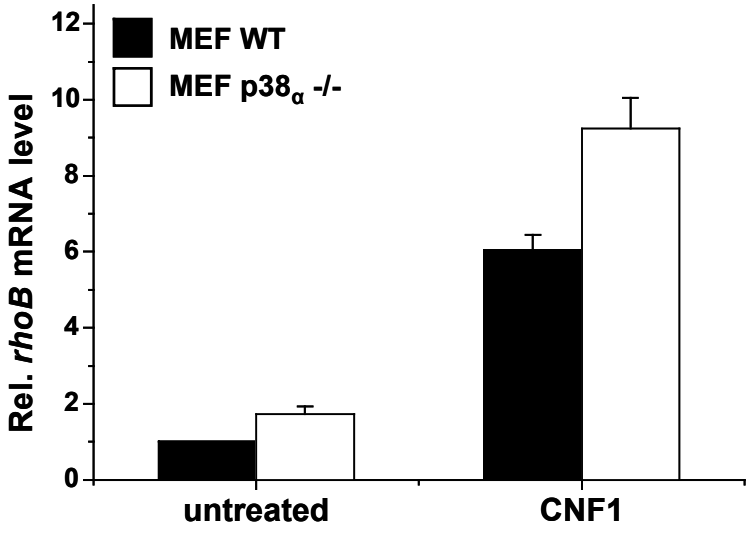

Fig. 5B: CNF1-induced up-regulation of *rhoB* mRNA is enhanced in p38$_\alpha$ -/- ME fibroblasts
WT (filled bars) or p38$_\alpha$ -/- (empty bars) ME fibroblasts were exposed to CNF1 (0.1 µg/ml) or left untreated for 4 h. Total mRNA was prepared and submitted to real-time RT-PCR. *rhoB* signals were normalised to beta-actin signals. The control level was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

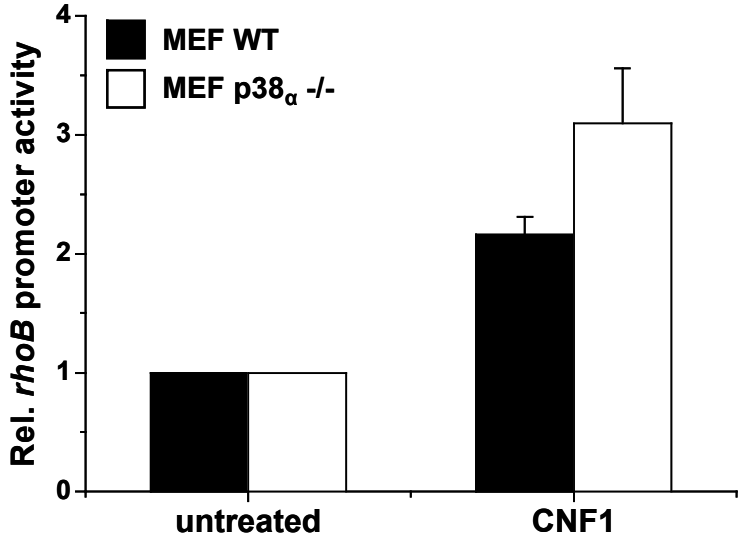

Fig. 5C: CNF1-induced activation of the *rhoB* promoter is enhanced in p38$_\alpha$ -/- ME fibroblasts
WT (filled bars) or p38$_\alpha$ -/- (empty bars) ME fibroblasts were transfected with 2 µg of the 3.5 kb *rhoB* promoter CAT construct for 14 h. CNF1 (0.1 µg/ml) was then added and incubation continued for 7 h. Cells were harvested and the CAT protein level was quantified. The CAT level of untreated cells was set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

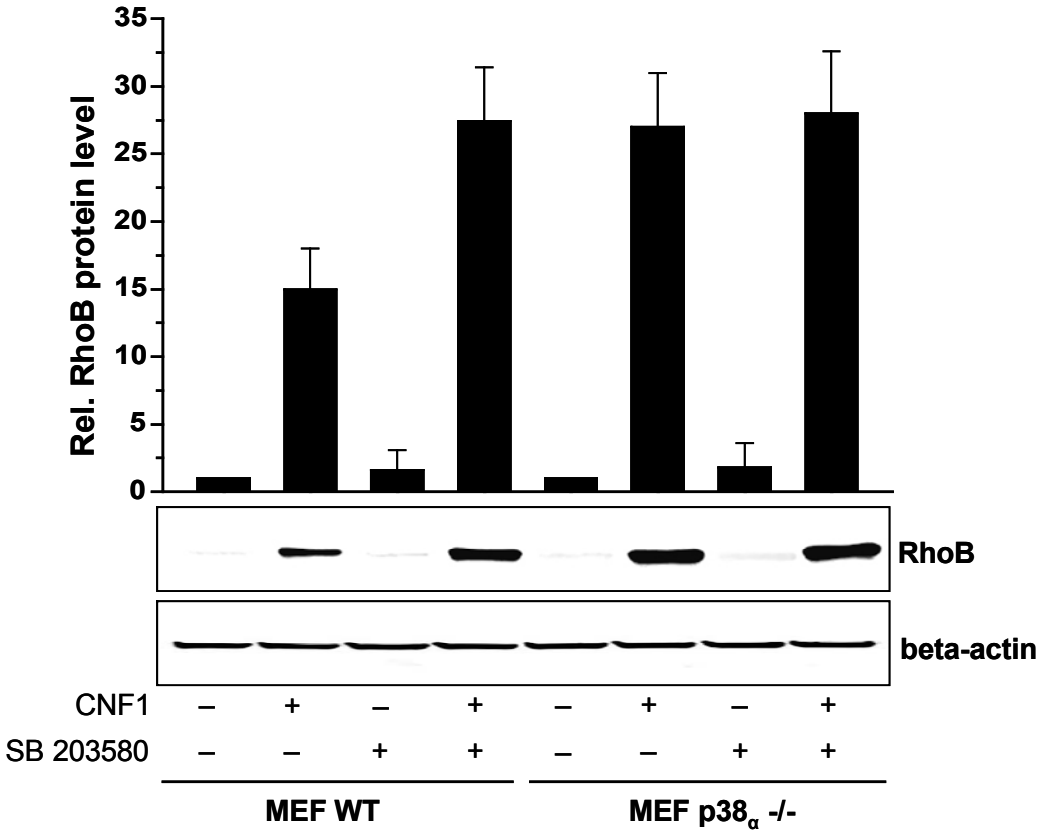

Fig. 5D: SB 203580 does not affect CNF1-induced RhoB up-regulation in p38$_\alpha$ -/- fibroblasts
WT or p38$_\alpha$ -/- ME fibroblasts were pre-treated with SB 203580 (10 µM) or left untreated for 1 h. CNF1 was then added as indicated and incubation continued for 4 h. Cells were lysed and submitted to immunoblot against RhoB and beta-actin. Signals were analysed densitometrically. RhoB signals were normalised to beta-actin signals. The control levels were set to 1.0. Results displayed are the mean + S.D. of three independent experiments.

4.5 RhoB is proteasomally degraded in CNF1-treated fibroblasts

RhoB is rapidly up-regulated upon stress stimulation and rapidly degraded, exhibiting a half-life period of 2 h (Lebowitz et al., 1995). Rho proteins are degraded in a proteasome-dependent manner in CNF1-treated cells (Doye et. al, 2002). We hypothesised that RhoB is degraded in CNF1-treated cells in a proteasome-dependent manner as well. To this end, cells were treated with CNF1 for 4 h (Fig. 6). Cycloheximide (CHX) was then applied and the RhoB level monitored for further 3 h. After addition of CHX, up-regulated RhoB was almost completely degraded within 1 h (Fig. 6). The half-life period was < 1 h. To check, whether RhoB was proteasomally degraded, fibroblasts were incubated with the proteasome inhibitor MG132 prior to CNF1 exposure (Fig. 6). RhoB was not degraded after addition of CHX in the presence of MG132, indicating that RhoB degradation was predominantly proteasome-dependent.

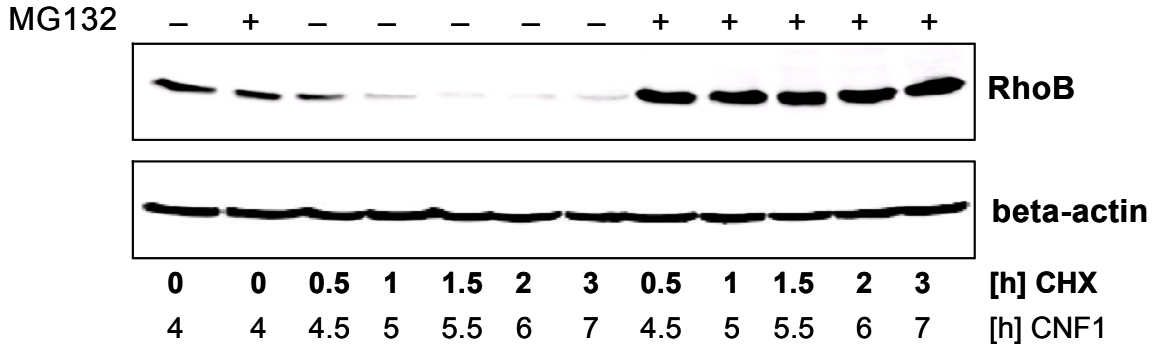

Fig. 6: RhoB is degraded in a proteasome-dependent manner in fibroblasts exposed to CNF1
Fibroblasts were pre-treated with MG132 (20 µM) or left untreated for 1 h. Cells were then exposed to CNF1 (1 µg/ml) for 4 h. Subsequently, cycloheximide (1 mM) was added and incubation continued for the periods indicated. Cells were lysed and submitted to immunoblot against RhoB and beta-actin. Western blots from representative experiments are shown (n = 3).

4.6 RhoB up-regulation is persistent in CNF1-treated fibroblasts

Up-regulation of immediate early gene products such as c-fos and c-jun is transient (Chen et al., 2001). To check, whether RhoB up-regulation was transient, the cellular protein level of RhoB was analysed after long-term treatment with CNF1 (Fig. 7). Western-Blot analysis revealed that the RhoB level was elevated for at least 48 h, independent of the applied CNF1 concentration (Fig. 7). In contrast, the cellular levels of Rac1 and RhoA in CNF1-treated cells were comparable to the levels in untreated fibroblasts after 24 h and 48 h (Fig. 7). In conclusion, although exhibiting a short half-life period (Fig. 6), the up-regulation of RhoB induced by CNF1 was persistent. Apparently, RhoB up-regulation was permanently triggered in CNF1-treated cells.

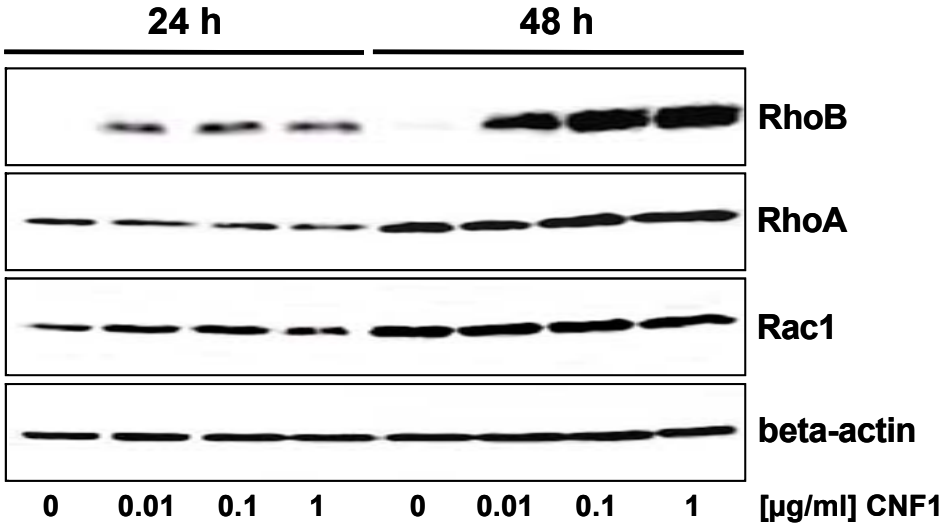

Fig. 7: RhoB up-regulation is persistent in CNF1-treated fibroblasts

Fibroblasts were treated with increasing concentrations of CNF1 for either 24 h or 48 h. Cells were lysed and submitted to immunoblot against RhoB, RhoA, Rac1, and beta-actin. Western blots from representative experiments are shown (n = 3).

4.7 CNF1 induces multinucleation and polyploidy in NIH3T3 fibroblasts

The most striking feature of CNF1-treatment of cultured cells is the formation of multinuclei (Oswald et al., 1989). We analysed CNF1-induced multinucleation in fibroblasts by fluorescence microscopy (Fig. 8A+B). The majority (70 %) of fibroblasts was multinucleated after toxin-treatment for 24 h (Fig. 8B).

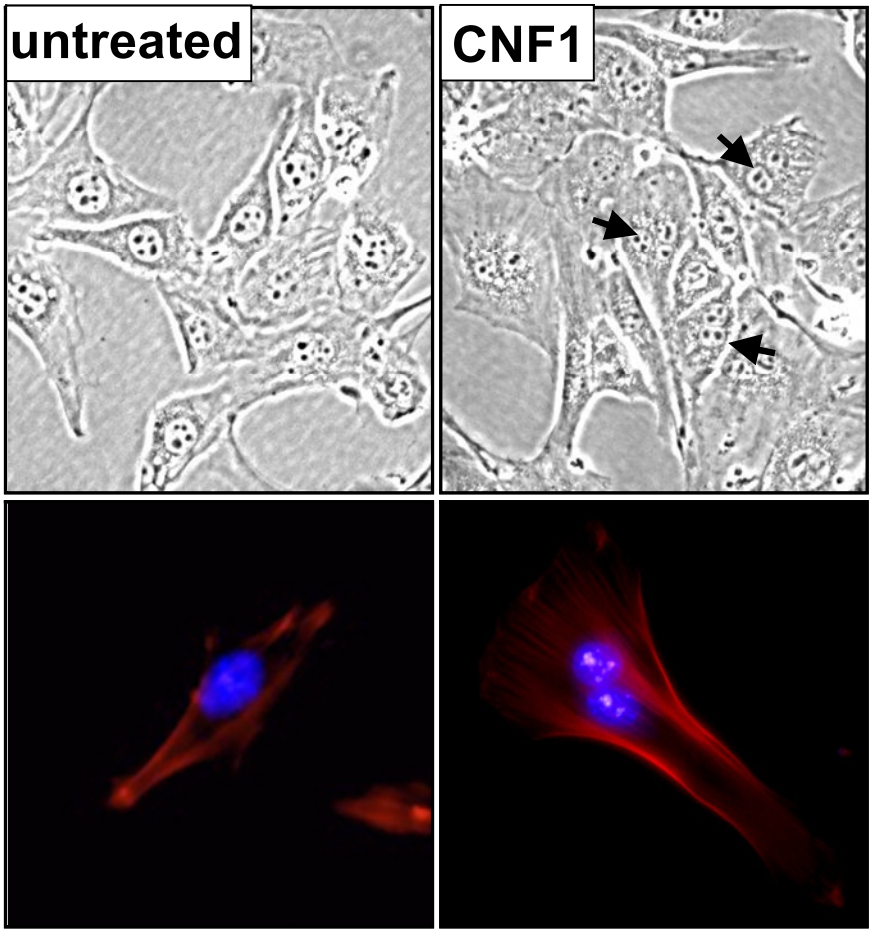

Fig. 8A: CNF1 induces multinucleation and stress fiber formation

Fibroblasts were exposed to CNF1 (1 µg/ml) or left untreated for 24 h. Cells were fixed and stained with rhodamine-phalloidin (30 µg/ml) and DAPI (0.1 µg/ml) to visualise F-actin and nuclei, respectively. Arrows indicate multinucleated cells (upper panels: phase contrast; lower panels; fluorescence images).

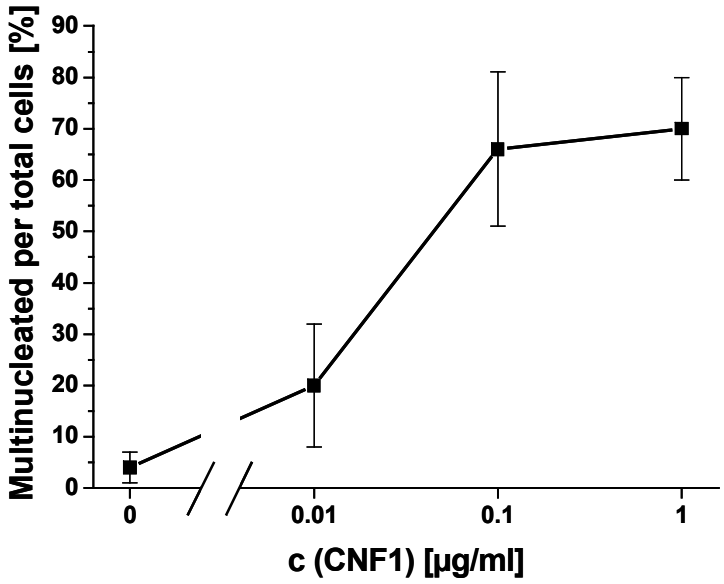

Fig. 8B: CNF1 induces multinucleation in a concentration-dependent manner

Fibroblasts were exposed to increasing concentrations of CNF1 for 24 h. The cells were fixed and stained with DAPI. Nuclei were visualised by fluorescence microscopy and multinucleation determined by counting. 200 cells were analysed for each experimental condition. Results displayed are the mean ± S.D. of three independent experiments.

Multinucleation implies an increased DNA content and is thus reflected by polyploidy. Polyploidy was analysed using flow cytometry applying the nuclear dye propidium iodide (PI) (Fig. 9A+B). CNF1 caused polyploidy in a time-dependent manner in fibroblasts (Fig. 9B). CNF1-treatment decreased the 2N population in a time-dependent manner (Fig. 9B). The 4N population transiently increased and subsequently diminished in response to CNF1. The percentage of cells with 8N DNA content increased in a time-dependent manner from 0 % to 50 %, reflecting CNF1-induced polyploidy. Note that further cells with a DNA content of 4N and > 4N – 8N were possibly multinucleated, explaining the lower number of polyploid cells (50 %) compared to multinucleated cells (70 %) (Fig. 8B+9B). Polyploidy depended on the deamidase activity, as deamidase-deficient CNF1-C866S failed to induce it (Fig. 9A). In untreated fibroblasts, the 2N population increased from 35 % to 70 % in a time-dependent manner, while the 4N population decreased from 30 % to 15 % (Fig. 9B). An 8N population was not observed. The redistribution between 2N and 4N after 72 h was likely due to density inhibition (Fig. 9B).

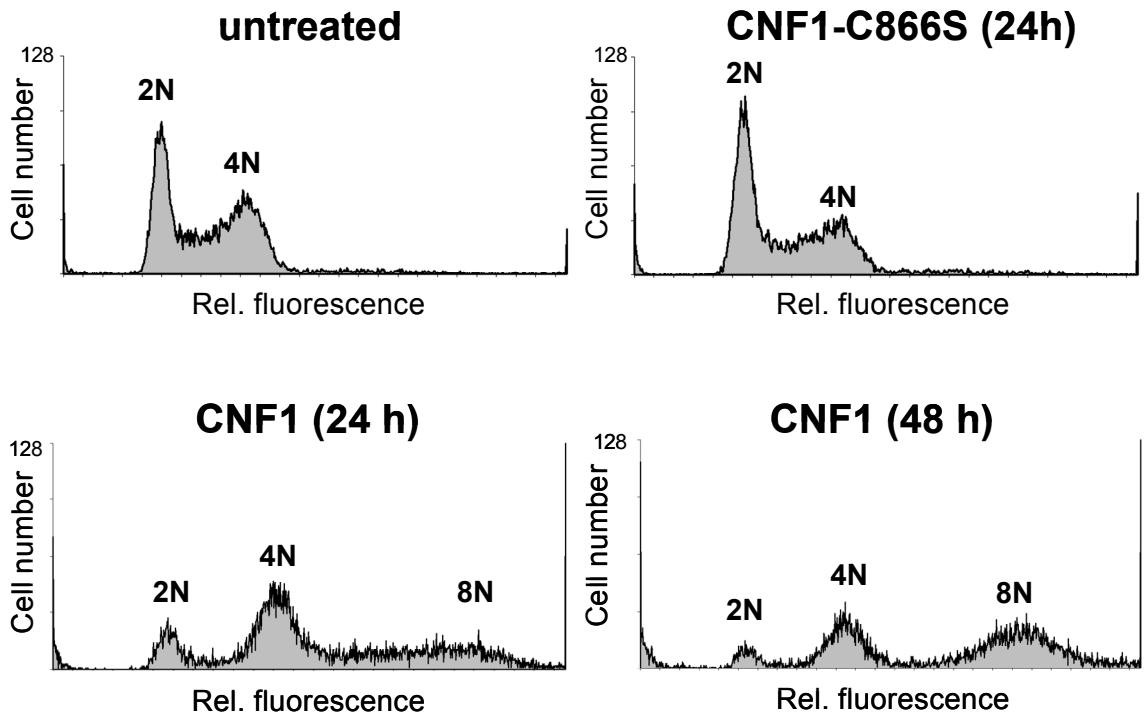

Fig. 9A: CNF1-induced polyploidy depends on its deamidase activity

Fibroblasts were exposed to either CNF1 (0.1 µg/ml) or CNF1-C866S (1 µg/ml) or left untreated for the indicated periods. After staining with propidium iodide (50 µg/ml), the DNA content of the cells was analysed by flow cytometry. 10^4 cells were analysed for each experimental condition. Histograms from representative experiments are shown (n = 3).

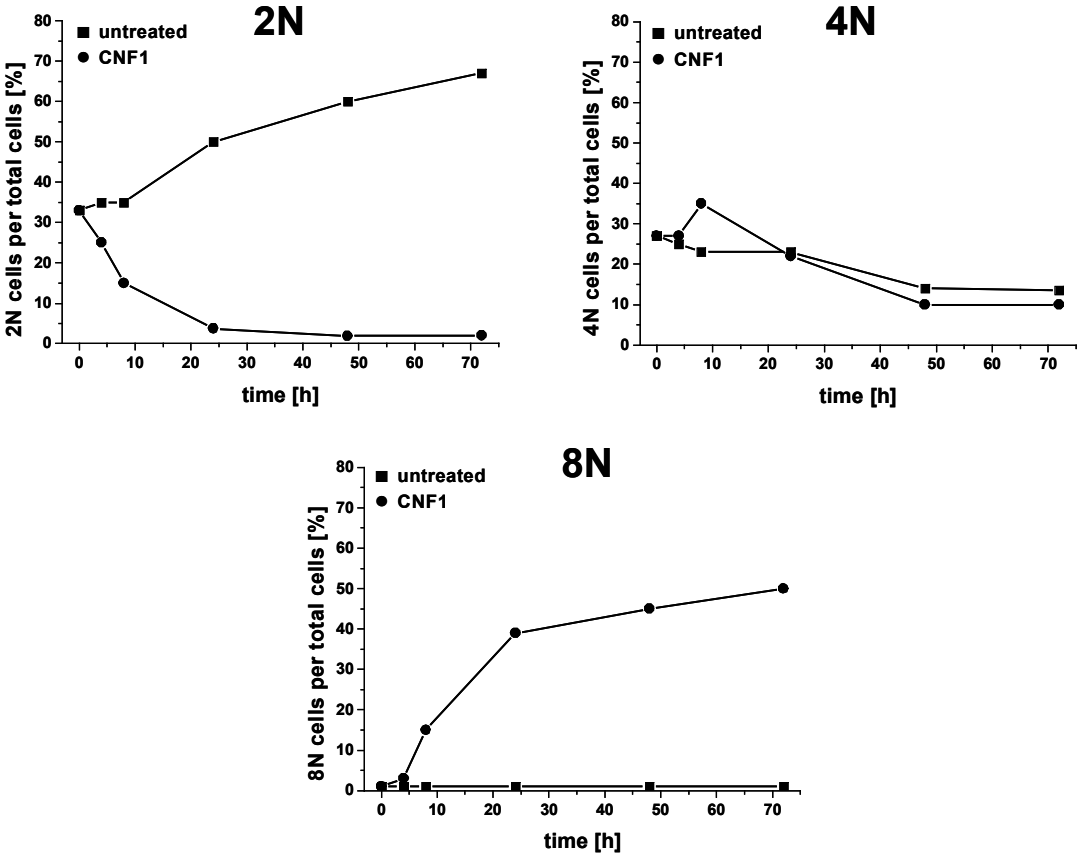

Fig. 9B: CNF1 induces polyploidy in a time-dependent manner

Fibroblasts were either exposed to CNF1 (0.1 µg/ml) (●) or left untreated (■) for the indicated periods. The cells were then stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Results displayed are the mean of three independent experiments.

To confirm that CNF1 blocks cell division without affecting nuclear division, we quantified the number of cells in untreated or in CNF1-treated samples in a time-dependent manner. The cell number of CNF1-treated fibroblasts remained almost unchanged, indicating that cell division was blocked (Fig. 10). In contrast, the number of untreated (proliferating) cells increased up to 48 h, until cell division was blocked by density inhibition (Fig. 10). Interestingly, the viability (determined by WST-1 assay) and the protein content (determined by Bradford test) comparably increased in CNF1-treated and untreated (proliferating) cells (Fig. 10). Thus, CNF1 caused an apparently increased viability and protein content of cells, while it inhibited cell division. In proliferating cells, the increased viability and protein content were based on an increased number of cells.

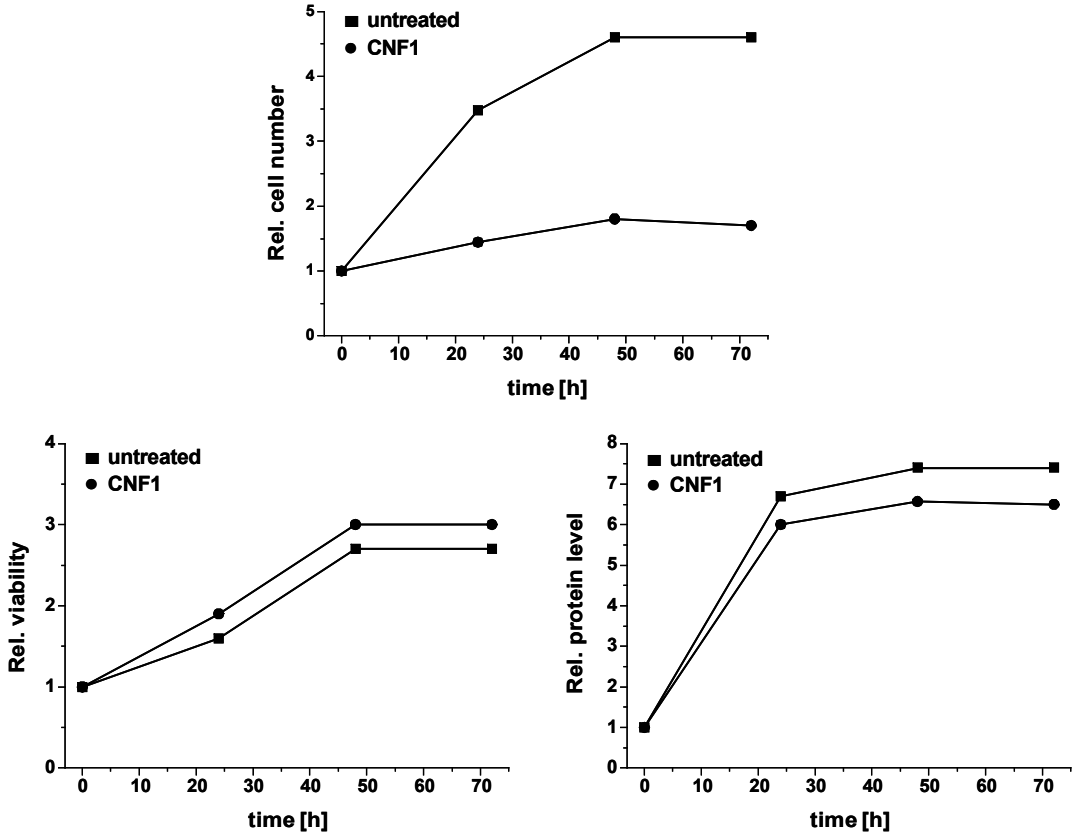

Fig. 10: CNF1 blocks cell division but increases viability and protein content of fibroblasts

Fibroblasts were exposed to CNF1 (0.1 µg/ml) (●) or left untreated (■) for the indicated periods. The cell number (upper panel) was determined using the Neubauer counting chamber. The cell number of untreated cells (0 h) was set to 1.0. Cell viability (left panel) was assessed by the WST-1 assay. The activity of untreated cells (0 h) was set to 1.0. The total protein content (right panel) was assessed by the Bradford test. The protein content of untreated cells (0 h) was set to 1.0. Results displayed are the mean of three independent experiments.

4.8 CNF1-induced polyploidy is p53-independent

The tumor suppressor p53 has been shown to induce cell cycle arrest and apoptosis in tetraploid cells ("tetraploidy checkpoint") (Andreassen et al., 2001). We hypothesised that p53 suppresses CNF1-induced polyploidy. Therefore, we utilised the human colon carcinoma cell lines HCT 116 wild type and HCT 116 p53 -/-. CNF1 caused an increase of the 8N and a decrease of the 2N and 4N populations in both wild type and p53 -/- cells in a time-dependent manner (Fig. 11). CNF1-induced polyploidy was comparable in p53 -/- and wild type cells (Fig. 11). Thus, CNF1-induced polyploidy was not suppressed by p53.

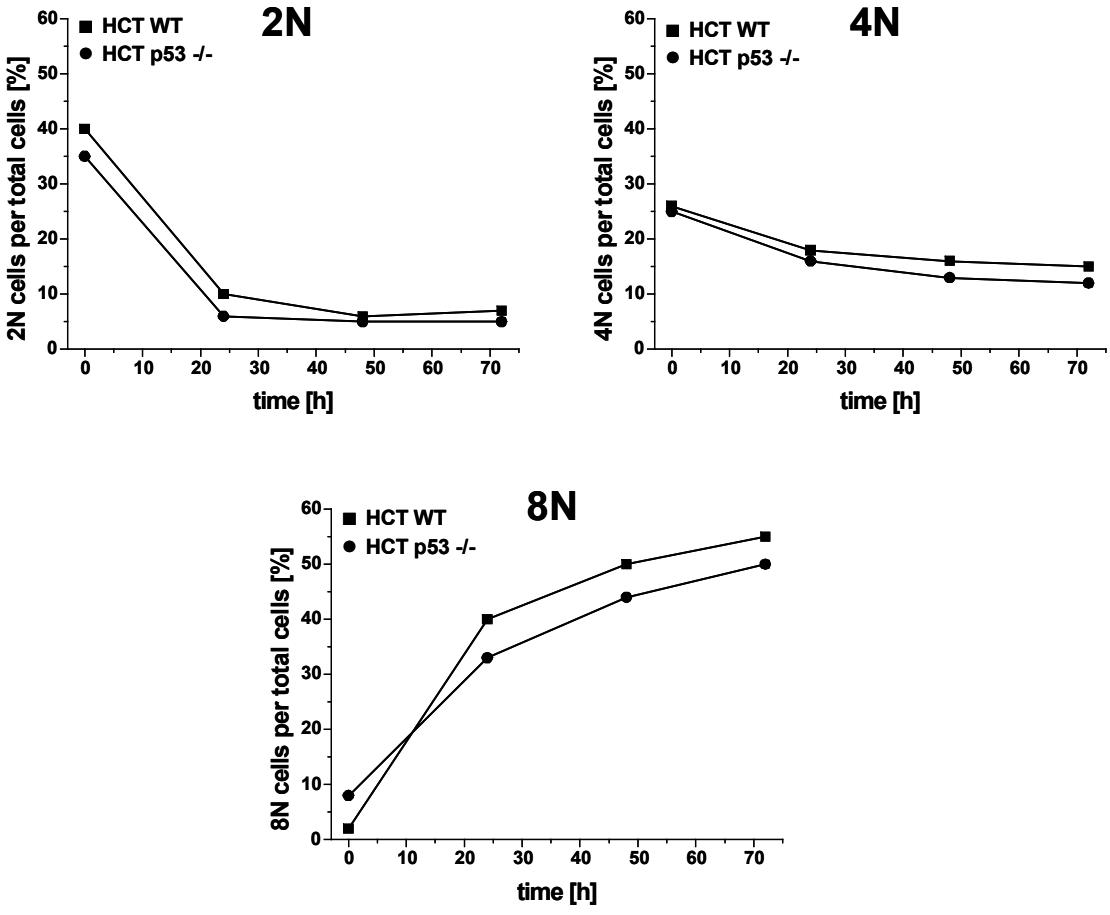

Fig. 11: CNF1-induced polyploidy is p53-independent

WT (■) and p53 -/- (●) HCTs were exposed to CNF1 (0.1 µg/ml) for the indicated periods. The cells were then stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Results displayed are the mean of three independent experiments.

4.9 p38 MAP kinase positively regulates the CNF1-induced polyploidy

p38 MAP kinase promotes cell cycle arrest in response to stress stimuli to prevent polyploidy (Mikule et al., 2007). We assumed that p38 MAP kinase suppresses CNF1-induced polyploidy. Surprisingly, inhibition of p38 by SB 203580 reduced the 8N population in CNF1-treated fibroblasts (Fig. 12A+B). To confirm this notion, polyploidy was analysed in CNF1-treated wild type and $p38_{\alpha}$ -/- fibroblasts (Fig. 12C). In line with our former observation, CNF1-induced polyploidy was less pronounced in $p38_{\alpha}$ -/- cells compared to wild type cells. Thus, p38 MAP kinase promoted rather than suppressed polyploidy in CNF1-treated fibroblasts.

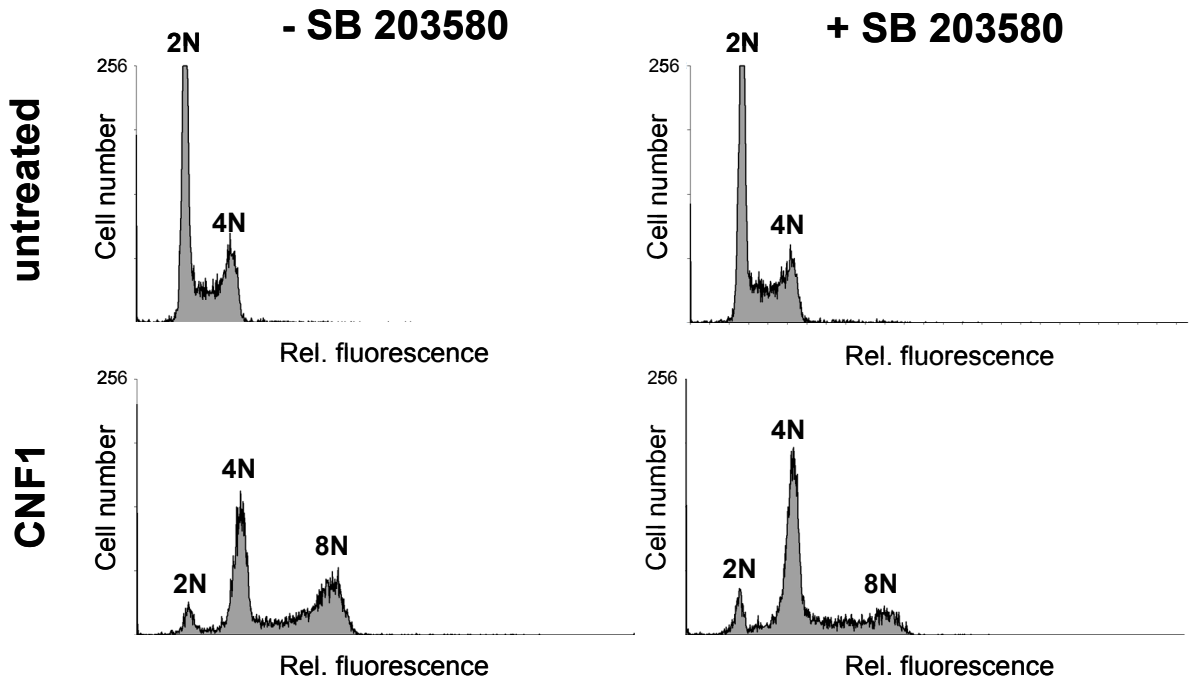

Fig. 12A: CNF1-induced polyploidy is reduced by SB 203580

Fibroblasts were pre-treated with SB 203580 (20 µM) or left untreated for 1 h. CNF1 (0.1 µg/ml) was then added and incubation continued for 24 h. The cells were subsequently stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Histograms from representative experiments are shown (n = 3).

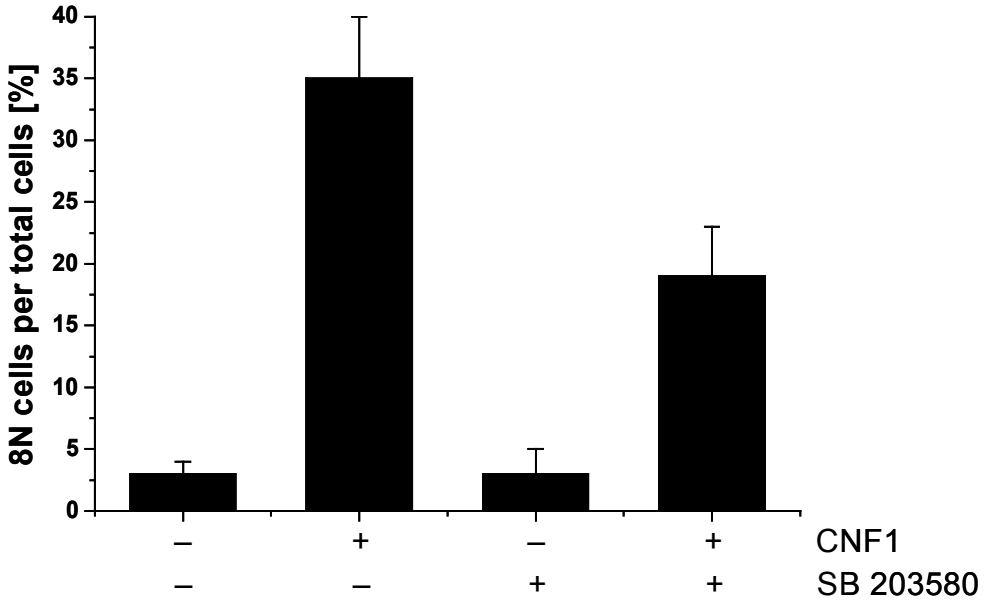

Fig. 12B: CNF1-induced polyploidy is reduced by SB 203580

NIH3T3 fibroblasts were pre-treated with SB 203580 (10 µM) or left untreated for 1 h. CNF1 (0.1 µg/ml) was then added and incubation continued for 24 h. The cells were subsequently stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Results displayed are the mean + S.D. of three independent experiments.

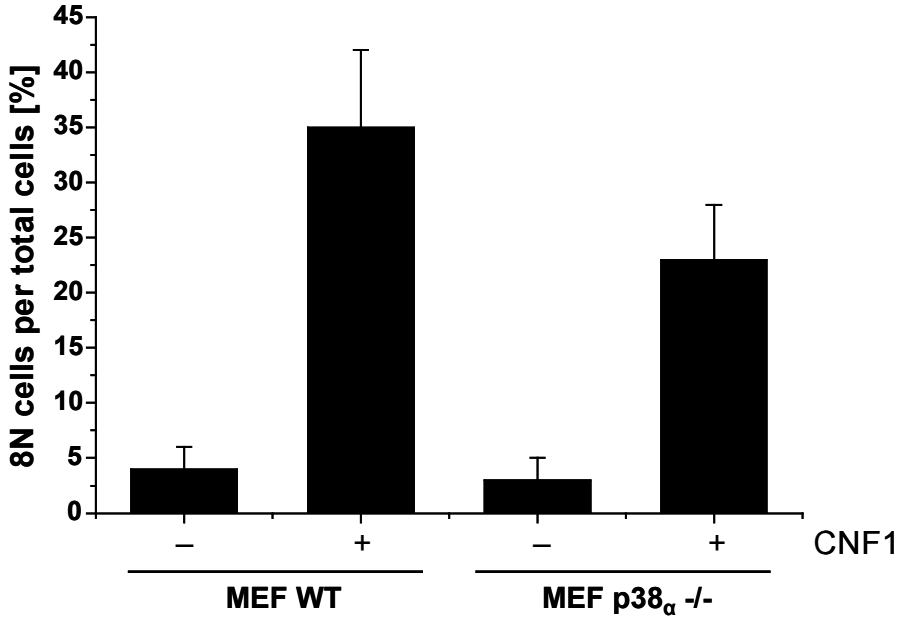

Fig. 12C: CNF1-induced polyploidy is reduced in $p38_\alpha$ -/- fibroblasts

WT or $p38_\alpha$ -/- fibroblasts were exposed to CNF1 (0.1 µg/ml) or left untreated for 24 h. The cells were then stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Results displayed are the mean + S.D. of three independent experiments.

4.10 RhoB suppresses CNF1-induced polyploidy

RhoB suppresses radiation-induced polyploidy in NIH3T3 fibroblasts (Milia et al., 2005). We hypothesised that RhoB suppresses CNF1-induced polyploidy as well. To this end, we exposed NIH3T3 fibroblasts, RhoB +/-, and RhoB -/- MEFs to CNF1 or CNF1-C866S for 24 h (Fig. 13A). CNF1 but not its inactive mutant CNF1-C866S caused a decrease of the 2N and an increase of the 8N population (exemplarily shown for RhoB -/- MEFs) (Fig. 13A). CNF1-induced polyploidy was further analysed in NIH3T3 fibroblasts, RhoB +/-, and RhoB -/- MEFs in a concentration-dependent manner after 24 h (Fig. 13B). CNF1 more efficiently increased the 8N population in RhoB -/- cells than in RhoB +/- or in NIH3T3 fibroblasts. This effect was reflected by a stronger decrease of the 2N and the 4N population in RhoB -/- cells compared to RhoB +/- and NIH3T3 fibroblasts (Fig. 13B). Thus, the efficacy of CNF1 to cause polyploidy was enhanced in the absence of RhoB, leading to the conclusion that RhoB suppresses polyploidy.

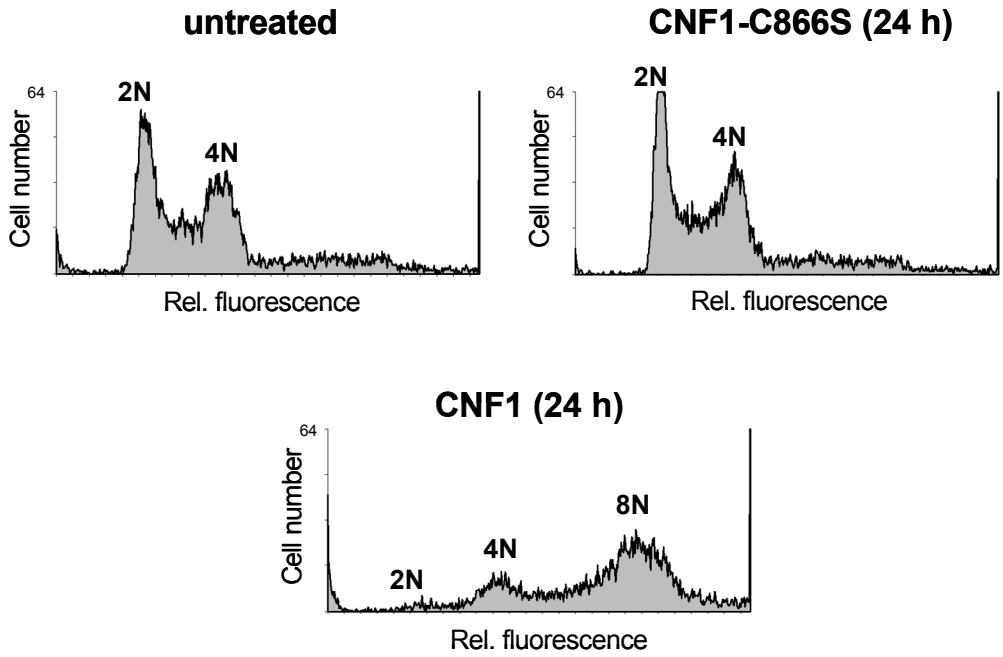

Fig. 13A: CNF1 induces polyploidy in RhoB -/- ME fibroblasts

RhoB -/- MEFs were exposed to either CNF1 (0.1 µg/ml) or CNF1-C866S (1 µg/ml) or left untreated for 24 h. The cells were then stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Histograms from representative experiments are shown (n = 3).

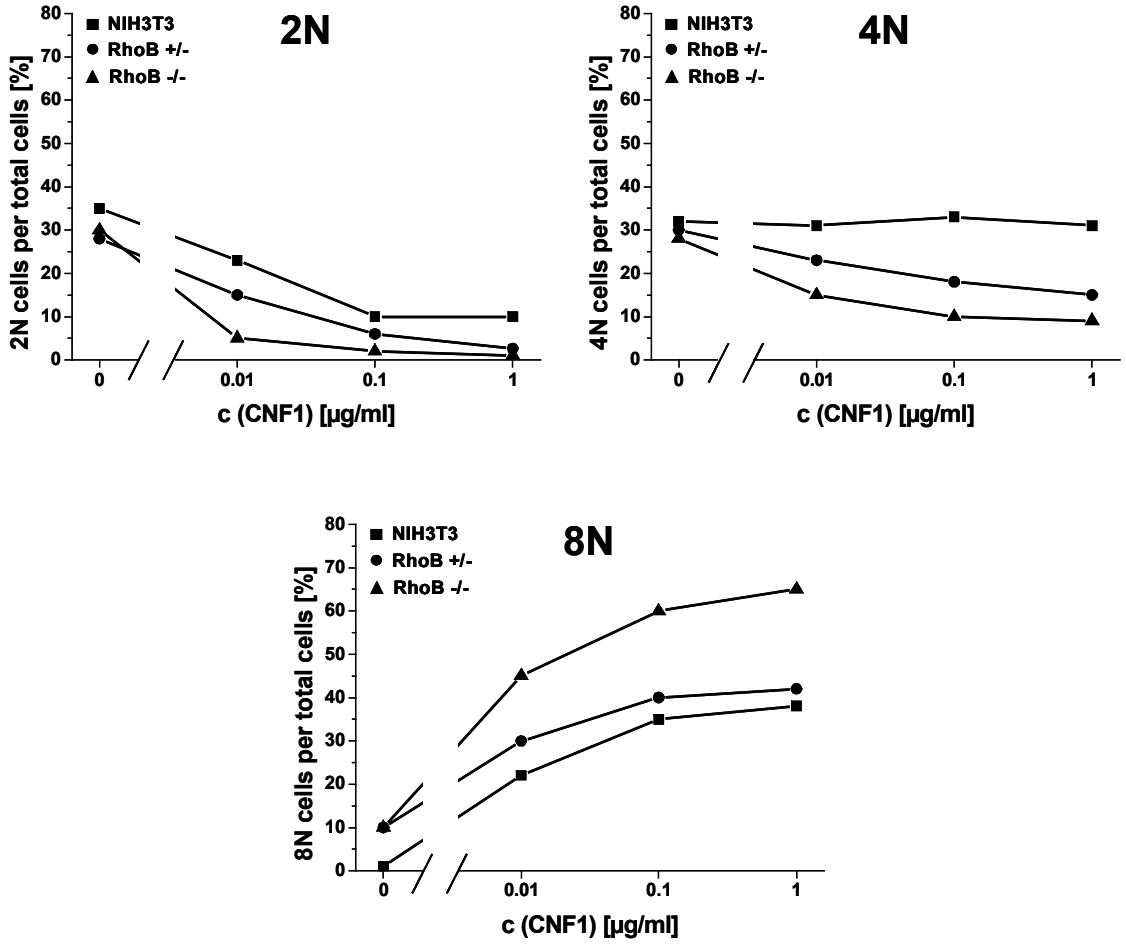

Fig. 13B: CNF1-induced polyploidy is enhanced in RhoB -/- ME fibroblasts

NIH3T3 (■), RhoB +/- (●), and RhoB -/- (▲) fibroblasts were exposed to increasing concentrations of CNF1 for 24 h. The cells were then stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Results displayed are the mean of three independent experiments.

We showed that p38 suppressed RhoB (Fig. 4+5). Therefore, we assumed that the p38-dependent enhancement of polyploidy was based on RhoB suppression.

Thus, polyploidy (8N DNA content) was determined in RhoB -/- cells either in the presence or in the absence of SB 203580 (Fig. 13C). SB 203580 reduced the CNF1-induced polyploidy in NIH3T3 fibroblasts but not in RhoB -/- cells (Fig. 13C). Thus, the enhanced CNF1-induced polyploidy in SB 203580-treated or $p38_{\alpha}$ -/- cells likely depended on RhoB suppression.

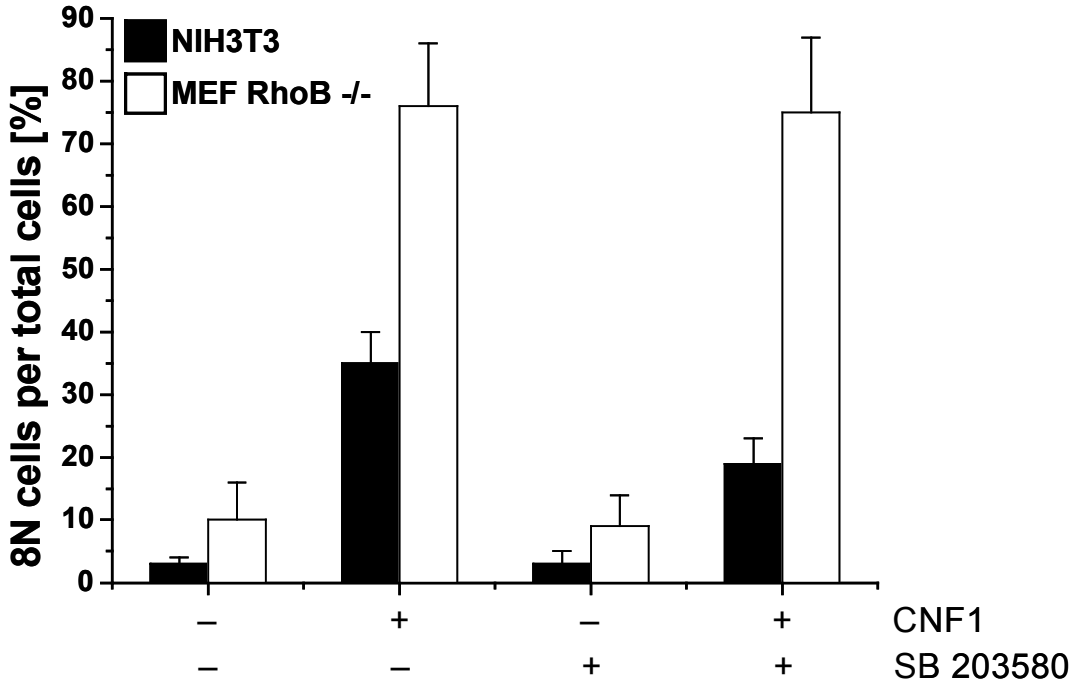

Fig. 13C: SB 203580 does not affect CNF1-induced polyploidy in RhoB -/- ME fibroblasts

NIH3T3 (filled bars) and RhoB -/- (empty bars) fibroblasts were pre-treated with SB 203580 (10 µM) or left untreated for 1 h. CNF1 (0.1 µg/ml) was then added and incubation continued for 24 h. The cells were subsequently stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Results displayed are the mean + S.D. of three independent experiments.

4.11 RhoB promotes survival of CNF1-treated cells

RhoB has been implicated to regulate cell survival as well as cell death pathways. Multinucleation and polyploidy are frequently associated with cell death. We investigated, whether CNF1 causes cell death in fibroblasts and whether RhoB is involved. Cell death was analysed as ratio of cells with subG1 (hypodiploid) DNA content per total cells. The DNA content of CNF1-treated NIH3T3, RhoB +/- and RhoB -/- cells was determined by flow cytometry. Long-term incubation (48 h) of fibroblasts with CNF1 caused an increased number of subG1 cells (< 2N) (Fig. 14A). RhoB -/- cells were more sensitive to CNF1-induced cell death than RhoB +/- cells and NIH3T3 fibroblasts (Fig. 14A). Apparently, the absence of RhoB sensitised cells to CNF1-treated cell death.

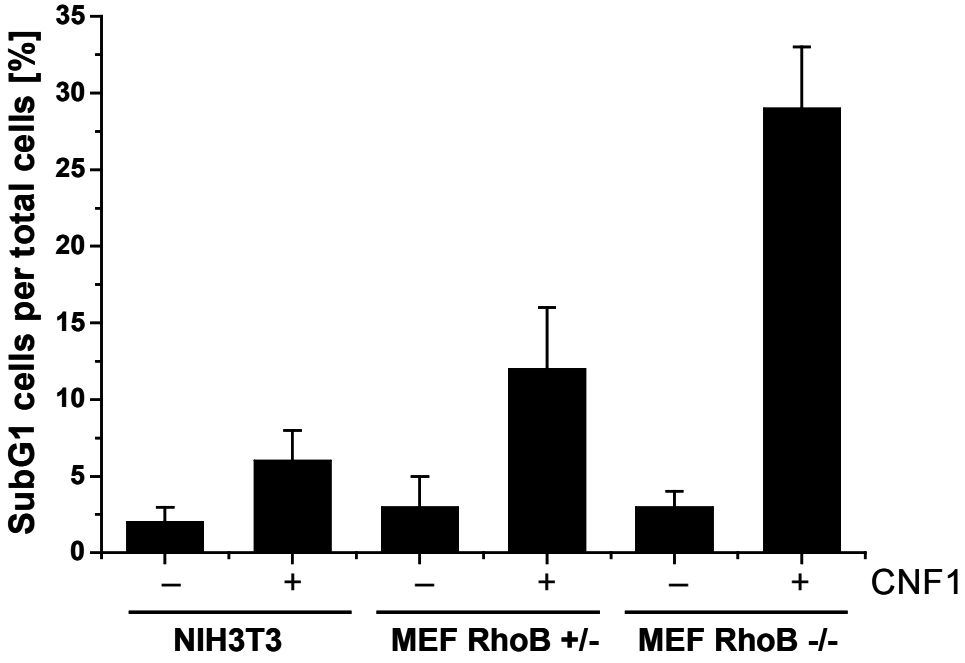

Fig. 14A: CNF1 increases the SubG1 population in RhoB -/- ME fibroblasts

NIH3T3, RhoB +/-, and RhoB -/- ME fibroblasts were exposed to CNF1 (0.1 µg/ml) or left untreated for 48 h. The cells were then stained with propidium iodide and analysed for DNA content by flow cytometry. 10^4 cells were analysed for each experimental condition. Results displayed are the mean + S.D. of three independent experiments.

To confirm this finding and further characterise CNF1-induced cell death, the number of Annexin V- and PI-positive cells was determined using flow cytometry. Annexin V-staining indicates phosphatidylserine exposure and thus the loss of membrane heterogeneity, a marker of early apoptotic cells. PI-staining reflects a loss of membrane integrity, a marker of late apoptotic or necrotic cells. CNF1 increased the number of Annexin V-positive (early apoptotic) and Annexin V + PI- positive (late apoptotic) RhoB -/- cells in a concentration-dependent manner after 48 h (Fig. 14B). In contrast, CNF1 hardly induced apoptosis in NIH3T3 and RhoB +/- (Fig. 14B). The absence of RhoB sensitised cells to CNF1-induced apoptotic cell death. Thus, these data suggested a pro-survival function of RhoB in CNF1-treated cells.

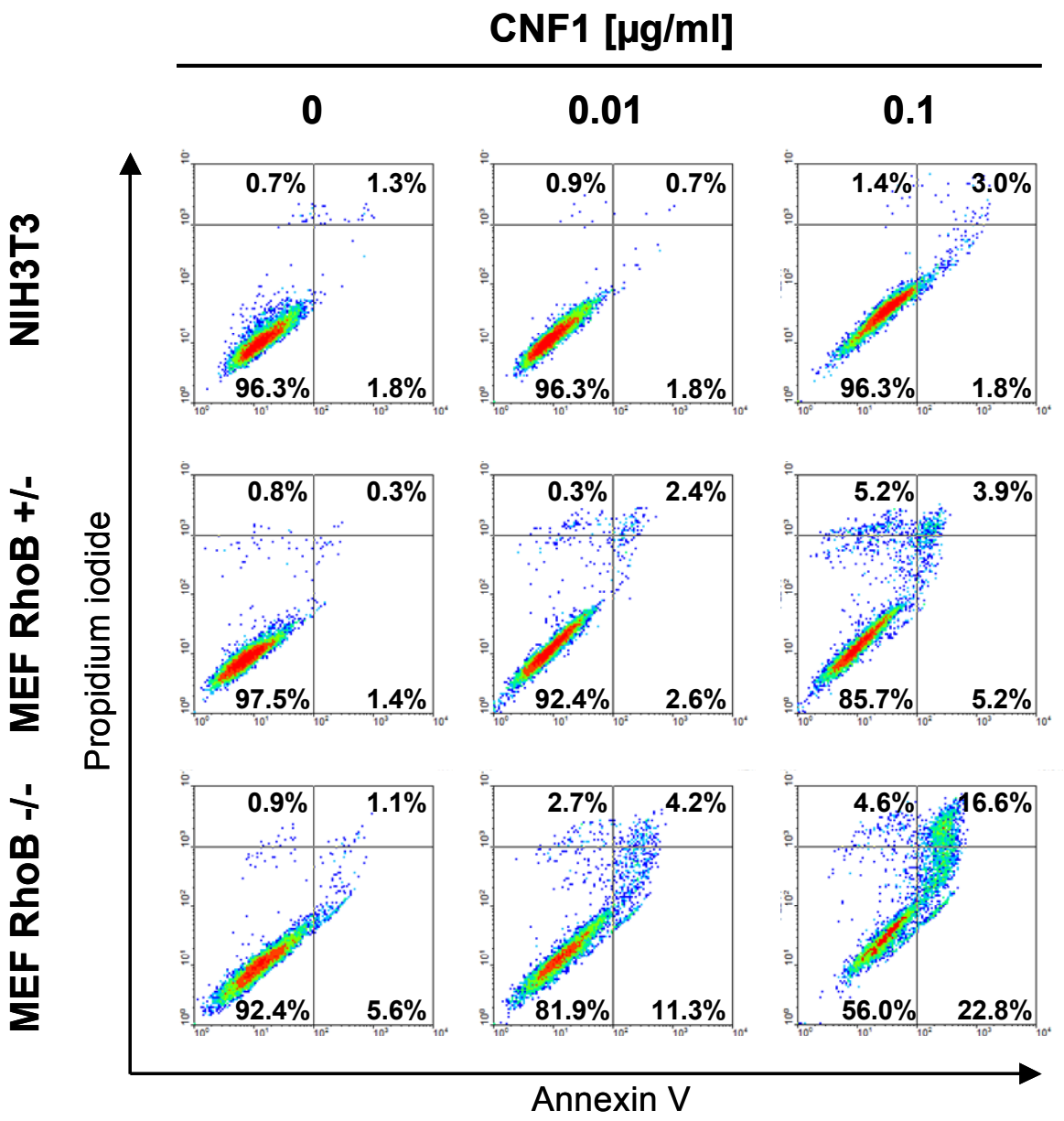

Fig. 14B: CNF1 causes phosphatidylserine exposure in RhoB -/- ME fibroblasts

NIH3T3, RhoB +/-, or RhoB -/- fibroblasts were treated with the indicated concentrations of CNF1 for 48 h. Cells were then stained with Annexin V-Alexa Fluor 488 and propidium iodide. Subsequent FACS analysis was performed by FACScalibur using the software CellQuest Pro (Becton Dickinson). Quantitative analysis was performed using WinMDI 2.8.

5 Discussion

5.1 Activation of *rhoB* by CNF1

RhoB is the only member of the Rho subfamily that is transcriptionally regulated. It is induced by cellular stress, e.g., genotoxic agents, and therefore classified as immediate early gene product (Jähner and Hunter, 1991). The basal expression level of RhoB is low in cells due to the RhoA- and (H/K/N)Ras-dependent suppression of the *rhoB* promoter activity (Jiang et al., 2004). Accordingly, inactivation of RhoA or (H/K/N)Ras by either geranylgeranyl-transferase/farnesyl-transferase inhibitors (Stamatakis et al., 2002) or Rho/Ras-inactivating bacterial protein toxins (Gerhard et al., 2005; Huelsenbeck et al., 2007) causes “de-suppression”, i.e., up-regulation of RhoB.

Transcriptional activation of *rhoB* also occurs in response to platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Jähner and Hunter, 1991). These growth factors activate Rac1 (Ridley et al., 1992). In fact, a Rac1-dependent activation of the *rhoB* promoter has been proposed (Fritz and Kaina, 1997). Thus, we hypothesised that Rac1 positively regulates RhoB. Initially, we confirmed that overexpression of (constitutively active) Rac1-Q61L caused an activation of the *rhoB* promoter (Fig. 1A). To analyse the Rac1-dependent RhoB up-regulation in detail, we applied the Rac1-activating cytotoxic necrotizing factor 1 (CNF1) from *E. coli*. CNF1 activated the *rhoB* promoter as well (Fig. 1B). CNF1 also activates Cdc42. In contrast to Rac1-Q61L, Cdc42-Q61L did not activate the *rhoB* promoter (Fig. 1A). Therefore, CNF1-induced activation of the *rhoB* promoter was most probably a consequence of Rac1 activation. Expectedly, RhoA-Q63L suppressed the *rhoB* promoter activity (Fig. 1A). Treatment with the isomeric CNF_Y from *Y. pseudotuberculosis*, which specifically activates RhoA, hardly increased the *rhoB* promoter activity (Fig. 1B). In conclusion, Rac1 but neither RhoA nor Cdc42 activates the *rhoB* promoter.

5.2 Up-regulation of *rhoB* mRNA and RhoB protein by CNF1

CNF1-induced activation of the *rhoB* promoter was reflected by an up-regulation of *rhoB* mRNA and RhoB protein (Fig. 2A+B). RhoB up-regulation was responsive to inhibition by actinomycin D and cycloheximide (Fig. 2B). Thus, RhoB up-regulation required both mRNA and protein *de novo* synthesis. The responsiveness to actinomycin D further indicated that changes in *rhoB* mRNA stability played a minor role at best in RhoB up-regulation. In CNF1-treated cells, RhoB is thus subject to linear regulation. An increase in *rhoB* promoter activity is reflected by increases of the mRNA and the protein level. Changes in mRNA or protein stability play minor roles. A comparable regulation has been described for IL-8 (Kuwahara et al., 2006). To our knowledge, this is the first detailed study on the regulation of RhoB encompassing regulation from the promoter to the protein level.

CNF1-induced activation of the *rhoB* promoter as well as RhoB protein up-regulation were susceptible to inhibition by NSC23766, an inhibitor of Rac1 (Fig. 3B+C). Thus, Rac1 activation was indeed the trigger of CNF1-induced RhoB up-regulation (Fig. V). NSC23766 inhibits the interaction of Rac1 with its GEF proteins, which in turn are activated by growth factors. We hypothesised that CNF1-induced Rac1 activation and subsequent RhoB up-regulation required the presence of growth factors in the serum. In serum-starved fibroblasts, both Rac1 activation and RhoB up-regulation were indeed reduced (Fig. 3B). Deamidation of Rho GTPases by CNF1 *per se* is supposed to be sufficient for their constitutive activation. Our findings, however, indicate that GEF-driven GTP-loading is still required and critical for short-term activation of Rac1 by CNF1 (4 h). Long-term activation of Rac1 by CNF1 seems not to require GEF-driven GTP loading. After 24 h, up-regulation of RhoB occurred independent of the presence of serum (data not shown). Spontaneous GTP loading is likely sufficient for long-term activation (auto-enhancing mechanism). In conclusion, deamidation is not sufficient for rapid Rac1 activation. The requirement for activation by GEFs challenges the paradigm that GTPase-deficient Rho proteins are *per se* constitutively active.

5.3 Role of c-Myc in CNF1-induced RhoB up-regulation

Rac1 regulates the transactivation of c-Myc in a JNK-dependent manner (Iavarone et al., 2003). We hypothesised that JNK and c-Myc are involved in the CNF1-induced up-regulation of RhoB. Both RhoB up-regulation and *rhoB* promoter activation were reduced by the c-Myc inhibitor (Z,E)-5-(4-Ethylbenzylidine)-2-thioxothiazolidin-4-one in CNF1-treated cells (Fig. 3C+E). This inhibitor blocks the interaction of c-Myc with its interaction partner Max. As a heterodimer, c-Myc-Max binds to DNA to activate gene expression (Yin et al., 2003). c-Myc activation is apparently an important intermediate step in the Rac1-dependent RhoB up-regulation (Fig. V). The *rhoB* promoter, however, does not possess the c-Myc-Max consensus sequence CAC(G/A)TG (Fritz and Kaina, 1997). Thus, c-Myc does not likely cause a direct transactivation of *rhoB*, suggesting the involvement of further transcription factors. As RhoB is up-regulated in CNF1-treated fibroblasts rapidly, c-Myc rather recruits a basal transcription factor instead of regulating *rhoB* via transactivation of a further inducible transcription factor. c-Myc has been shown to interact with the CCAAT-box binding, basal transcription factor NF-Y without affecting its ability to bind to DNA (Izumi et al., 2001). The *rhoB* promoter possesses a CCAAT-box, which is critical for *rhoB* activation induced by UV-light (Fritz and Kaina, 2001). Furthermore, NF-Y has been suggested to bind to the *rhoB* promoter (Fritz and Kaina, 2001). Thus, it is conceivable that c-Myc regulates the *rhoB* promoter activity via NF-Y.

5.4 Role of p38 MAP kinase in CNF1-induced RhoB up-regulation

p38 MAP kinase regulates gene expression by distinct mechanisms: (i) transcriptional, via phosphorylation of transcription factors that regulate promoter activity; (ii) posttranscriptional, via alteration of mRNA stability or by affecting the mRNA translation into protein (Schieven, 2005). Rac1 and Cdc42 positively regulate p38 MAP kinase (Kyriakis and Avruch, 2001). Accordingly, CNF1 has been shown to activate p38 (Munro et al., 2004). We observed that RhoB up-regulation was enhanced by inhibition of p38 with its pharmacological inhibitor SB 203580 (Fig. 4A-C). Accordingly, RhoB up-regulation was enhanced in $p38_{\alpha}$ -/- MEF compared to WT

MEF (Fig. 5A-C). These findings were reflected at the *rhoB* promoter level, as the *rhoB* promoter activity was enhanced by inhibition or in the absence of p38 (Fig. 4C+5C). SB 203580 was without effect in CNF1-treated $p38_{\alpha}$ -/- MEF, confirming the specificity of SB 203580 (Fig. 5D). Thus, we conclude that p38 negatively regulates the CNF1-induced RhoB up-regulation at the transcriptional rather than post-transcriptional level (Fig. V). This is a striking finding, as p38 usually activates gene transcription, as shown for ATF-2 (Raingeaud et al., 1996). p38 MAP kinase, however, negatively regulates the level and activity of ERK (Page et al., 2001).

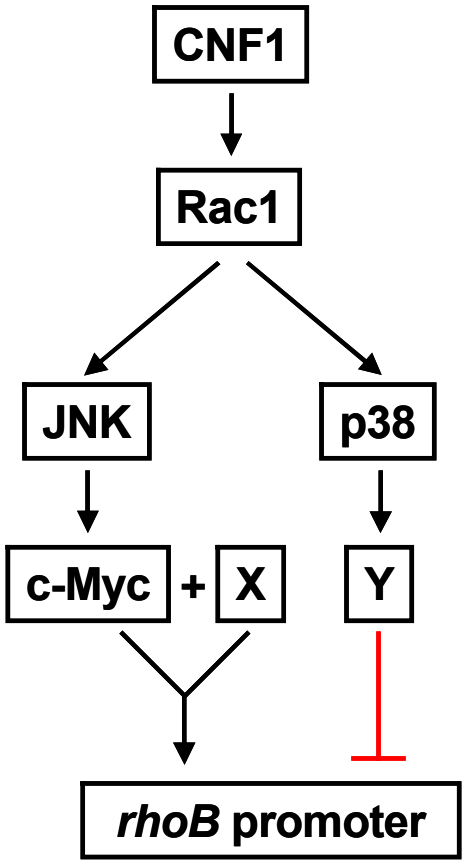

Fig. V: Model of RhoB up-regulation by CNF1

RhoB is up-regulated by CNF1 in a Rac1-dependent manner by transcriptional activation. c-Myc is activated by CNF1 in a Rac1-JNK-dependent manner. Activation of c-Myc is an important intermediate step in the Rac1-dependent RhoB up-regulation. A basal transcription factor (X) is likely recruited by c-Myc to cause activation of the *rhoB* promoter. CNF1 causes activation of p38 MAP kinase in a Rac1-dependent manner. p38 negatively regulates the CNF1-induced RhoB up-regulation at the transcriptional level. Thereby, an inhibitory transcription factor (Y) may be involved.

5.5 State of activity of RhoB in CNF1-treated cells

Rho proteins exert their biological functions only in the active, GTP-bound, state. We found that CNF1-induced RhoB up-regulation correlated with its activation (Fig. 3A). Even though CNF_Y failed to cause RhoB up-regulation, it caused an activation of RhoB (Fig. 3B). This finding suggests that RhoB may be a yet non-identified cellular substrate of CNF_Y.

Both RhoB mRNA and protein are labile, with half-life periods of approximately 20 min and 2 h, respectively (Fritz et al., 1995; Lebowitz et al., 1995). Inhibition of the 26S proteasome by MG132 showed that RhoB is degraded in CNF1-treated cells in a proteasome-dependent manner (Fig. 6). In CNF1-treated cells, the half-life period of RhoB was < 1 h (Fig. 6). Thus, the half-life period was reduced in CNF1-treated cells compared to cells treated with FTI (Lebowitz et al., 1995). Deamidation by CNF1 sensitises Rho proteins to proteasomal degradation (Doye et al., 2002). RhoB has been shown to be deamidated by CNF1 *in vitro* (G. Schmidt, personal communication). The enhanced degradation of RhoB in CNF1-treated cells provides a hint that RhoB may not only be an *in vitro* but also a cellular substrate of CNF1.

As opposed to other immediate early gene products, the up-regulation of RhoB by CNF1 was not transient but long-lasting in CNF1-treated cells (Fig. 7). This finding was remarkable, since its half-life period was < 1 h in CNF1-treated cells. The persistent RhoB up-regulation is likely based on permanent Rac1 activation and thus permanent activation of the *rhoB* promoter.

5.6 CNF-induced polyploidy

CNFs cause multinucleation of cultured cells (Oswald et al., 1989). This effect is based on their ability to modulate the eukaryotic cell cycle, leading to their classification as cyclomodulins (Oswald et al., 2005). We observed multinuclei in fibroblasts exposed to CNF1. Multinucleation is associated with an increased DNA content (polyploidy). Binucleated cells, however, appear as cells with 4N DNA content and cannot be distinguished from cells in the G2- or M-phase of the cell

cycle. 8N cells occur in response to CNF1 and its homologue CNF2 (Denko et al., 1997; Nougayrede et al., 2005). We determined the number of 8N cells to quantify polyploidy. These cells are at least binucleated and are rarely observed in untreated fibroblasts. Initially, we confirmed that CNF1 increased the 8N population in fibroblasts, reflecting multinucleation (Fig. 9A+B). The formation of multinucleated cells suggests failure in cytokinesis. Although the formation of multinuclei is described as hallmark of CNF-treatment, CNFs have been suggested to promote cell proliferation (Nougayrede et al., 2005). Cell proliferation, however, is characterised by ongoing cellular division, resulting in an increasing cell number. To clarify this contradiction, we determined cell proliferation by two distinct methods: 1. Determination of the cell number; 2. Application of the generally accepted proliferation assay WST-1, which measures the viability in terms of activity of cellular dehydrogenases. The cell number remained almost constant after CNF1-exposure, while it increased in the untreated (proliferating) cell population (Fig. 10). Thus, CNF1 blocks cell proliferation. The increase in viability (WST-1 assay) of untreated and CNF1-treated cells was comparable (Fig. 10). Thus, in CNF1-treated cells, the viability per cell increased. This may be due to an increase in cell size. In untreated cells, the increased viability reflected the increased cell number (Fig. 10). Thus, the WST-1 assay misleadingly indicates that proliferation continues during CNF1-treatment. The activity of cellular dehydrogenases does not necessarily correlate with cell proliferation. Therefore, the WST-1 assay is not suitable to determine proliferation of cells exposed to cyclomodulins. In conclusion, CNF1 blocks cell proliferation. Inhibition of cytokinesis with ongoing karyokinesis causes polyploidy.

It has been proposed that CNF1-induced multinucleation is based on Rac1 activation (Malorni and Fiorentini, 2006). The homologue CNF_Y from *Yersinia pseudotuberculosis*, which selectively deamidates RhoA, however, also causes polyploidy (Hoffmann et al., 2004). This led us to the hypothesis that RhoA rather than Rac1 deamidation is critical for CNF1-induced polyploidy. RhoA plays a critical role during cytokinesis (Fig. VI) (Maddox and Oegema, 2003). During the first step of cytokinesis furrow ingression occurs (Fig. VI). RhoA is activated by its GEF Ect2.

Active, GTP-bound, RhoA, which is localised to the plasma membrane, is responsible for the formation of the contractile ring. Furthermore, RhoA blocks the actin-severing activity of cofilin during furrow ingression (Fig. VI). Completion of cytokinesis, however, requires RhoA inactivation induced by Mgc GAP (Fig. VI). Mgc GAP is localised to the interzonal microtubule bundles that form between the segregating chromosomes. Contraction of the cleavage furrow around the microtubules brings RhoA into the vacinity of Mgc GAP, leading to RhoA inactivation. RhoA inactivation in turn is accompanied by activation of cofilin. Cofilin causes disassembly of the contractile ring to terminate cell division (Fig. VI) (Maddox and Oegema, 2003). We therefore propose that CNFs impair cytokinesis by blocking the Mgc-dependent RhoA inactivation (Fig. VI). As a consequence, cofilin remains inactive and is therefore incapable of completing cytokinesis, resulting in polyploidy. In this line, disruption of Mgc GAP has been shown to cause polyploidy (Minoshima et al., 2003).

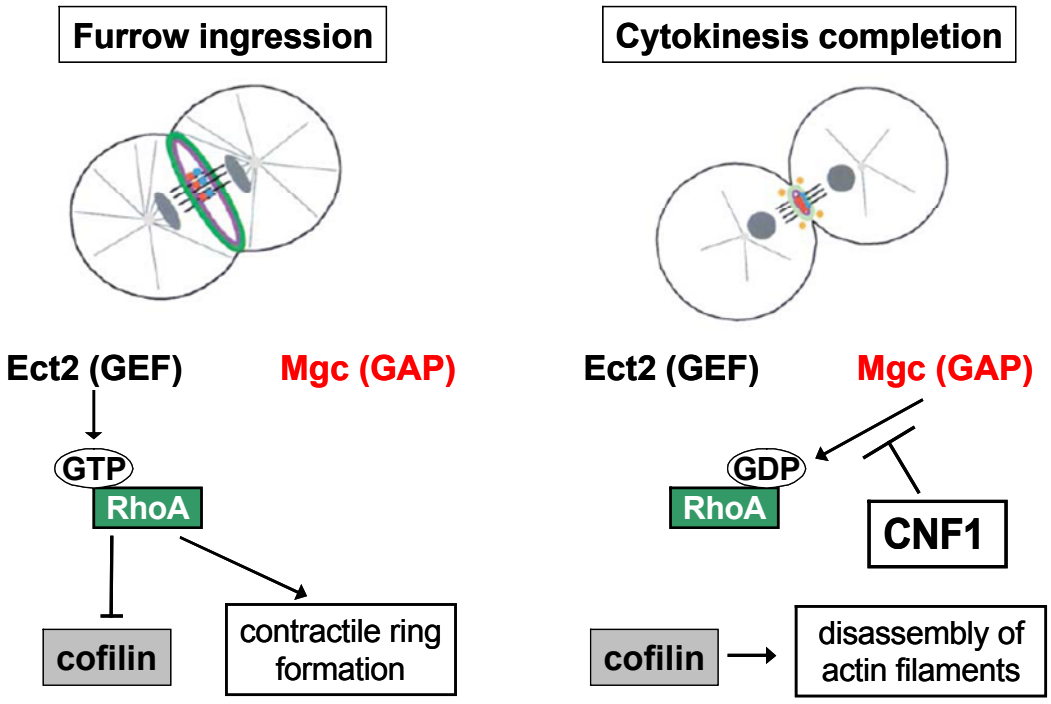

Fig. VI: Model of CNF1-induced polyploidy

During the first step of cytokinesis (furrow ingression) RhoA is kept in the active, GTP-bound, conformation by its GEF Ect2. RhoA, which is localised to the plasma membrane, is responsible for the formation of the contractile ring. Contraction of the cleavage furrow leads to RhoA inactivation by Mgc GAP. RhoA inactivation in turn causes cofilin activation. Disassembly of actin filaments induced by cofilin completes cytokinesis. CNF1 blocks the Mgc-stimulated RhoA inactivation. It thereby inhibits cytokinesis completion, leading to polyploidy (adapted from Maddox and Oegema, 2003).

5.7 Involvement of p53 in the CNF1-induced polyploidy

After failed cytokinesis, tetraploid cells can enter the G1 phase (Rieder and Maiato, 2004). p53 has been suggested to cause G1-arrest or apoptosis of tetraploid cells to prevent genomic instability (Andreassen et al., 2001). This “tetraploidy checkpoint” provides an opportunity to eliminate potentially dangerous cells. We hypothesised that p53 suppresses CNF1-induced polyploidy (Table II). CNF1 increased the number of 8N cells in HCT 116 wild type and HCT 116 p53 -/- with comparable kinetics (Fig. 11). Thus, tetraploidy *per se* does not cause a p53-dependent G1-arrest or apoptosis. Several other studies also provide evidence that tetraploid or polyploid cells are capable of proliferation, challenging the idea of a tetraploidy checkpoint (Guidotti et al., 2003; Storchova and Pellman, 2004). In uroepithelial cells, CNF1 has been suggested to cause a G2/M-arrest, corresponding to G1-arrest of tetraploid cells (Falzano et al., 2006). We observed the occurrence of a population of cells with 8N DNA content in several cell lines including NIH3T3 fibroblasts, ME fibroblasts, and the human colon carcinoma cell line HCT 116 (Fig. 9B+11+12C). In J774A.1 macrophages and in the epithelial cell line Vero, however, CNF1 caused an increase of the 4N population without an occurrence of an 8N population (data not shown). Thus, a possible cell cycle arrest in response to CNF1 appears to be cell type specific but does not likely depend on p53.

Table II: Effect of inhibition of p53, p38, and RhoB on CNF1-induced polyploidy

	effect on polyploidy (literature)	**expected effect on polyploidy** (after inhibition/genetic deletion)	**observed effect on polyploidy** (after inhibition/genetic deletion)
p53	↓	↑	↔
p38	↓	↑	↓
RhoB	↓	↑	↑

5.8 Involvement of the p38 MAP kinase in the CNF1-induced polyploidy

p38 MAP kinase is induced by cellular stress, such as ultraviolet radiation, osmotic shock, or pro-inflammatory cytokines (Schieven, 2005; Seo et al., 2004). p38 blocks cell proliferation and tumorigenesis by causing senescence or apoptosis (Han and Sun, 2007). We hypothesised that p38 suppresses CNF1-induced polyploidy (Table II). Inhibition of p38 by SB 203580 suppressed CNF1-induced polyploidy (Fig. 12A+B). Accordingly, polyploidy was reduced in $p38_{\alpha}$ MAP kinase -/- cells compared to wild type cells (Fig. 12C). Thus, p38 promotes rather than suppresses CNF1-induced polyploidy (Table II). p38 triggered neither cell cycle arrest nor apoptosis in CNF1-treated fibroblasts. This finding is contrary to the general anti-proliferative and pro-apoptotic role of p38. We hypothesise that this unexpected effect of p38 is based on its suppressive effect on RhoB (Fig. 4+5).

5.9 Role of RhoB in the CNF1-induced polyploidy

We suggested above that RhoA deamidation causes cytokinesis failure, resulting in polyploidy. The RhoA homologue RhoB protects fibroblasts from radiation-induced multinucleation associated with abberant nuclei (Milia et al., 2005). In response to CNF1-treatment, multinucleated cells exhibited nuclei of equal size but no abberant nuclei (micronulei). RhoB, however, also suppressed the CNF1-induced type of multinucleation (reflected by polyploidy) (Fig. 13B). The p38-dependent enhancement of polyploidy we observed is most likely based on RhoB suppression, as inhibition of p38 was without effect on RhoB -/- cells (Fig. 13C).

RhoB has been proposed to protract the radiation induced G2 arrest, by which means it suppresses centrosome overduplication (Milia et al., 2005). Centrosomes are key regulators of the mitotic spindle. Centrosome overduplication contributes to the assembly of multipolar spindles, thereby disturbing division of chromosomes (Sato et al., 2000). Centrosome abnormalities are often associated with the occurrence of abberant nuclei rather than polyploidy (Pihan et al., 2003). Thus, this model is not suitable to explain the suppressive effect of RhoB in CNF1-induced

polyploidy. A further role of centrosomes in cytokinesis completion has been described (Piel et al., 2001). After formation of the cleavage furrow, one centrosome moves into the intracellular bridge. This effect is required for cytokinesis completion (abscission) (Piel et al., 2001). The Rho-kinase p160ROCK, a downstream effector of RhoB, is involved in the regulation of centrosomes (Milia et al., 2005). p160ROCK is required for centrosome positioning (Chevrier et al., 2002). RhoB may control the movement of the centrosome into the intracellular bridge in a p160ROCK-dependent manner, thereby promoting abscission.

5.10 Role of RhoB in survival of CNF1-treated cells

RhoB might be involved in the physiological arrest- and repair-mechanisms after stress stimuli. As a janus-faced protein, RhoB appears to be involved in the decision of the cell to undergo DNA-repair or apoptosis (Fritz and Kaina, 2000). Recent data have highlighted the protective role of CNF1 against UV-B-induced apoptosis (Miraglia et al., 2007). The Rac-stimulated PI3K/Akt pathway that in turn leads to NF-κB-dependent Bcl-2 expression is essential for the protective effect (Miraglia et al., 2007). RhoB activates NF-κB (Rodriguez et al., 2007), which in turn promotes the transcription of pro-survival factors such as Bcl-2 (Buchholz et al., 2005). Furthermore, RhoB controls trafficking of the pro-survival protein kinase Akt (Adini et al., 2003). We hypothesised that RhoB plays an essential role in the regulation of survival in CNF1-treated cells. CNF1 caused cell death in the absence of RhoB (Fig. 14A+B), suggesting a pro-survival function of RhoB.

We cannot rule out that polyploidy *per se* causes cell death. The pro-survival function of RhoB in CNF1-treated cells may thus be based on inhibition of polyploidy (Fig. VII).

5.11 Conclusions

We characterised a new Rac1-dependent pathway, which causes up-regulation of RhoB. The pathway involves c-Myc as an acivator and p38 MAP kinase as a suppressor (Fig. V). Based on published data we expected that inhibition of RhoB, p38, and p53 enhances CNF1-induced polyploidy (Table II). We found that p53 has no effect on CNF1-induced polyploidy (Fig. VII). p38 promotes polyploidy due to suppression of RhoB. RhoB negatively regulates polyploidy (Fig. VII).

CNF1 is an important virulence factor of uropathgenic *E. coli* (Rippere-Lampe et al., 2001). Inhibition of cytokinesis by CNF1 likely inhibits renewal of epithelial cells. Lack of renewal may result in prolonged colonisation and persistence of uropathogenic bacteria in the epithel. RhoB may be required for the survival of the mammalian target cells.

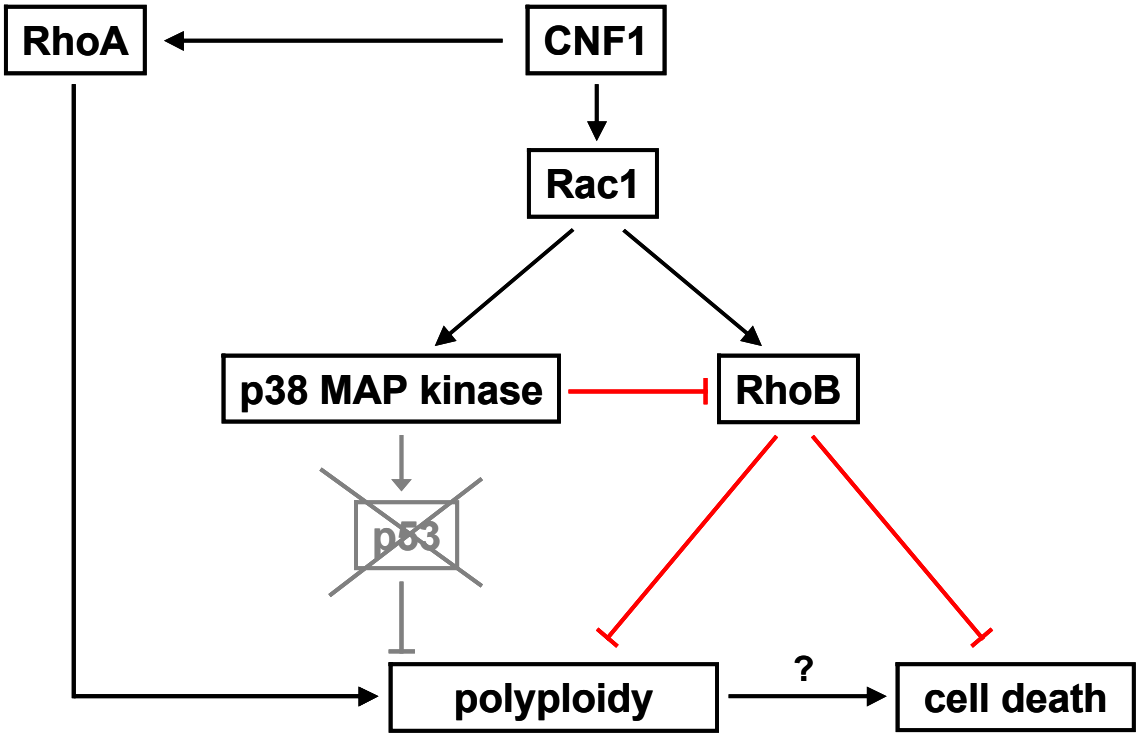

Fig. VII: Role of p53, p38, and RhoB in CNF1-induced polyploidy

CNF1-induced deamidation of RhoA causes polyploidy. Activation of Rac1 by CNF1 causes RhoB up-regulation and p38 MAP kinase activation. p53, which is downstream of p38 (Ganem and Pellman, 2007), has no effect on CNF1-induced polyploidy. p38 promotes polyploidy due to suppression of RhoB. RhoB negatively regulates polyploidy. Polyploidy may result in cell death. RhoB suppresses cell death in CNF1-treated cells.

6 Perspective

We showed that c-Myc and p38 MAP kinase are involved in the Rac1-dependent RhoB up-regulation. The molecular mechanism of the regulation of the *rhoB* promoter remains to be solved. Based on sequence analysis of the *rhoB* promoter, diverse sequences for the binding sites of transcription factors were identified (Fig. VIII) (Fritz and Kaina, 1997). Besides a TATA box, the *rhoB* promoter contains consensus sequences for SP1, p53, AP-2, AP-4, PEA3, XRE, and CF1. The identification of those transcription factors involved in the activation/inactivation of the *rhoB* promoter will give hints on signalling pathways involved. Four deletion mutants of the *rhoB* promoter construct are available to perform this analysis (Fig. VIII).

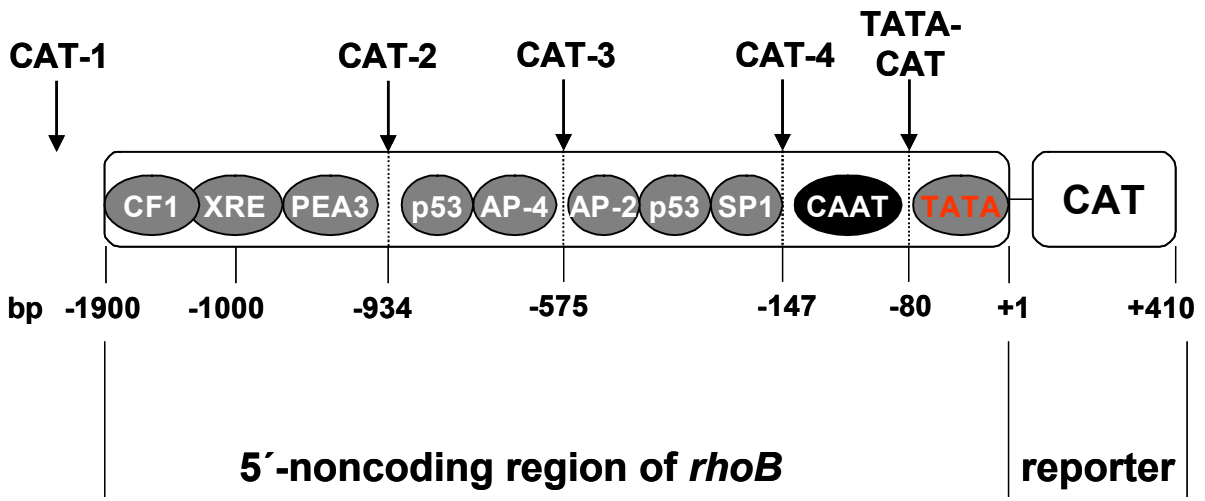

Fig. VIII: Schematic representation of the deletion mutants of the *rhoB* promoter construct with putative binding sites for transcription factors

The *rhoB* promoter contains consensus sequences for SP1, p53, AP-2, AP-4, PEA3, XRE, and CF1. Four deletion mutants of the *rhoB* promoter construct are available to identify transcription factors, which are involved in the regulation of *rhoB*, by reporter gene assay.

7 Abbreviations

aa	amino acid
AP-2	activating protein-2
AP-4	activating protein-4
ATF-2	activating transcription factor-2
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma 2
CF1	common factor 1
BSA	bovine serum albumin
C3bot	*Clostridium botulinum* C3 exoenzyme
C21	Rho-binding domain of Rhotekin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CGT	clostridial glucosylating toxin
CHX	cycloheximide
CNF1	*Escherichia coli* cytotoxic necrotizing factor 1
CNF_Y	*Yersinia pseudotuberculosis* cytotoxic necrotizing Y
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DD	deamidase domain
DNA	deoxyribonucleic acid
DNT	*Bordetella* dermonecrotic toxin
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FCS	fetal Calf Serum
Fig.	figure
FT	farnesyl-transferase
FTI	farnesyl-transferase inhibitor
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GGT	geranylgeranyl-transferase
GGTI	geranylgeranyl-transferase inhibitor
GST	glutathione S-transferase

GTP	guanosine triphosphate
HCT	human colon carcinoma cells
HeLa	cervical cancer cells (taken from Henrietta Lacks)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSPG	heparan sulfate proteoglycan
IL	interleukine
JNK	c-Jun N-terminal kinases
kb	kilo base pairs
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MEF	mouse embryonic fibroblast
MKK	mitogen-activated protein kinase kinase
mRNA	messenger RNA
NAD	nicotinamid adenin dinucleotide
NF-κB	nuclear factor-κB
NF-Y	nuclear factor-Y
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBS	phospate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	propidium iodide
PMSF	phenylmethylsulphonyl fluoride
Ras	Rat sarcoma
RBD	receptor binding domain
Rel.	relative
Rho	Ras homologous
RhoB-F	farnesylated RhoB
RhoB-GG	geranylgeranylated RhoB
RNA	ribonucleic acid
ROCK	Rho-associated kinase
ROS	reactive oxygen species
rpm	rotations per minute
RT	reverse transcription
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SP1	specificity protein 1
TcdA	*Clostridium difficile* toxin A

TcsL	*Clostridium sordellii* lethal toxin
TMD	transmembrane domain
TNF-α	tumor necrosis factor-α
Tris	tris(hydroxymethyl)aminomethane
UPEC	uro-pathogenic *Escherichia coli*
UV	ultraviolet
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt
WT	wild type
w/v	weight/volume
XRE	xenobiotic response element

SI units and non-SI units accepted for use with SI were applied.

The one-letter and three-letter abbreviation codes for amino acids were used.

The bases of the DNA were abbreviated according to the IUB (International Union of Biochemistry) tentative rules.

8 Reference List

Adamson,P., Marshall,C.J., Hall,A., and Tilbrook,P.A. 1992a. Post-translational modifications of $p21^{rho}$ proteins. *J. Biol. Chem.* 267:20033-20038.

Adamson,P., Paterson,H.F., and Hall,A. 1992b. Intracellular localization of the $p21^{rho}$ proteins. *J. Cell Biol.* 119:617-627.

Ader,I., Toulas,C., Dalenc,F., Delmas,C., Bonnet,J., Cohen-Jonathan,E., and Favre,G. 2002. RhoB controls the 24 kDa FGF-2-induced radioresistance in HeLa cells by preventing post-mitotic cell death. *Oncogene* 21:5998-6006.

Adini,I., Rabinovitz,I., Sun,J.F., Prendergast,G.C., and Benjamin,L.E. 2003. RhoB controls Akt trafficking and stage-specific survival of endothelial cells during vascular development. *Genes Dev.* 17:2721-2732.

Andreassen,P.R., Lohez,O.D., Lacroix,F.B., and Margolis,R.L. 2001. Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. *Mol. Biol. Cell* 12:1315-1328.

Aspenström,P., Fransson,A., and Saras,J. 2004. Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem. J.* 377:327-337.

Blumenthal,B., Hoffmann,C., Aktories,K., Backert,S., and Schmidt,G. 2007. The cytotoxic necrotizing factors from Yersinia pseudotuberculosis and from Escherichia coli bind to different cellular receptors but take the same route to the cytosol. *Infect. Immun.* 75:3344-3353.

Boureux,A., Furstoss,O., Simon,V., and Roche,S. 2005. Abl tyrosine kinase regulates a Rac/JNK and a Rac/Nox pathway for DNA synthesis and Myc expression induced by growth factors. *J. Cell Sci.* 118:3717-3726.

Boyer,L., Travaglione,S., Falzano,L., Gauthier,N.C., Popoff,M.R., Lemichez,E., Fiorentini,C., and Fabbri,A. 2004. Rac GTPase instructs nuclear factor-kappaB activation by conveying the SCF complex and IkBalpha to the ruffling membranes. *Mol. Biol. Cell* 15:1124-1133.

Boyer,L., Turchi,L., Desnues,B., Doye,A., Ponzio,G., Mege,J.L., Yamashita,M., Zhang,Y.E., Bertoglio,J., Flatau,G., Boquet,P., and Lemichez,E. 2006. CNF1-induced ubiquitylation and proteasome destruction of activated RhoA is impaired in Smurf1-/- cells. *Mol. Biol. Cell* 17:2489-2497.

Brest,P., Turchi,L., Le'Negrate,G., Berto,F., Moreilhon,C., Mari,B., Ponzio,G., and Hofman,P. 2004. *Escherichia coli* cytotoxic necrotizing factor 1 inhibits intestinal epithelial wound healing in vitro after mechanical injury. *Infect. Immun.* 72:5733-5740.

Buchholz,T.A., Garg,A.K., Chakravarti,N., Aggarwal,B.B., Esteva,F.J., Kuerer,H.M., Singletary,S.E., Hortobagyi,G.N., Pusztai,L., Cristofanilli,M., and Sahin,A.A. 2005. The nuclear transcription factor kappaB/bcl-2 pathway correlates with pathologic complete response to doxorubicin-based neoadjuvant chemotherapy in human breast cancer. *Clin. Cancer Res.* 11:8398-8402.

Buetow,L., Flatau,G., Chiu,K., Boquet,P., and Ghosh,P. 2001. Structure of the Rho-activating domain of *Escherichia coli* cytotoxic necrotizing factor 1. *Nat. Struct. Biol.* 8:584-588.

Bustelo,X.R., Sauzeau,V., and Berenjeno,I.M. 2007. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays* 29:356-370.

Canguilhem,B., Pradines,A., Baudouin,C., Boby,C., Lajoie-Mazenc,I., Charveron,M., and Favre,G. 2005. RhoB protects human keratinocytes from UVB-induced apoptosis through epidermal growth factor receptor signaling. *J. Biol. Chem.* 280:43257-43263.

Caprioli,A., Falbo,V., Roda,L.G., Ruggeri,F.M., and Zona,C. 1983. Partial purification and characterization of an *escherichia coli* toxic factor that induces morphological cell alterations. *Infect. Immun.* 39:1300-1306.

Chen,J.Y., Hsu,P.C., Hsu,I.L., and Yeh,G.C. 2001. Sequential up-regulation of the c-fos, c-jun and bax genes in the cortex, striatum and cerebellum induced by a single injection of a low dose of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in C57BL/6 mice. *Neurosci. Lett.* 314:49-52.

Chevrier,V., Piel,M., Collomb,N., Saoudi,Y., Frank,R., Paintrand,M., Narumiya,S., Bornens,M., and Job,D. 2002. The Rho-associated protein kinase p160ROCK is required for centrosome positioning. *J. Cell Biol.* 157:807-817.

Chiariello,M., Marinissen,M.J., and Gutkind,J.S. 2001. Regulation of c-myc expression by PDGF through Rho GTPases. *Nat. Cell Biol.* 3:580-586.

Chung,J.W., Hong,S.J., Kim,K.J., Goti,D., Stins,M.F., Shin,S., Dawson,V.L., Dawson,T.M., and Kim,K.S. 2003. 37-kDa laminin receptor precursor modulates cytotoxic necrotizing factor 1-mediated RhoA activation and bacterial uptake. *J. Biol. Chem.* 278:16857-16862.

Contamin,S., Galmiche,A., Doye,A., Flatau,G., Benmerah,A., and Boquet,P. 2000. The p21 Rho-activating toxin cytotoxic necrotizing factor 1 is endocytosed by a clathrin-independent mechanism and enters the cytosol by an acidic-dependent membrane translocation step. *Mol. Biol. Cell* 11:1775-1787.

Cox,A.D., and Der,C.J. 2002. Ras family signaling: therapeutic targeting. *Cancer Biol. Ther.* 1:599-606.

Denko,N., Langland,R., Barton,M., and Lieberman,M.A. 1997. Uncoupling of S-phase and mitosis by recombinant cytotoxic necrotizing factor 2 (CNF2). *Exp. Cell Res.* 234:132-138.

Doye,A., Mettouchi,A., Bossis,G., Clement,R., Buisson-Touati,C., Flatau,G., Gagnoux,L., Piechaczyk,M., Boquet,P., and Lemichez,E. 2002. CNF1 exploits the ubiquitin-proteasome machinery to restrict Rho GTPase activation for bacterial host cell invasion. *Cell* 111:553-564.

Egerer,M., Giesemann,T., Jank,T., Satchell,K.J., and Aktories,K. 2007. Auto-catalytic cleavage of *Clostridium difficile* toxins A and B depends on cysteine protease activity. *J. Biol. Chem.* 282:25314-25321.

Falbo,V., Pace,T., Picci,L., Pizzi,E., and Caprioli,A. 1993. Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *escherichia coli*. *Infect. Immun.* 61:4909-4914.

Falzano,L., Filippini,P., Travaglione,S., Miraglia,A.G., Fabbri,A., and Fiorentini,C. 2006. *Escherichia coli* cytotoxic necrotizing factor 1 blocks cell cycle G2/M transition in uroepithelial cells. *Infect. Immun.* 74:3765-3772.

Falzano,L., Fiorentini,C., Donelli,G., Michel,E., Kocks,C., Cossart,P., Cabanié,L., Oswald,E., and Boquet,P. 1993. Induction of phagocytic behaviour in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. *Molecular Microbiology* 9:1247-1254.

Falzano,L., Quaranta,M.G., Travaglione,S., Filippini,P., Fabbri,A., Viora,M., Donelli,G., and Fiorentini,C. 2003. Cytotoxic necrotizing factor 1 enhances reactive oxygen species-dependent transcription and secretion of proinflammatory cytokines in human uroepithelial cells. *Infect. Immun.* 71:4178-4181.

Fiorentini,C., Falzano,L., Fabbri,A., Stringaro,A., Logozzi,M., Travaglione,S., Contamin,S., Arancia,G., Malorni,W., and Fais,S. 2001. Activation of rho GTPases by cytotoxic necrotizing factor 1 induces macropinocytosis and scavenging activity in epithelial cells. *Mol. Biol. Cell* 12:2061-2073.

Flatau,G., Lemichez,E., Gauthier,M., Chardin,P., Paris,S., Fiorentini,C., and Boquet,P. 1997. Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* 387:729-733.

Fritz,G., and Kaina,B. 1997. rhoB encoding a UV-inducible ras-related small GTP-binding protein is regulated by GTPases of the Rho family and independent of JNK, ERK, and p38 MAP kinase. *J. Biol. Chem.* 272:30637-30644.

Fritz,G., and Kaina,B. 2000. Ras-related GTPase RhoB forces alkylation-induced apoptotic cell death. *Biochem. Biophys. Res. Commun.* 268:784-789.

Fritz,G., and Kaina,B. 2001. Transcriptional activation of the small GTPase gene rhoB by genotoxic stress is regulated via a CCAAT element. *Nucleic Acids Res.* 29:792-798.

Fritz,G., Kaina,B., and Aktories,K. 1995. The Ras-related small GTP-binding protein RhoB is immediate- early inducible by DNA damaging treatments. *J. Biol. Chem.* 270:25172-25177.

Fujisawa,K., Madaule,P., Ishizaki,T., Watanabe,G., Bito,H., Saito,Y., Hall,A., and Narumiya,S. 1998. Different regions of Rho determine Rho-selective binding of different classes of Rho target molecules. *J. Biol. Chem.* 273:18943-18949.

Ganem,N.J., Pellman,D. 2007. Limiting the proliferation of polyploid cells. Cell 131(3):437-440

Gao,Y., Dickerson,J.B., Guo,F., Zheng,J., and Zheng,Y. 2004. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc. Natl. Acad. Sci. U. S. A* 101:7618-7623.

Genth,H., Aktories,K., and Just,I. 1999. Monoglucosylation of RhoA at Threonine-37 blocks cytosol-membrane cycling. *J. Biol. Chem.* 274:29050-29056.

Genth,H., Gerhard,R., Maeda,A., Amano,M., Kaibuchi,K., Aktories,K., and Just,I. 2003. Entrapment of Rho ADP-ribosylated by *Clostridium botulinum* C3 exoenzyme in the Rho-guanine nucleotide dissociation inhibitor-1 complex. *J. Biol. Chem.* 278:28523-28527.

Gerhard,R., Schmidt,G., Hofmann,F., and Aktories,K. 1998. Activation of Rho GTPases by *Escherichia coli* cytotoxic necrotizing factor 1 increases intestinal permeability in Caco-2 cells. *Infect. Immun.* 66:5125-5131.

Gerhard,R., Tatge,H., Genth,H., Thum,T., Borlak,J., Fritz,G., and Just,I. 2005. *Clostridium difficile* toxin A induces expression of the stress-induced early gene product RhoB. *J. Biol. Chem.* 280:1499-1505.

Guidotti,J.E., Bregerie,O., Robert,A., Debey,P., Brechot,C., and Desdouets,C. 2003. Liver cell polyploidization: a pivotal role for binuclear hepatocytes. *J. Biol. Chem.* 278:19095-19101.

Hall,A. 1998. Rho GTPases and the actin cytoskeleton. *Science* 279:509-514.

Hall,A., and Nobes,C.D. 2000. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 355:965-970.

Han,J., and Sun,P. 2007. The pathways to tumor suppression via route p38. *Trends Biochem. Sci.* 32:364-371.

Herrmann,C., Ahmadian,M.R., Hofmann,F., and Just,I. 1998. Functional consequences of monoglucosylation of H-Ras at effector domain amino acid threonine-35. *J. Biol. Chem.* 273:16134-16139.

Hoffmann,C., Pop,M., Leemhuis,J., Schirmer,J., Aktories,K., and Schmidt,G. 2004. The *Yersinia pseudotuberculosis* cytotoxic necrotizing factor (CNFY) selectively activates RhoA. *J. Biol. Chem.* 279:16026-16032.

Hoffmann,C., and Schmidt,G. 2004. CNF and DNT. *Rev. Physiol Biochem. Pharmacol.* 152:49-63.

Huelsenbeck,J., Dreger,S.C., Gerhard,R., Fritz,G., Just,I., and Genth,H. 2007. Upregulation of the Immediate Early Gene Product RhoB by Exoenzyme C3 from *Clostridium limosum* and Toxin B from *Clostridium difficile*. *Biochemistry* 46:4923-4931.

Iavarone,C., Catania,A., Marinissen,M.J., Visconti,R., Acunzo,M., Tarantino,C., Carlomagno,M.S., Bruni,C.B., Gutkind,J.S., and Chiariello,M. 2003. The platelet-derived growth factor controls c-myc expression through a JNK- and AP-1-dependent signaling pathway. *J. Biol. Chem.* 278:50024-50030.

Izumi,H., Molander,C., Penn,L.Z., Ishisaki,A., Kohno,K., and Funa,K. 2001. Mechanism for the transcriptional repression by c-Myc on PDGF beta-receptor. *J. Cell Sci.* 114:1533-1544.

Jaffe,A.B., and Hall,A. 2005. Rho GTPases: biochemistry and biology. *Annu. Rev. Cell Dev. Biol.* 21:247-269.

Jähner,D., and Hunter,T. 1991. The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor and platelet-derived growth factor in rat fibroblasts. *Mol. Cell. Biol.* 11:3682-3690.

Jiang,K., Sun,J., Cheng,J., Djeu,J.Y., Wei,S., and Sebti,S. 2004. Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis. *Mol. Cell Biol.* 24:5565-5576.

Just,I., Selzer,J., Hofmann,F., Green,G.A., and Aktories,K. 1996. Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J. Biol. Chem.* 271:10149-10153.

Just,I., Selzer,J., Wilm,M., Von Eichel-Streiber,C., Mann,M., and Aktories,K. 1995a. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* 375:500-503.

Just,I., Wilm,M., Selzer,J., Rex,G., Von Eichel-Streiber,C., Mann,M., and Aktories,K. 1995b. The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *J. Biol. Chem.* 270:13932-13936.

Kamon,H., Kawabe,T., Kitamura,H., Lee,J., Kamimura,D., Kaisho,T., Akira,S., Iwamatsu,A., Koga,H., Murakami,M., and Hirano,T. 2006. TRIF-GEFH1-RhoB pathway is involved in MHCII expression on dendritic cells that is critical for CD4 T-cell activation. *EMBO J.* 25:4108-4119.

Kim,K.J., Chung,J.W., and Kim,K.S. 2005. 67-kDa laminin receptor promotes internalization of cytotoxic necrotizing factor 1-expressing *Escherichia coli* K1 into human brain microvascular endothelial cells. *J. Biol. Chem.* 280:1360-1368.

Kuwahara,I., Lillehoj,E.P., Lu,W., Singh,I.S., Isohama,Y., Miyata,T., and Kim,K.C. 2006. Neutrophil elastase induces IL-8 gene transcription and protein release through p38/NF-{kappa}B activation via EGFR transactivation in a lung epithelial cell line. *Am. J. Physiol Lung Cell Mol. Physiol* 291:L407-L416.

Kyriakis,J.M., and Avruch,J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* 81:807-869.

Lebowitz,P.F., Davide,J.P., and Prendergast,G.C. 1995. Evidence that farnesyltransferase inhibitors supress ras transformation by interfering with rho activity. *Mol. Cell. Biol.* 15:6613-6622.

Lemonnier,M., Landraud,L., and Lemichez,E. 2007. Rho GTPase-activating bacterial toxins: from bacterial virulence regulation to eukaryotic cell biology. *FEMS Microbiol. Rev.* 31:515-534.

Lerm,M., Pop,M., Fritz,G., Aktories,K., and Schmidt,G. 2002. Proteasomal degradation of cytotoxic necrotizing factor 1- activated Rac. *Infection and Immunity* 70:4053-4058.

Lerm,M., Schmidt,G., Goehring,U.-M., Schirmer,J., and Aktories,K. 1999. Identification of the region of Rho involved in substrate recognition by *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1)*. *J. Biol. Chem.* 274:28999-29004.

Lockman,H.A., Gillespie,R.A., Baker,B.D., and Shakhnovich,E. 2002. *Yersinia pseudotuberculosis* produces a cytotoxic necrotizing factor. *Infect. Immun.* 70:2708-2714.

Maddox,A.S., and Oegema,K. 2003. Closing the GAP: a role for a RhoA GAP in cytokinesis. *Mol. Cell* 11:846-848.

Malorni,W., and Fiorentini,C. 2006. Is the Rac GTPase-activating toxin CNF1 a smart hijacker of host cell fate? *FASEB J.* 20:606-609.

Mazieres,J., Tillement,V., Allal,C., Clanet,C., Bobin,L., Chen,Z., Sebti,S.M., Favre,G., and Pradines,A. 2005. Geranylgeranylated, but not farnesylated, RhoB suppresses Ras transformation of NIH-3T3 cells. *Exp. Cell Res.* 304:354-364.

Michaelson,D., Silletti,J., Murphy,G., D'Eustachio,P., Rush,M., and Philips,M.R. 2001. Differential Localization of Rho GTPases in Live Cells. Regulation by hypervariable regions and rho gdi binding. *J. Cell Biol.* 152:111-126.

Mikule,K., Delaval,B., Kaldis,P., Jurcyzk,A., Hergert,P., and Doxsey,S. 2007. Loss of centrosome integrity induces p38-p53-p21-dependent G1-S arrest. *Nat. Cell Biol.* 9:160-170.

Milia,J., Teyssier,F., Dalenc,F., Ader,I., Delmas,C., Pradines,A., Lajoie-Mazenc,I., Baron,R., Bonnet,J., Cohen-Jonathan,E., Favre,G., and Toulas,C. 2005. Farnesylated RhoB inhibits radiation-induced mitotic cell death and controls radiation-induced centrosome overduplication. *Cell Death. Differ.* 12:492-501.

Minoshima,Y., Kawashima,T., Hirose,K., Tonozuka,Y., Kawajiri,A., Bao,Y.C., Deng,X., Tatsuka,M., Narumiya,S., May,W.S., Jr., Nosaka,T., Semba,K., Inoue,T., Satoh,T., Inagaki,M., and Kitamura,T. 2003. Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. *Dev. Cell* 4:549-560.

Miraglia,A.G., Travaglione,S., Meschini,S., Falzano,L., Matarrese,P., Quaranta,M.G., Viora,M., Fiorentini,C., and Fabbri,A. 2007. Cytotoxic necrotizing factor 1 prevents apoptosis via the Akt/IkappaB kinase pathway: role of nuclear factor-kappaB and Bcl-2. *Mol. Biol. Cell* 18:2735-2744.

Munro,P., Flatau,G., Doye,A., Boyer,L., Oregioni,O., Mege,J.L., Landraud,L., and Lemichez,E. 2004. Activation and proteasomal degradation of rho GTPases by cytotoxic necrotizing factor-1 elicit a controlled inflammatory response. *J. Biol. Chem.* 279:35849-35857.

Nobes,C.D., and Hall,A. 1995. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81:53-62.

Nougayrede,J.P., Taieb,F., De,R.J., and Oswald,E. 2005. Cyclomodulins: bacterial effectors that modulate the eukaryotic cell cycle. *Trends Microbiol.* 13:103-110.

Olofsson,B. 1999. Rho Guanine Dissociation Inhibitors: Pivotal Molecules in Cellular Signalling. *Cell. Signal.* 11:545-554.

Oswald,E., de Rycke,J., Guillot,J.F., and Boivin,R. 1989. Cytotoxic effect of multinucleation in HeLa cell cultures associated with the presence of Vir plasmid in *Escherichia coli* strains. *FEMS Microbiol. Lett.* 58:95-100.

Oswald,E., Nougayrede,J.P., Taieb,F., and Sugai,M. 2005. Bacterial toxins that modulate host cell-cycle progression. *Curr. Opin. Microbiol.* 8:83-91.

Page,K., Li,J., and Hershenson,M.B. 2001. p38 MAP kinase negatively regulates cyclin D1 expression in airway smooth muscle cells. *Am. J. Physiol Lung Cell Mol. Physiol* 280:L955-L964.

Pei,S., Doye,A., and Boquet,P. 2001. Mutation of specific acidic residues of the CNF1 T domain into lysine alters cell membrane translocation of the toxin. *Mol. Microbiol.* 41:1237-1247.

Piel,M., Nordberg,J., Euteneuer,U., and Bornens,M. 2001. Centrosome-dependent exit of cytokinesis in animal cells. *Science* 291:1550-1553.

Pihan,G.A., Wallace,J., Zhou,Y., and Doxsey,S.J. 2003. Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res.* 63:1398-1404.

Popoff,M.R., Chaves,O.E., Lemichez,E., Von Eichel-Streiber,C., Thelestam,M., Chardin,P., Cussac,D., Chavrier,P., Flatau,G., Giry,M., Gunzburg,J., and Boquet,P. 1996. Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. *J. Biol. Chem.* 271:10217-10224.

Raingeaud,J., Whitmarsh,A.J., Barrett,T., Derijard,B., and Davis,R.J. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell Biol.* 16:1247-1255.

Ridley,A.J. 2001. Rho GTPases and cell migration. *J. Cell Sci.* 114:2713-2722.

Ridley,A.J., and Hall,A. 1994. Signal transduction pathways regulating Rho-mediated stress fibre formation: Requirement for a tyrosine kinase. *EMBO J.* 13:2600-2610.

Ridley,A.J., Paterson,H.F., Johnston,C.L., Diekmann,D., and Hall,A. 1992. The small GTP-binding protein rac regulates growth factor- induced membrane ruffling. *Cell* 70:401-410.

Rieder,C.L., and Maiato,H. 2004. Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev. Cell* 7:637-651.

Rippere-Lampe,K.E., O'Brien,A.D., Conran,R., and Lockman,H.A. 2001. Mutation of the gene encoding cytotoxic necrotizing factor type 1 (cnf(1)) attenuates the virulence of uropathogenic *Escherichia coli*. *Infect. Immun.* 69:3954-3964.

Rodriguez,P.L., Sahay,S., Olabisi,O.O., and Whitehead,I.P. 2007. ROCK I-mediated activation of NF-kappaB by RhoB. *Cell Signal.* 19:2361-2369.

Rojas,R., Ruiz,W.G., Wang,E., Kinlough,C.L., Poland,P.A., Hughey,R.P., Dunn,K.W., and Apodaca,G. 2004. RhoB-dependent modulation of early endocytic traffic in Madin-Darby canine kidney cells. *J. Biol. Chem.*

Sander,E.E., van Delft,S., ten Klooster,J.P., Reid,T., Van der Kammen,R.A., Michiels,F., and Collard,J.G. 1998. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol.* 143:1385-1398.

Sato,N., Mizumoto,K., Nakamura,M., and Tanaka,M. 2000. Radiation-induced centrosome overduplication and multiple mitotic spindles in human tumor cells. *Exp. Cell Res.* 255:321-326.

Schieven,G.L. 2005. The biology of p38 kinase: a central role in inflammation. *Curr Top. Med Chem.* 5:921-928.

Schmidt,G., Sehr,P., Wilm,M., Selzer,J., Mann,M., and Aktories,K. 1997. Gln63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor 1. *Nature* 387:725-729.

Schmidt,G., Selzer,J., Lerm,M., and Aktories,K. 1998. The Rho-deamidating cytotoxic-necrotizing factor CNF1 from *Escherichia coli* possesses transglutaminase activity - cysteine-866 and histidine-881 are essential for enzyme activity . *J. Biol. Chem.* 273:13669-13674.

Sehr,P., Joseph,G., Genth,H., Just,I., Pick,E., and Aktories,K. 1998. Glucosylation and ADP-ribosylation of Rho proteins - Effects on nucleotide binding, GTPase activity, and effector-coupling. *Biochemistry* 37:5296-5304.

Sekine,A., Fujiwara,M., and Narumiya,S. 1989. Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. *J. Biol. Chem.* 264:8602-8605.

Seo,M., Cho,C.H., Lee,Y.I., Shin,E.Y., Park,D., Bae,C.D., Lee,J.W., Lee,E.S., and Juhnn,Y.S. 2004. Cdc42-dependent mediation of UV-induced p38 activation by G protein betagamma subunits. *J. Biol. Chem.* 279:17366-17375.

Stamatakis,K., Cernuda-Morollón,E., Hernández-Perera,O., and Pérez-Sala,D. 2002. Isoprenylation of RhoB is necessary for its degradation. *J. Biol. Chem.* 277:43396.

Storchova,Z., and Pellman,D. 2004. From polyploidy to aneuploidy, genome instability and cancer. *Nat. Rev. Mol. Cell Biol.* 5:45-54.

Wadsworth,P. 2005. Cytokinesis: Rho marks the spot. *Curr. Biol.* 15:R871-R874.

Wennerberg,K., and Der,C.J. 2004. Rho-family GTPases: it´s not only Rac and Rho (and I like it). *J. Cell Sci.* 117:1301-1312.

Wennerberg,K., Rossman,K.L., and Der,C.J. 2005. The Ras superfamily at a glance. *J. Cell Sci.* 118:843-846.

Wittinghofer,A., and Pai,E.F. 1991. The structure of *ras* protein: a model for a universial molecular switch. *Trends Biochem Sci.* 16:382-387.

Yin,X., Giap,C., Lazo,J.S., and Prochownik,E.V. 2003. Low molecular weight inhibitors of Myc-Max interaction and function. *Oncogene* 22:6151-6159.

Zalcman,G., Closson,V., Linarès-Cruz,G., Lerebours,F., Honoré,N., Tavitian,A., and Olofsson,B. 1995. Regulation of Ras-related RhoB protein expression during the cell cycle. *Oncogene* 10:1935-1945.

9 Curriculum vitae

persönliche Angaben

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Schulbildung

1987-1991: Grundschule Bissendorf

1991-1993: Orientierungsstufe Bissendorf

1993-2000: Gymnasium Mellendorf

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10/2000: Beginn des Studiums der Biochemie (Diplom) an der Leibniz Universität Hannover

12/2002: Vordiplom an der Leibniz Universität Hannover Biochemie, Gesamtnote „gut“

01/2006: Diplom in Biochemie an der Leibniz Universität Hannover, Gesamtnote „sehr gut“

Titel der Diplomarbeit: „Unterschiedliche Rho-GTPasen bestimmen den zytopathischen und den zytotoxischen Effekt clostridialer glucosylierender Toxine “, angefertigt unter Anleitung von Prof. I. Just / PD Dr. H. Genth in der Abteilung für Toxikologie der Medizinischen Hochschule Hannover

Seit 03/2006: Doktorarbeit zum Thema „Hochregulation des „immediate early gene“ Produkts RhoB durch den zytotoxisch nekrotisierenden Faktor 1 aus *E. coli*“ unter Anleitung von Prof. I. Just / PD Dr. H. Genth in der Abteilung für Toxikologie der Medizinischen Hochschule Hannover

Wissenschaftliche Publikationen

S.C. Dreger, H. Genth, I. Just, J. Huelsenbeck, G. Fritz, G. Schmidt: Up-regulation of the immediate early gene product RhoB by *Escherichia coli* cytotoxic necrotizing factor 1. Manuscript in preparation.

S.C. Dreger, J. Huelsenbeck, R. Gerhard, F. Hofmann, I. Just, H. Genth: Killing of rat basophilic leukemia cells by Lethal Toxin from *Clostridium sordellii*: Requirement of (H/K/N)Ras glucosylation. Biochemistry. Returned for modification.

H.Genth, **S.C. Dreger**, J. Huelsenbeck, I. Just: *Clostridium difficile* toxins: More than mere inhibitors of Rho proteins. Int J Biochem Cell Biol. Review 2008 Jan 5;40(4):592-597.

J. Huelsenbeck, **S.C. Dreger**, R. Gerhard, G. Fritz, I. Just, H. Genth: Up-regulation of the immediate early gene product RhoB by exoenzyme C3 and toxin B from *Clostridium difficile*. Biochemistry 2007 Apr 24;46(16):4923-31.

J. Huelsenbeck, **S. Dreger**, R. Gerhard, H. Barth, I. Just, H. Genth: Difference in the cytotoxic effects of toxin B from *Clostridium difficile* strain VPI10463 and toxin B from variant *Clostridium difficile* strain 1470. Infect Immun. 2007 Feb;75(2):801-9.

Kurzartikel (Meeting Abstracts)

Vorträge

Potsdam (Germany), 22nd – 26th June 2008, FEBS/ECF Workshop: Mechanics and Dynamics of the Cytokeleton.
S.C. Dreger, M. May, G. Schmidt, I. Just, H. Genth.
Title: Inhibition of cytokinesis by bacterial pathogenicity factors affecting the activity of Rho proteins

Mainz (Germany), 11th – 13th March 2008, 49th Spring Meeting of the German Society of Pharmacology and Toxicology.
S.C. Dreger, J. Huelsenbeck, G. Fritz, I. Just, G. Schmidt, H. Genth.
Title: Up-regulation of the immediate-early gene product RhoB by the cytotoxic necrotizing factor 1 from *Escherichia coli*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 377: p. 27, 96, Suppl. 1 MAR 2008

Mainz (Germany), 11th – 13th March 2008, 49th Spring Meeting of the German Society of Pharmacology and Toxicology.
J. Huelsenbeck, **S.C. Dreger**, F. Schulz, M. Gaestel, I. Just, G. Fritz, H. Genth.
Title: Upregulation of the immediate-early gene product RhoB by the Ras-glucosylating *Clostridium sordellii* lethal toxin. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 377: p. 27, 97, Suppl. 1 MAR 2008

Freiburg (Germany), 29th February –2nd March 2008, 2nd Schauinsland-Meeting, DFG Priority Program SPP 1150: Signal Pathways to the Cytoskeleton and Bacterial Pathogenesis
S.C. Dreger
Title: CNF1-induced up-regulation of RhoB

Mainz (Germany), 13th – 15th March 2007, 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
S. Dreger, J. Isermann, J. Huelsenbeck, F. Hofmann, I. Just, H. Genth.
Title: Inactivation of H-/K-/N-Ras is the basis of the cytotoxic effect of *Clostridium sordellii* lethal toxin. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 36, 140, Suppl. 1 MAR 2007

Mainz (Germany), 13th – 15th March 2007, 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
J. Huelsenbeck, **S. Dreger**, F. Hofmann, I. Just, H. Genth.
Title: Different cytotoxic effects of reference toxin B and variant toxin B from *Clostridium difficile*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 36, 141, Suppl. 1 MAR 2007

Posterpräsentationen

Mainz (Germany), 11th – 13th March 2008, 49th Spring Meeting of the German Society of Pharmacology and Toxicology.
B. Hirsch**, S.C. Dreger**, J. Huelsenbeck, I. Just, G. Schmidt, H. Genth.
Effect of the cytotoxic necrotizing factors from *Escherichia coli* and *Yersinia pseudotuberculosis* on cell division and cell viability. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 377: p. 29, 109, Suppl. 1 MAR 2008

Mainz (Germany), 11th – 13th March 2008, 49th Spring Meeting of the German Society of Pharmacology and Toxicology.
I. Bock**, S.C. Dreger**, G. Schmidt, H. Genth.
Formation of multi-nuclei by the cytotoxic necrotizing factor 1 from *Escherichia coli*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 377: p. 29, 110, Suppl. 1 MAR 2008

Maribor (Slovenia), 6th – 9th June 2007, 2nd International Clostridium difficile Symposium.
S. Dreger, M. Isermann, J. Huelsenbeck, F. Hofmann, R. Gerhard, J. Just, H. Genth. Difference in the Protein Substrate Specificity of *Clostridium difficile* Toxin B from Serotype F Strain 1470 and *Clostridium sordellii* Lethal Toxin.

Maribor (Slovenia), 6th – 9th June 2007, 2nd International Clostridium difficile Symposium.
J. Huelsenbeck, **S. Dreger**, R. Gerhard, J. Just, H. Genth.
Active RhoB is Required for the Cytotoxic Effect of Clostridium difficile Toxin B.

Mainz (Germany), 13th – 15th March 2007, 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
H. J. Meyer, **S. Dreger**, G. Fritz, K. Aktories, G. Schmidt, H. Genth.
Up-regulation of RhoB by the Cytotoxic Necrotizing Factor 1 from *Escherichia coli*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 38, 151, Suppl. 1 MAR 2007

Nottingham (United Kingdom), 21th – 25th June 2006, 5th International Meeting on the Molecular Biology and Pathogenesis of the Clostridia.
H. Genth, **S. Dreger**, J. Huelsenbeck, I. Just.
Distinct dynamics of the cytopathic and cytotoxic effect induced by *Clostridium difficile* toxin B.

Mainz (Germany), 4th – 6th April 2006, 47th Spring Meeting of the German Society of Pharmacology and Toxicology.
S. Dreger, J. Huelsenbeck, I. Just, H. Genth.
The Cytopathic Activity of Clostridium difficile Toxin B Includes Up-Regulation of the Immediate Early-Gene RhoB and Caspase-3-Activation. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 186, Suppl. 1 MAR 2006

Berlin (Germany), 13th – 15th October 2005, 9th International Dahlem Symosium on Cellular Signal Recognition and Transduction.
H. Genth, J. Huelsenbeck, **S. Dreger**, F. Hofmann, R. Gerhard.
Clostridial factors associated to *Clostridium difficile* toxin A and toxin B responsible for their cytotoxic activity.

Mainz (Germany), 15th – 17th March 2005, 46th Spring Meeting of the German Society of Pharmacology and Toxicology.
J. Huelsenbeck, **S. Dreger**, B. Hartmann, R. Gerhard, H. Genth.
Up-regulation of RhoB protein by glucosylating toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R118-R118 493 Suppl. 1 FEB 2005