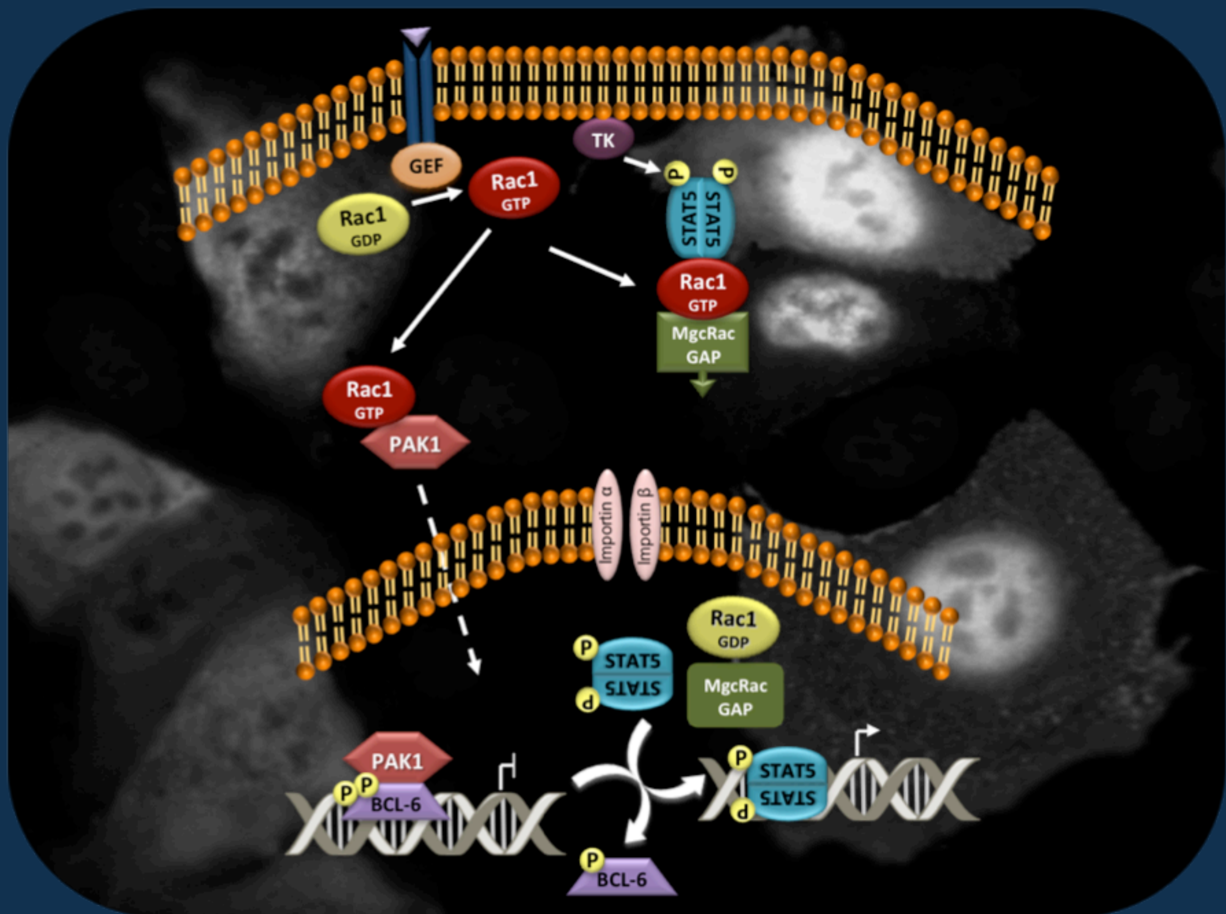
The role of Rac1-modulated gene transcription in tumorigenesis

Patrícia Alexandra Sousa Barros



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
October, 2012

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Knowledge Creation

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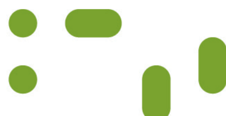

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WORK PERFORMED AT

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SUPERVISORS

Doutor Peter Jordan

Head of Oncobiology laboratory (INSA)

Prof. Júlia Costa (Institutional Supervisor)

Head of Glycobiology laboratory (ITQB-UNL)

The opinions expressed in this thesis are the exclusive responsibility of the author.

A todos aqueles que não deixaram de acreditar

À luzinha que me acompanha…

“The merit of all things lies in their difficulty.”

Alexandre Dumas

Summary

Gene expression regulation is a dynamic and multi-step process, in which transcription plays a major role. Transcription initiation depends on binding of transcription regulators to DNA elements located in promoter or enhancer regions, a process often controlled by signalling pathways. One such pathway is regulated by Rac1, a member of the Rho family of small GTPases involved in cell proliferation, adhesion and migration. In this work, novel links between Rac1 signalling and transcriptional regulation in colorectal tumour cells are described. First, it is shown that Rac1 activation leads to PAK1-mediated phosphorylation of the transcriptional repressor BCL-6 in colorectal cancer cells, inactivating its repressor function. In the presence of active Rac1, BCL-6 redistribution within the nucleus, a reduction in its affinity to chromatin and increased expression of the endogenous target genes *NFKB1* and *CD44*, and of a BCL-6-controlled luciferase reporter construct were observed. Next, it was found that Rac1 signalling promotes gene transcription by inducing a transcriptional switch from the repressor BCL-6 to the activator STAT5A at the promoter of certain target genes. Using chromatin immunoprecipitation, it is demonstrated in different colorectal cell lines that active Rac1 promotes release of BCL-6 with concomitant nuclear translocation and binding of STAT5A at the same promoter site. Three endogenous cell-cycle-related genes (*CCND2*, *CDKN2B*, *SUMO1*) were identified to be inversely regulated by BCL-6 and STAT5A and shown to respond to Rac1 signalling with promoter occupancy switches that correlate directly with changes in their expression levels.

This work provides new mechanistic insights into how Rac1 signalling modulates gene transcription through the switching between

transcription factors and contributes to uncovering the implications of deregulated Rac1 activity in cancer.

Sumário

A expressão génica é um processo essencial à vida, determinando a forma como todas as funções biológicas são executadas, pelo que qualquer alteração à forma como os genes são expressos tem implicações para a célula e encontra-se muitas vezes ligada à ocorrência de doenças (Maston *et al*., 2006), como por exemplo o cancro. A expressão génica é um processo que se desenrola em vária etapas, começando na transcrição do gene, passando pelo processamento do mRNA (por exemplo, no *splicing* e/ou na poliadenilação), pelo transporte do mRNA para o citoplasma, pela sua tradução e mesmo pelas modificações pós-transducionais que conferem à proteína resultante a sua forma e funcionalidades finais (Singer and Green, 1997; Maston *et al*., 2006). Embora a regulação apertada de cada um destes passos, bem como a sua sincronia, seja fundamental para o funcionamento correcto da célula, muitos autores defendem que o ponto crítico na regulação da expressão génica se encontra no início da transcrição (Maston *et al*., 2006; Hager *et al*., 2009).

O início da transcrição depende da ligação de factores reguladores, activadores ou repressores, a sequências de DNA particulares localizadas no promotor do gene ou em regiões vizinhas designadas vulgarmente por *enhancer regions*. Por sua vez, a activação destes factores de transcrição é, em grande parte, controlada por vias de sinalização celular que respondem a estímulos intracelulares e extracelulares de ordem diversa (Orphanides and Reinberg, 2002; Venters and Pugh, 2009). Algumas dessa vias de sinalização são moduladas por Rac1, um membro da família *Rho* de GTPases de baixa massa molecular, que está envolvido na regulação de diversos processos celulares, incluíndo a proliferação, sobrevivência, adesão e migração

celulares. A desregulação da expressão e/ou actividade de Rac1 pode resultar numa sinalização celular anormal e tem sido frequentemente associado a condições patológicas diversas (Bosco *et al*., 2009).

Neste trabalho são descritos novos pontos de ligação entre a sinalização de Rac1 e a regulação transcricional em linhas celulares de cancro colorectal.

Em primeiro lugar, demonstrou-se que o repressor transcricional BCL-6 é regulado negativamente pela sinalização de Rac1. Para tal, utilizou-se como repórter uma construção do gene da luciferase sob o controlo dum promotor artificial responsivo a BCL-6. Verificou-se que em células colorectais DLD-1 a actividade deste repórter de luciferase aumentava na presença de Rac1 constitutivamente activo e que, pelo contrário, era reprimida na presença de um inibidor da activação endógena de Rac1 (NSC23766). Adicionalmente, verificou-se que a expressão de genes endógenos regulados por BCL-6, como *NFKB1* e *CD44*, aumentava ou diminuía de acordo com a activação de Rac1. Em seguida, verificou-se que a activação de Rac1 também afectava a distribuição sub-nuclear de BCL-6, passando de uma localização característica em *foci* nucleares para uma distribuição difusa e mais homogénea pelo nucleoplasma. Este resultado foi corroborado por ensaios de fraccionamento celular que mostraram que, na presença de Rac1, o factor BCL-6 perde a sua afinidade para o DNA, ocorrendo uma transição de BCL-6 da fracção nuclear insolúvel, ligada à cromatina, para uma fracção nuclear solúvel. Seguidamente, analisou-se qual o mecanismo que podia estar subjacente à inactivação de BCL-6 por parte de Rac1. Após se ter verificado que não se tratava de uma interacção directa entre as duas proteínas, avaliou-se a participação de outras proteínas, nomeadamente, outras Rho GTPases (RhoA, Cdc42, Rac1b) e

efectores de Rac1 (JNK, PAK), através de ensaios de luciferase. Constatou-se que apenas a cinase PAK tinha um efeito semelhante ao induzido por Rac1 e que a sua activação era necessária para produzir as alterações observadas na actividade de BCL-6. Por último, demonstrou-se que a isoforma alfa da cinase PAK (PAK1) fosforila directamente BCL-6 *in vitro* e *in vivo*, identificando-se esta cinase como o mediador da regulação negativa de Rac1 sobre BCL-6 em células colorectais. Desta forma, caracterizou-se uma nova via de sinalização – Rac1/PAK1/BCL-6 – que relaciona Rac1 com a regulação transcricional.

Sabia-se de estudos anteriores que, em células epiteliais, a sinalização de Rac1 é necessária à activação de outros factores de transcrição, nomeadamente do activador trancricional STAT5. (Kawashima *et al*., 2006), Curiosamente, as sequências de DNA reconhecidas por BCL-6 são bastante semelhantes às sequências de ligação de STAT5 (Dent *et al*., 1997; Horvath, 2000), pelo que colocámos a hipótese de BCL-6 e STAT5 poderem desempenhar papéis opostos na regulação transcricional de alguns genes alvo e a sinalização de Rac1 poder estar a coordenar a troca (*switch*) entre estes dois factores a nível dos promotores desses mesmos genes. Assim, utilizou-se a técnica de imunoprecipitação da cromatina (ChIP) para avaliar a ligação de STAT5 e BCL-6 ao promotor da construção repórter acima referida e mostrou-se que, de facto, a activação de Rac1 promove a remoção de BCL-6 do promotor, ao mesmo tempo, que aumenta a ligação de STAT5. Paralelamente, verificou-se que, em células colorectais, a activação de Rac1 estimula a translocação nuclear de STAT5 e a sua ligação à cromatina. Para entender a relevância fisiológica deste *switch* entre BCL-6 e STAT5 caracterizaram-se os níveis de activação endógenos de Rac1, PAK1, STAT5 e BCL-6 em várias linhas celulares colorectais, com

vista a eleger os modelos celulares mais adequados para a identificação de genes alvo da nova via de sinalização Rac1/PAK1/BCL-6/STAT5. Tendo-se constatado que em duas dessas linhas (DLD-1 e HT29) o estado de activação da via de sinalização Rac1/PAK1 se correlacionava com o nível de fosforilação de BCL-6 e STAT5, procedeu-se ao silenciamento, por interferência de RNA (RNAi), da expressão endógena de BCL-6 ou STAT5 e analisou-se, por PCR em tempo-real, o efeito da supressão destes factores na expressão de 84 genes, relacionados com o ciclo celular, presentes num *array* comercial. Desta forma, identificaram-se três genes (*CCND2*, *CDKN2B*, *SUMO1*) inversamente regulados por BCL-6 e STAT5 e confirmou-se por ChIP que ambos os factores de transcrição se ligam aos promotores destes genes. Além disso, demonstrou-se que estes genes respondem à sinalização de Rac1 e PAK1 com um *switch* entre STAT5/BCL-6 na ocupação do promotor e que tal se correlaciona directamente com as alterações observadas ao nível da sua expressão.

Em conclusão, os resultados expostos nesta tese proporcionam um melhor entendimento dos mecanismos moleculares através dos quais a sinalização via Rac1 consegue modular a expressão génica, contribuindo com novos dados para o esclarecimento das implicações da desregulação da actividade de Rac1 no cancro.

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List of Abbreviations

aa	Amino acid
AID	Autoinhibitory domain
AP-1	Activator protein 1
Asn (N)	Asparagine residue
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BAD	BCL-2 antagonist of cell death
bp	Base pair
BCL	B-cell lymphoma
BRE	TFIIB-Recognition Element
bZIP	Basic leucine zipper
ca	Constitutively active
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CKI	CDK inhibitor
CRIB	Cdc42/Rac interactive binding domain
C-terminal	Carboxyl-terminal
Ctrl	Control
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DCE	Downstream core element
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco’s minimal essential medium
DNA	Deoxyribonucleic acid
DPE	Downstream promoter element
DPI	NADPH-oxidase inhibitor diphenyleneiodonium chloride
DTT	Dithiothreitol
E (Glu)	Glutamic acid residue
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELK	ETS-like transcription factor
Epub	Electronic publishing
ERK	Extracellular signal-regulated kinase
F*	Forward
FBS	Foetal bovine serum
GAP	GTPase-activating protein
GC	Germinal centre
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate

GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GH	Growth hormone
Gln (Q)	Glutamine residue
Gly (G)	Glycine residue
GPCR	G protein-coupled receptor
GTF	General transcription factor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HA	Epitope tag derived from the human influenza hemagglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HLH	Helix-loop-helix
HNSCC	Head and neck squamous cell carcinoma
Ig	Immunoglobulin
Inr	Initiator element
IP	Immunoprecipitation
IPA-3	PAK inhibitor III
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K (Lys)	Lysine residue
kb	Kilobase
kDa	Kilodalton
kd	Kinase dead
LCR	Locus control regions
Leu (L)	Leucine residue
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
mRNA	Messenger RNA
MTE	Motif ten element
MUTYH	MutY homolog
MW	Molecular weight marker
Myc	Epitope tag derived from the *MYC* gene product
NADPH	Nicotinamide adenine dinucleotide phosphatase
NES	Nuclear export sequence
NIH	National Institutes of Health
NF-κB	Nuclear factor kappa-light-chain-gene-enhancer of activated B cells
NLS	Nuclear localization sequence
NP-40	Nonidet P-40
Ns	Non-specific

NS	Non-soluble
NSC23766	Rac1 inhibitor
nt	Nucleotide
N-terminal	Amino terminal
PAK	p21-activated protein kinase
PAGE	Polyacrylamide gel electrophoresis
PBD	p21-binding domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PIC	Preinitiation complex
Pol	RNA polymerase
POU	Pit-Oct-Unc
POZ	Poxvirus and zinc finger
PRL	Prolactin
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real time PCR
R (Arg)	Arginine residue
R*	Reverse
Rac1	Ras-related C3 botulinum toxin substrate 1
Ras	Rat sarcoma
Rb	Retinoblastoma protein
REM	Rho effector homology domain
Rho	Ras homologous
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK	Rho-associated coiled-coil-containing protein kinase
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
RTKN	Rhotekin
RT-PCR	Reverse transcription-PCR
S	Soluble
SDS	Sodium dodecyl sulfate
SH	Src-homology
siRNA	Small interfering RNA
snRNA	Small nuclear RNA genes
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
SV40	Simian virus 40
Ta	Annealing temperature

TAD	Transactivation domain
TAF	TBP-associated factor
TBS	Tris-buffered saline
TBP	TATA-box-binding protein
Thr (T)	Threonine residue
TK	Tyrosine kinase
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
TSS	Transcription start site
Tyr	Tyrosine residue
Val (V)	Valine residue
WASP	Wiscott-Aldrich syndrome protein
WB	Western blot
wt	Wild-type
α-	Anti
μCi	MicroCurie

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Chapter 1

General Introduction

All living cells need to constantly assess the surrounding environment to communicate and collect information about their state and requirements in order to make accurate decisions. Thus, the ability to convert extracellular signals into specific internal cellular responses is fundamental for cell survival and proper development (Karin, 1992; Downward, 2001).

Signal transduction is the field that studies the mechanisms by which biological information is transferred, from the level of individual cells to the whole organism. In general, these include a multitude of sequential biochemical reactions and interacting molecular cascades – signalling transduction pathway – that are initiated with the activation of a specific cell-surface receptor by an extracellular stimulus. The signal is then conducted into the cell, from the receptor to intermediate molecules through changes in their conformation and activity, until the final target in the cytoplasm or in the nucleus is reached and an outcome for the cell is produced (Persidis, 1998; Downward, 2001).

Due to the diversity and complexity of signalling pathways, their influence covers almost every aspect of cell life, including cell division, apoptosis, cytoskeletal organization, metabolism and gene expression (Pawson and Nash, 2000). The modulation of gene expression is an important consequence since it can generate long-lasting responses that, in turn, will affect several biological functions (Karin, 1992).

The relationship between signalling dysfunctions and several diseases, such as cancer, does not surprise and is widely recognized, increasing the interest and possible impact of dissecting and understanding these cellular networks (Karin, 1992; Persidis, 1998; Downward, 2001). A good example of this straight connection is the Ras superfamily of small guanosine triphosphatases (GTPases). On one hand,

these proteins are key elements of several signalling pathways and on the other hand play crucial roles in human oncogenesis, in particularly their founding members, the Ras (Rat sarcoma) proteins (Hernández-Alcoceba *et al*., 2000; Wennerberg *et al*., 2005).

1.1. Rho GTPases

Rho (Ras homologous) GTPases form a subgroup of the Ras superfamily of small GTPases (Wennerberg *et al*., 2005), highly conserved amongst all eukaryotic organisms, from yeasts, to plants, up to mammals (Wherlock and Mellor, 2002; Boureux *et al*., 2007). Similar to Ras, they are intracellular signalling molecules that can respond to diverse stimuli and regulate a wide variety of cellular processes including cell size, proliferation, survival, cell adhesion, cell polarity, membrane trafficking, cytoarchitecture and transcriptional activation (Van Aelst and D'Souza-Schorey, 1997; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005).

The Rho-family is composed by monomeric G proteins of low molecular weight (~20-30 kDa) (Van Aelst and D'Souza-Schorey, 1997; Cotteret and Chernoff, 2002) with the capacity to bind with high affinity to the guanine nucleotides, guanosine diphosphate (GDP) or guanosine triphosphate (GTP) and to hydrolyze GTP (GTPase activity) (Vetter and Wittinghofer, 2001). This attribute is due to a conserved element within Ras-like proteins, the GTPase domain, that comprises a set of G-boxes with consensus amino acid sequences for GTP binding and hydrolysis (Vetter and Wittinghofer, 2001; Wennerberg *et al*., 2005). The presence of a Rho-specific insert in the GTPase domain, involved in the recognition of effector proteins and regulators, distinguishes Rho-family members (Wennerberg and Der, 2004; Boureux *et al*., 2007). Another particular

feature of this family is that most members undergo post-translational modifications at their carboxyl (C-) terminus by the addition of prenyl groups (such as farnesyl or geranylgeranyl). These function as lipid anchors that facilitate association with membranes and thus subcellular localization, essential for Rho GTPases biological functions (Wennerberg and Der, 2004; Wennerberg *et al*., 2005; Bos *et al*., 2007).

1.1.1. Organization and regulation

Currently, after phylogenetic and evolutionary studies, the human Rho-family counts 22 members, 2 of which being variants of Rac1 and Cdc42 originated by alternative splicing, distributed into 8 subfamilies according to sequence similarity: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac1b [Rac1 splice variant], Rac2, Rac3, RhoG), Cdc42 (Cdc42, G25K [Cdc42 brain-specific C-terminal splice variant], TC10/RhoQ, TCL/RhoJ), RhoD/F (RhoD, Rif/RhoF), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoU/V (Wrch-1/RhoU, Chp/RhoV), RhoH and RhoBTB (RhoBTB1, RhoBTB2) (Fig. 1) (Aspenström *et al*., 2007; Boureux *et al*., 2007). Out of these subfamilies, Rnd, Wrch-1/Chp, RhoH and RhoBTB have particularities in terms of structure, function and regulation that make them atypical compared to the Rho, Rac (with exception of Rac1b), Cdc42 and RhoD/RhoF subfamilies that follow the classical activation cycle (see below) (Aspenström *et al*., 2007).

Like other small GTPases, Rho proteins function as molecular switches, cycling between an inactive (GDP-bound) and an active (GTP-bound) conformational state (Vetter and Wittinghofer, 2001; Etienne-Manneville and Hall, 2002). These conformational changes produce alterations in their binding affinity for downstream effectors or regulatory

proteins and are mainly localized in two short and flexible loop structures of the GTPase domain, designated as *Switch I* and *Switch II* (Vetter and Wittinghofer, 2001; Wennerberg *et al.*, 2005). Hence, the active conformation favours the interaction with target proteins, inducing downstream signalling events that cease with the hydrolysis of GTP and the return of the GTPase to its inactive state (Bishop and Hall, 2000; Hernández-Alcoceba *et al.*, 2000; Etienne-Manneville and Hall, 2002).

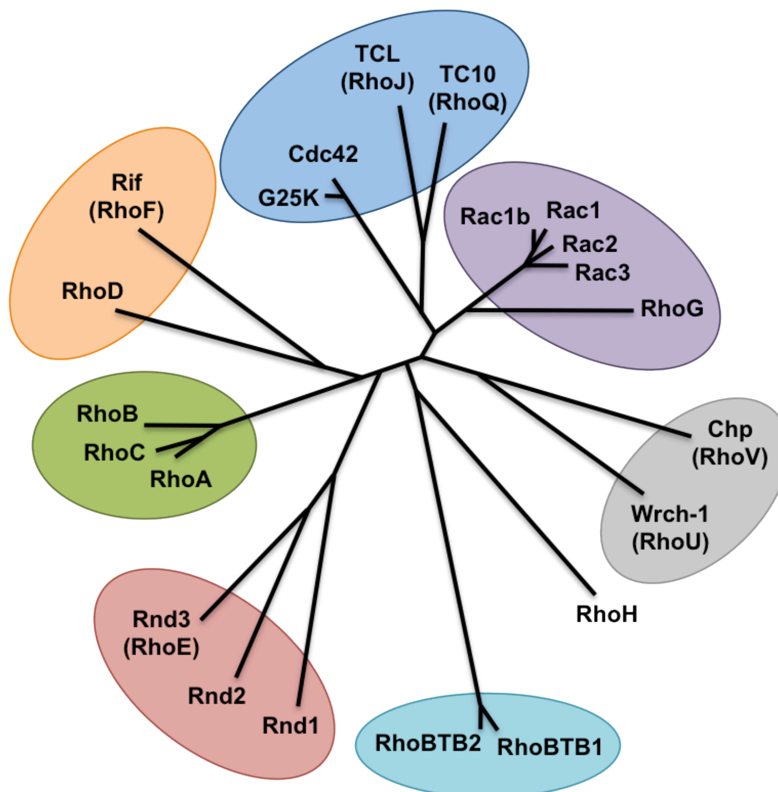

Figure 1: Phylogenetic tree representation of the Rho GTPases family. The 22 members are distributed into 8 groups – Rho, Rac, Cdc42, RhoD/F, Rnd, RhoU/V, RhoH and RhoBTB – according to sequence and phylogenetic similarity (adapted from Aspenström *et al.*, 2007).

Although GDP-GTP exchange reactions could occur spontaneously in the cell, the dissociation rate of bound GDP as well as the hydrolysis of GTP inherent to GTPases activity is extremely slow and poorly efficient, and therefore requires the specific regulatory proteins described in the following to catalyze the process. Additionally, these proteins participate in the spatial regulation of the activation/inactivation cycle (Takai *et al*., 2001; Vetter and Wittinghofer, 2001; Schmidt and Hall, 2002; Bos *et al*., 2007).

Guanine nucleotide exchange factors (GEFs) work as positive regulators of Rho GTPases activation. After a specific signal is relayed by cell receptors, GEFs are recruited to cellular membranes, where they bind small GTPases, enhance the release of bound GDP and promote its replacement by GTP, that is found in a much higher concentration in the cytosol than GDP. With the opposite role, GTPase-activating proteins (GAPs) bind to small GTPases and stimulate their intrinsic GTPase activity to hydrolyse the bound GTP and convert active Rho proteins into inactive GDP-bound forms (Fig. 2) (Van Aelst and D'Souza-Schorey, 1997; Kaibuchi *et al*., 1999; Schmidt and Hall, 2002; Bos *et al*., 2007). An additional level of regulation is provided through guanine nucleotide dissociation inhibitors (GDIs) that were initially described as simple inhibitors of Rho GTPases activity but whose function is now recognized to be much more complex (Dovas and Couchman, 2005). GDIs can inhibit Rho GTPase activation in several ways. First, by intervening at the GDP-GTP exchange step, preventing the dissociation of GDP and action of GEFs and therefore maintaining the protein in its inactive state. And second, by acting at the GTP hydrolytic step, blocking both the endogenous and GAP-catalyzed GTPase activity, impeding interactions with effectors. In addition, GDIs can modulate the distribution of Rho

GTPases between membranes and cytoplasm. This occurs by masking their prenyl modification and extracting inactive Rho proteins from cell membranes, producing soluble high-affinity complexes that keep them sequestered in the cytoplasm, away from their sites of activation at the membranes. In response to specific cues, the complex Rho GTPase-GDI is targeted to the plasma membrane where GDI is displaced and the GTPase can be activated again (reviewed in DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005; Garcia-Mata *et al*., 2011). In parallel with regulating Rho protein cycling, GDIs contribute also to the maintenance of a cytoplasmic pool of inactive Rho GTPases that, in response to a signal, can rapidly be translocated to a cell membrane to be activated (Fig. 2) (Van Aelst and D'Souza-Schorey, 1997; Kaibuchi *et al*., 1999; DerMardirossian and Bokoch, 2005; Garcia-Mata *et al*., 2011).

To date, a large number of Rho-GEFs (~70) and Rho-GAPs (~80) have been identified in the human genome and these have shown to be functionally redundant (Schmidt and Hall, 2002; Rossman *et al*., 2005; Bos *et al*., 2007; Heasman and Ridley, 2008). Indeed, multiple GEFs, GAPs and GDIs participate in the activation/inactivation cycle of the same Rho GTPase. This diversity of regulators and apparent redundancy pointed to cell-specific expression and specific receptor pathway-dependency (Scita *et al*., 2000; Ellenbroek and Collard, 2007). Thus, the activation state of Rho proteins is tightly regulated and occurs in a cell-type and pathway-dependent manner, depending upon the balance of the regulators (GEFs, GAPs and GDIs) at any given moment, and this determines its downstream signalling (Raptis *et al*., 2011).

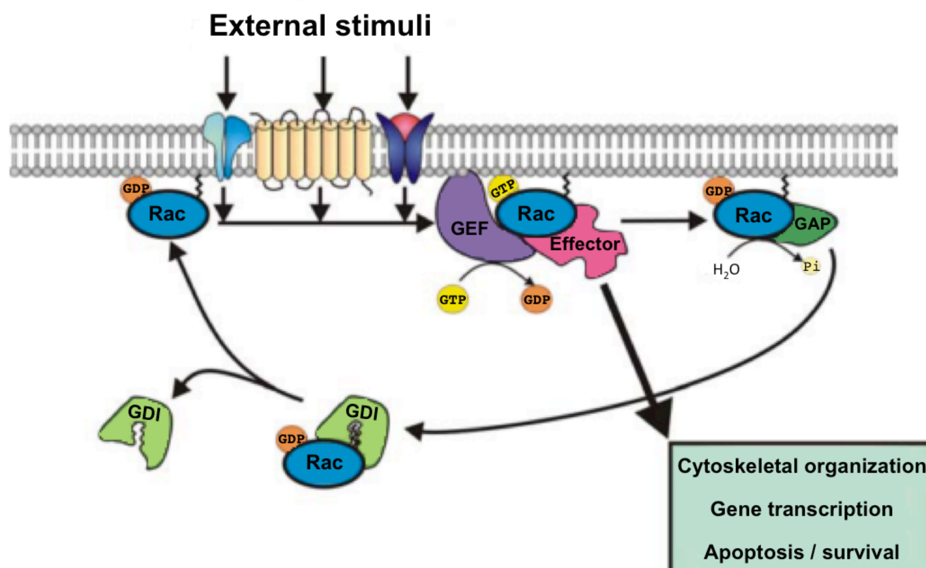

Figure 2: The cycle of activation/inactivation of Rho GTPases. Following a specific extracellular stimulus, guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, enabling the interaction of GTPases with specific effectors leading to cellular responses. In opposite, GTPase-activating proteins (GAPs) inactivate GTPases by stimulating their intrinsic GTPase activity. GDP-bound GTPases are maintained mainly cytoplasmic by guanine nucleotide dissociation inhibitors (GDIs) that masks the C-terminal tail required for plasma membrane localization. Upon dissociation of the GDI, GTPases translocate back to the plasma membrane, where they can be activated by GEFs (adapted from Ellenbroek and Collard, 2007).

1.1.2. Biological functions

Most of the functional information available on Rho-family proteins has come from studies on the best-characterized members RhoA, Rac1 and Cdc42 (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002). These were first described for their distinct effects on the actin cytoskeleton. Experiments with quiescent Swiss 3T3 fibroblasts showed that the activation of RhoA induced the formation of stress fibres, elongated actin bundles that transverse the cells and promote cell

attachment to the extracellular matrix through focal adhesions. In turn, active Rac1 promoted the assembly of a meshwork of actin filaments at the cell periphery to produce *lamellipodia* and membrane ruffles. The activation of Cdc42 produced actin-rich, finger-like cytoplasmic extensions called filopodia, which are probably involved in the recognition of the extracellular environment (Hall, 1998). These highly specific effects on the actin cytoskeleton were observed in many other cell types, including epithelial and endothelial cells, astrocytes and lymphocytes (Hall, 1998; Etienne-Manneville and Hall, 2002), pointing to a series of well-defined signal transduction pathways controlled by each GTPase, leading to both the formation and the organization of actin filaments (Jaffe and Hall, 2005).

The control of actin cytoskeleton rearrangements allows Rho proteins to influence various cytoskeleton-dependent processes, such as cell migration, cytokinesis, morphogenesis, cell adhesion and polarity, tissue architecture, phagocytosis and axon guidance (Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002). In addition to the strong role in actin dynamics regulation, Rho GTPases have been associated to a wide variety of biological functions (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005).

The diversity and complexity of Rho functional properties is consistent with the large number of target proteins with which they bind and interact, exerting their effects. Each Rho-family protein has binding affinity for multiple effectors, some expressed in specific cellular context, and some effectors are recognized by multiple family members (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002). Until now, over 70 effector proteins have been identified (Ellenbroek and Collard, 2007),

including protein kinases, lipid kinases, lipases, oxidases, phosphatases and scaffold proteins (Cotteret and Chernoff, 2002; Jaffe and Hall, 2005). Among these, protein kinases that act by phosphorylation of downstream target proteins, constitute an important group with some well-characterized elements, like Rho-associated coiled-coil-containing protein kinases (ROCKs or RHO kinases) that bind to active RhoA and p21-activated kinases (PAKs) that bind to active Rac1 and Cdc42 (Sahai and Marshall, 2002; Ellenbroek and Collard, 2007). Many of Rac1 and Cdc42 effectors (e.g. PAK, Wiskott-Aldrich-syndrome protein – WASP) contain a conserved GTPase-binding consensus site, the Cdc42/Rac interactive binding domain (CRIB), to which the GTP-bound forms of Rac1 and Cdc42 bind specifically and together with additional binding regions contribute to a productive interaction. A characteristic binding region called amino (N-) terminal Rho effector homology domain (REM) is also found in some RhoA effectors, like rhotekin (RTKN) (Bishop and Hall, 2000). The CRIB and REM binding domains derived from various downstream effectors have been exploited as very useful biochemical tools to study the activation of Rho GTPases (Aspenström *et al*., 2004).

Much of our insight into the biological activities of individual Rho GTPases has come from overexpression studies in cell lines with dominant-negative and constitutively active mutants. Dominant-negative mutants were created through the substitution of the amino acid (aa) Thr for Asn (T17N for Rac1 or Cdc42 and T19N for RhoA). This point mutation allows binding of GEFs but inhibits downstream interactions with effector proteins, generating a non-productive complex that competes with endogenous proteins for binding to GEFs. Amino acid substitutions of Gly to Val or Gln to Leu (G12V or Q61L for Rac1 or Cdc42 and G14V or Q63L for RhoA) produce constitutively active mutants, that signal

continuously to their effectors due to blockage of intrinsic and GAP-promoted GTP hydrolysis. Although quite useful and informative, this approach lacks in specificity, since some GEFs and GAPs do not distinguish between the members of the Rho-family, so that these mutants can affect other GTPase pathways and thus results need to be interpreted with caution (Bishop and Hall, 2000; Heasman and Ridley, 2008; Spiering and Hodgson, 2011). More recently, other strategies such as RNA interference (RNAi) and gene knockout in mice have allowed selective inactivation of different Rho GTPases and their regulators and subsequent analysis of *in vivo* function, but they too have limitations, once again due to functional redundancy between closely related Rho proteins (Tybulewicz and Henderson, 2009; Hall and Lalli, 2010).

1.1.3. Association with cancer

Cancer can be generally seen as a disease involving dynamic changes in the genome. It is a complex, multi-step process that reflects the accumulation of acquired genetic and epigenetic alterations that result in alterations of key signalling pathways, and thus in the progressive transformation of normal cells into malignant derivatives (Gray and Collins, 2000; Hanahan and Weinberg, 2000; Cairns, 2009). According to Hanahan and Weinberg (2000), tumorigenesis requires six essential alterations to normal cell physiology: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion to apoptosis, unlimited replicative potential, sustained angiogenesis for nutrient supply, and ability to invade neighbouring tissues and metastasize.

The discovery of activating mutations that converted proto-oncogenes into oncogenes, conferring them with gain of function and thus

causing many of the perturbations in cell growth and differentiation seen in cancer cells, revolutionized cancer research and boosted it to search for more mutations (Hanahan and Weinberg, 2000; Cairns, 2009). In fact, Rho-family proteins were initially cloned on the basis of their similarity to the *RAS* oncogenes (Sahai and Marshall, 2002). *RAS* is one of the most important oncogenes in humans, mutated in about 30% of cancers of different origins (Hernández-Alcoceba *et al*., 2000), contributing to several aspects of the malignant phenotype, including the deregulation of tumour-cell growth, programmed cell death and invasiveness, and the ability to induce new blood-vessel formation (Downward, 2003). However, to date, the hypothesis that Rho proteins could also present homologous activating mutations in their coding sequence has not been confirmed (Benitah *et al*., 2004; del Pulgar *et al*., 2005). Nevertheless, Rho GTPases were found to play *in vitro* an essential role in Ras-induced transformation (Boettner and Van Aelst, 2002; Sahai and Marshall, 2002; Karlsson *et al*., 2009). This finding and all the cellular functions assigned to Rho GTPases with impact on tumour formation and progression, like the regulation of polarisation, migration, proliferation and survival of cells, strengthened the link between aberrant Rho signalling and cancer. Although recent whole exome sequencing efforts have revealed an oncogenic Rac1 mutation in codon 29 of 5-9% of melanoma cases (Hodis *et al*., 2012; Krauthammer *et al*., 2012), Rho signalling deregulation appears to be occurring at the level of GTPase expression or its activation by mutation in their upstream regulators or downstream effectors (Ellenbroek and Collard, 2007).

In fact, it has been reported that Rho proteins expression or activity is frequently altered in human cancers or cancer-derived cell lines (Ellenbroek and Collard, 2007; Vega and Ridley, 2008). For example, overexpression of RhoA has been observed in breast, colon, lung (Fritz *et*

al., 1999), and gastric cancer, as well as in head and neck squamous cell carcinoma (HNSCC), bladder and testicular cancer (Ellenbroek and Collard, 2007). Studies that compared malignant breast tissue with benign tissue showed that Rac1 protein levels were elevated in malignant breast tissue, suggesting that increased Rac activity promotes breast cancer development (Fritz *et al*., 1999). Also, the highly active splice variant of Rac1, Rac1b, was found overexpressed in some tumour types (Jordan *et al*., 1999; Schnelzer *et al*., 2000). Altered expression of Rho GTPases can take place at the messenger RNA (mRNA) level or at the protein level and have been linked to prognosis and development of diseases (Benitah *et al*., 2004; del Pulgar *et al*., 2005; Ellenbroek and Collard, 2007).

In addition to aberrant expression of Rho GTPases, also altered expression and mutations of regulatory proteins (GEFs, GAPs and GDIs), as well of effector proteins (e.g. ROCK; PAK) have been described for various human tumours (del Pulgar *et al*., 2005; Ellenbroek and Collard, 2007; Vega and Ridley, 2008). It is not clear how the altered expression of these various proteins influences Rho GTPase activity in cancer and indeed whether the connection between these upregulated proteins and the associated Rho GTPases is relevant for tumour progression (Vega and Ridley, 2008).

Nowadays, the literature reports contributions of Rho proteins to most steps of cancer initiation and progression. Although best characterized for their effects on the cytoskeleton and cell adhesion and these implied most likely an effect in cell migration and invasion, the function of Rho GTPases is not restricted to these events and they can affect tumour cells through regulation of gene expression, cell growth and survival, intracellular transport of signalling molecules or modifying the interaction of cancer cells with surrounding stromal cells (Sahai and

Marshall, 2002; Ridley, 2004; Ellenbroek and Collard, 2007; Vega and Ridley, 2008).

The initiation of tumour formation reflects the coordination of uncontrolled proliferation and the evasion of apoptosis (Ridley, 2004; Ellenbroek and Collard, 2007). Rho proteins contribute to cell survival by either promoting or antagonizing apoptosis in a cell type-specific manner (Sahai and Marshall, 2002; Karlsson *et al*., 2009). The increased production of superoxides and subsequent activation of nuclear factor kappa-light-chain-gene-enhancer of activated B cells (NF-κB) is one of the mechanism by which cells are protected from apoptosis, however a mechanism that involves phosphorylation of the apoptotic regulator BCL-2 antagonist of cell death (BAD) by PAK has also been proposed (Sahai and Marshall, 2002).

Cell cycle progression is tightly regulated and follows an ordered progression of molecular events, involving the activation of cell cycle regulatory molecules, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs) (Matsumura *et al*., 1999). Genetic analysis of human tumours has revealed that some of the molecules most often altered in cancer are those involved in the control of the G1/S transition of the cell cycle, a time when cells become committed to a new round of cell division. One of the primary events in the early G1 phase, dependent of extracellular mitogenic signals, is the synthesis of D-type cyclins (mainly cyclin D1) (Ortega *et al*., 2002). D-type cyclins family is composed of three closely related proteins, cyclin D1, D2 and D3, expressed in a wide variety of organs in a tissue-specific manner (Friedrichsen *et al*., 2003). These cyclins associate with CDK4 or 6, which up-regulates the kinase catalytic activity, and leads to the phosphorylation and partial inactivation of the key substrate retinoblastoma protein (Rb). Consequently, members of

transcription factor E2F family are released from growth-inhibitory Rb complexes and activated, leading to transcription of genes important for S-phase activity, such as cyclin E. Next, cyclin E interacts with and activates CDK2 kinase, leading to hyperphosphorylation of Rb proteins, which is required for proper G1/S transition and S-phase entry. The G1/S transition is also negatively regulated by CKIs that bind to cyclin-CDK complexes and inhibit their activity. Based on their sequence homology and specificity of action, CKIs are divided into two families: INK4 and Cip/Kip. Members of the INK4 family of CKIs, namely $p16^{INK4a}$ (CDKN2A), $p15^{INK4b}$ (CDKN2B), $p18^{INK4c}$ (CDKN2C), and $p19^{INK4d}$ (CDKN2D), specifically inhibit the activity of CDK4 and CDK6 by preventing cyclinD binding. On the other hand, the Cip/Kip family members, that include $p21^{CIP1/WAF1}$ (CDKN1A), $p27^{KIP1}$ (CDKN1B), and $p57^{KIP2}$ (CDKN1C), act more broadly and inhibit the activity of preformed cyclin–CDK complexes (reviewed in Obaya and Sedivy, 2002; Ortega *et al*., 2002).

Genetic alterations or abnormal expression of numerous cell cycle components have been implicated in tumorigenic processes. In particular, given the critical role and the potential to disrupt the cell cycle, cyclin D1 and CKIs have been assigned as oncogenes and tumour suppressors, respectively (Obaya and Sedivy, 2002; Mermelshtein *et al*., 2005). Multiple pathways seem to link Rho proteins to the control of cyclin D1 levels. Many of these involve the activation of protein kinases, leading to the subsequent modulation of transcription factor activity. Cyclin D1 transcription is controlled by ETS, activator protein 1 (AP-1) and NF-κB transcription factors (Albanese *et al*., 1995; Shaulian and Karin, 2001; Hinz *et al*., 2002), the activity of which is regulated by RhoA, Rac1 and Cdc42 (Perona *et al*., 1997; Sahai and Marshall, 2002). Rho GTPases can also regulate the activities of CKIs. RhoA signalling modulates the levels

of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. Downregulation of $p21^{CIP1/WAF1}$ levels by active RhoA is crucial for oncogenic *RAS* to promote cell-cycle entry (Olson *et al*., 1998). Inhibition of $p27^{KIP1}$ seems to require RhoA activity, but it is not clear whether this is a direct effect or achieved through effects on cyclin E-CDK complexes, which can promote $p27^{KIP1}$ degradation (Sahai and Marshall, 2002; Coleman *et al*., 2004).

1.2. Rac1

The GTPase Rac1 is probably the founder member of the Rho family (Boureux *et al*., 2007). Initially discovered as Ras-related C3 botulinum toxin substrate 1 (Didsbury *et al*., 1989) is nowadays considered to be a canonical member of Rho-family and one of the most studied (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002; Bosco *et al*., 2009). Together, Rac1 with its splice variant Rac1b, Rac2, Rac3 and RhoG comprise the Rac subfamily of Rho GTPases, sharing significant sequence identity (more than 80% between the Rac isoforms) and diverging essentially in the C-terminal region (Wennerberg and Der, 2004).

Rac1 is ubiquitously expressed (Didsbury *et al*., 1989) and its promoter presents characteristics similar to a housekeeping gene: a small size, the lack of a TATA-box and a CCAAT-box, an high GC content (74,2%), a CpG island surrounding the transcription initiation sites, and a number of known consensus sequences for transcription factors, such as Sp1, c-Jun/c-Fos (AP-1, AP-2, AP-4), E2F-2, Ikaros2, MZF1 and the oncogene *ETS1* (Matos *et al*., 2000). Rac1 is encoded by the *RAC1* gene, whose deletion results in early embryonic lethality (Sugihara *et al*., 1998),

evidencing the critical role of the signalling pathways in which this GTPase is involved.

Indeed, beyond the first insights into Rac1 cellular function as a regulator of actin cytoskeleton reorganization, it has been implicated in a myriad of processes that go from cell proliferation, apoptosis, motility, membrane trafficking and superoxide production to transcriptional regulation (Jaffe and Hall, 2005). These functions are mediated through interaction with specific effectors. One such effector, for example, is the protein kinase PAK that becomes activated upon direct interaction with GTP-Rac1. Activated Rac1 also stimulates transcription factors and gene expression, for example following its activation of the c-Jun N-terminal kinase (JNK) cascade (Coso *et al*., 1995), or of the transcription factor NF-κB (Perona *et al*., 1997). The pathway that links Rac1 to NF-κB involves the production of reactive oxygen species (ROS) and occurs in epithelial cells via NOX1 during Rac1-induced mitogenesis (Sulciner *et al*., 1996; Joneson and Bar-Sagi, 1998; Park *et al*., 2004). Rac1-stimulated ROS formation activates NF-κB leading to increased cyclin D1 expression and subsequent cell cycle progression (Guttridge *et al*., 1999; Hinz *et al*., 1999; Joyce *et al*., 1999), independent of the extracellular signal-regulated kinase (ERK) or JNK kinase cascade (Lamarche *et al*., 1996).

1.2.1. The efector PAK

Among the first described and best-characterized effectors of Rac1 are the PAK kinases (Manser *et al*., 1994).

PAKs are a highly conserved group of serine/threonine kinases represented, in mammals, by six isoforms (PAK1 to PAK6) subdivided into two groups according with their biochemical and structural features.

Group I is constituted by PAK1 (αPAK), PAK2 (γPAK) and PAK3 (βPAK), whereas PAK4, PAK5 and PAK6 belong to group II (Jaffer and Chernoff, 2002; Bokoch, 2003).

The group I PAKs, on which we will focus, comprise an N-terminal regulatory domain and a highly conserved C-terminal catalytic domain. The regulatory domain includes a conserved p21-binding domain (PBD), partially overlapped by an autoinhibitory domain (AID), crucial for controlling basal kinase activity. The PBD is responsible for the overall binding of the active forms of Rac1/Cdc42 (the CRIB – Cdc42/Rac1-interactive binding – domain [aa 75-90 in PAK1] is included in this region and contributes for the binding). Structural and biochemical data has shown that PAKs exists in cells as homodimers in a *trans*-autoinibitory conformation, in which the AID of one molecule inhibits the catalytic domain of the other (Fig. 3) (Jaffer and Chernoff, 2002; Bokoch, 2003).

The activation mechanism of PAKs can be GTPase-dependent or independent, although the molecular mechanisms underlying this last have not been clear yet. In a GTPase-dependent activation mechanism, the binding of active Rac1 or Cdc42 to the PBD disrupts PAK dimerization and releases the inhibition, allowing autophosphorylation of the threonine residue (T423 for PAK1) in the activation loop of the catalytic domain. Phosphorylation of this site activates PAK and is important for maintaining relief from autoinhibition, even in the absence of the GTPase, and for full kinase activity (Fig. 3). An acidic substitution of this residue (T423E for PAK1) renders PAK constitutively active. Activated PAKs can phosphorylate multiple substrates or interact with other proteins and through them modulate a range of biological activities, including the regulation of cytoskeletal dynamics and cell motility, stimulation of cell proliferation, pro- and anti-apoptotic signals and regulation of gene

expression (reviewed in Jaffer and Chernoff, 2002; Bokoch, 2003; Dummler *et al.*, 2009). Deregulation of these cellular processes can promote tumorigenesis and in fact, overexpression and/or hyperactivation of PAK family members, have been detected in several human tumours. In breast cancer, for example, deregulation of PAK1 is well documented and correlates with increased invasiveness and survival of these cancer cells (Kumar *et al.*, 2006). PAK1 expression was also found increased during malignant progression on human colorectal cancer (Gururaj *et al.*, 2005).

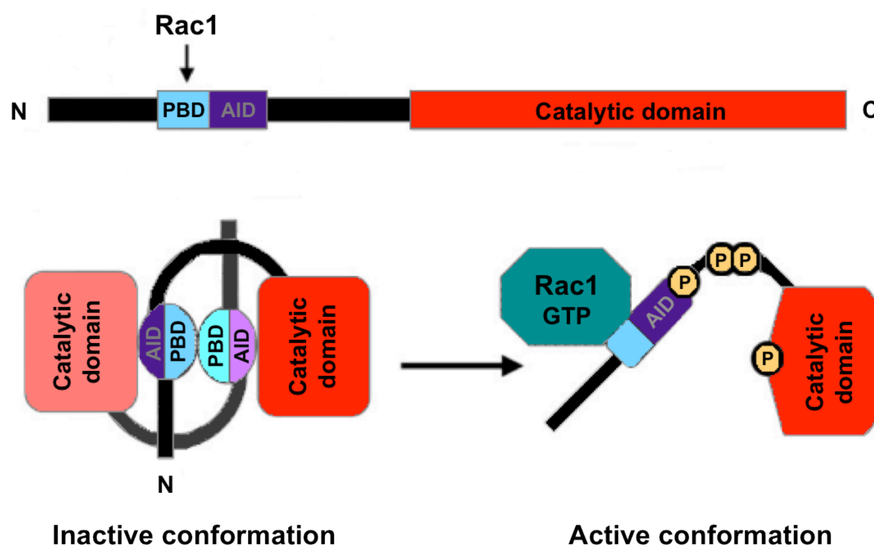

Figure 3: Domain structure and activation mechanism of group I PAKs. The group I Paks contain a N-terminal regulatory domain and a C-terminal catalytic domain. The regulatory domain includes a conserved p21-binding domain (PBD), partially overlapped by an autoinhibitory domain (AID), crucial for controlling basal kinase activity. PAKs are maintained in an inactive, autoinhibited dimeric complex, in which the AID of one molecule inhibits the catalytic domain of the other. The binding of an active form of Rac1 to the PBD disrupts PAK dimerization and releases the inhibition, allowing autophosphorylation and activation of the kinase (adapted from Parrini *et al.*, 2005).

1.2.2. Rac1b

Recently, it was found that the *RAC1* gene encoded a second isoform, designated Rac1b, through an alternative splicing event. Rac1b transcripts can be amplified from a variety of normal epithelial tissues, with a stronger prevalence in colon-derived samples, but is normally less abundant that the Rac1 transcripts (Jordan *et al*., 1999). Curiously, Rac1b was found overexpressed in colorectal (Jordan *et al*., 1999), breast (Schnelzer *et al*., 2000) and lung tumours (Liu *et al*., 2012; Stallings-Mann *et al*., 2012; Zhou *et al*., 2012) both at the RNA and protein levels, when compared to levels in benign tissue. This result was very interesting and suggested a role of Rac1b in tumorigenesis.

The GTPase Rac1b is the result of the inclusion of an additional exon 3b, located between exons 3 and 4 of *RAC1* gene, into the Rac1 mRNA. Thus, Rac1b transcript contains an additional 57 nucleotides (nt) that encodes an in frame insertion of 19 amino acids between Rac1 residues 75 and 76, positioned immediately C-terminal to the *Switch II* domain (Jordan *et al*., 1999). As already stated, the *Switch II* domain (Rac1 residues 60–76) along with the *Switch I* domain (Rac1 residues 30–38), constitute the regions that change in conformation during GDP-GTP cycling and consequently, contribute for interaction with regulators and effectors (Vetter and Wittinghofer, 2001; Wennerberg *et al*., 2005).

Rac1b was shown to be a highly activated variant. The analysis of the total endogenous level of Rac1b protein versus the activated GTP-bound fraction revealed that, although present in small amounts in cells, the amount of active Rac1b is surprisingly high, and even can exceed the amount of active Rac1 (Matos *et al*., 2003).

The high activation level of Rac1b is due to several differences. Rac1b is unable to interact with Rho-GDI and consequently to cycle between the plasma membrane and the cytoplasm, which leaves it persistently associated with membranes, in a favoured position to become activated (Matos *et al*., 2003). Additionally, Rac1b shows impaired intrinsic GTPase activity *in vitro* (Schnelzer *et al*., 2000), yet maintaining GAP responsiveness *in vivo* (Matos *et al*., 2003) and *in vitro* (Fiegen *et al*., 2004; Singh *et al*., 2004). And it also reveals an increased intrinsic nucleotide exchange rate (GDP to GTP) *in vitro* (Schnelzer *et al*., 2000). Curiously, however, Rac1b failed to activate several classical Rac1 pathways, as the formation of *lamellipodia*, the activation of the protein kinase PAK, or the stimulation of JNK pathway. Because Rac1b retained the ability to stimulate the classical NF-κB pathway it seems to be selective in its downstream signalling properties (Matos *et al*., 2003).

1.3. Gene expression regulation

For all living cells the accurate execution of biological processes such as development, homeostasis, differentiation or adaptation to the environment requires a precise and coordinated set of steps that depend on the proper spatial and temporal expression of genes. Thus, deregulation of gene expression is often linked with the occurrence of diseases (Emerson, 2002; Maston *et al*., 2006). Cancer, for example, is essentially a disease of disordered gene expression, driven by the accumulation of genetic and epigenetic alterations that gradually transform normal cells into cancer cells (Gray and Collins, 2000; Hanahan and Weinberg, 2000).

Eukaryotic gene expression is a highly complex and dynamic process that involves several steps, including transcription of the gene, mRNA processing (e.g. splicing, polyadenylation), transport of the fully processed mRNA to the cytoplasm, translation into a protein and post-translational modifications that confer the protein its mature form (Singer and Green, 1997; Maston *et al*., 2006). Gene expression can be specifically regulated at any point of the process, however, the critical step seems to be at the level of transcription initiation (Maston *et al*., 2006; Hager *et al*., 2009).

1.3.1. Eukaryotic gene transcription

Transcription is, in a simple definition, the biochemical process by which information is transferred from DNA to RNA. However, this process it is all but simple, involving distinct stages – initiation, elongation and termination – and a large number of regulatory proteins (Venters and Pugh, 2009).

Transcription initiation is triggered by the binding of transcription factors to specific DNA sequences located in *cis*-regulatory elements, such as gene promoters or enhancers. In turn, transcription factors activation is controlled by many signal transduction pathways, which respond to distinct cellular and environmental signals (Orphanides and Reinberg, 2002; Bilu and Barkai, 2005; Venters and Pugh, 2009). The significance of this regulatory process is even more highlighted by the fact that about 10% of the human gene products are predicted to have DNA-binding properties (Kim and Park, 2011). Therefore, extracellular signals are believed to constitute the main regulatory source for transcriptional regulation.

In eukaryotes, this process is carried out by three different DNA-dependent RNA polymerases: RNA polymerase (Pol) I, II and III. RNA Pol I synthesises ribosomal RNA (rRNA) and RNA Pol III produces transfer RNAs (tRNA) and some small RNAs, while RNA Pol II is responsible for transcribing protein-coding genes into mRNAs and producing small nuclear RNA genes (snRNA) (Cramer *et al*., 2008).

In case of RNA Pol II, the factors involved in the transcription of genes can be classified into three groups: general transcription factors (GTFs), promoter-specific activator or repressor proteins, and co-activators or co-repressors. GTFs, in contrast to sequence-specific regulators that are targeted to a discrete set of genes, are broadly utilized by the cell at many genes and can be sufficient for accurate transcription initiation *in vitro*. GTFs are constituted by RNA Pol II and a variety of auxiliary components, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. Additionally to these factors it is required a highly conserved, large multisubunit complex, the Mediator. GTFs assemble on the core promoter in an ordered manner to form a transcription preinitiation complex (PIC), which directs RNA polymerase II to the transcription start site (TSS). The first step in PIC assembly is binding of TFIID, a multisubunit complex consisting of TATA-box-binding protein (TBP) and a set of tightly bound TBP-associated factors (TAFs). Transcription then proceeds through a series of steps, including promoter melting, clearance, and escape, before a fully functional RNA Pol II elongation complex is formed (Lemon and Tijan, 2000; Maston *et al*., 2006; Venters and Pugh, 2009).

The assembly of a PIC on the core promoter is sufficient to direct only low levels of accurately initiated transcription from DNA templates *in vitro*, a process generally referred to as basal transcription. Transcriptional activity is further regulated by a second class of factors, termed activators

or repressors. In general, these transcription factors are sequence-specific DNA-binding proteins whose recognition sites are usually present in sequences upstream of the core promoter. Many activators and repressors interact with other proteins known as respectively, co-activators or co-repressors, that do not have DNA-binding activity but help the transcription factor to execute its function (Gaston and Jayaraman, 2003; Maston *et al*., 2006).

Both activators and co-activators can further stimulate transcription by promoting the alteration of chromatin structure in the vicinity of the promoter. Three classes of protein associated with the RNA Pol II are involved in this remodelling of chromatin: histone-modifying enzymes, chromatin-binding proteins, and ATP-dependent nucleosome-remodelling proteins. Activators and co-activators can recruit one or more of these proteins to a promoter and the resulting chromatin remodelling can alter histone-DNA interactions, nucleosome-nucleosome interactions, and/or re-position nucleosomes relative to transcription factor binding sites (Lemon and Tijan, 2000; Gaston and Jayaraman, 2003; Venters and Pugh, 2009).

Transcriptional repression is of two types: general or global repression and gene-specific repression. General repression occurs when a repressor protein or complex either sequesters or modifies a central component of the PIC or a component of RNA Pol II, so that it is unavailable for transcription. Thus, general repression will downregulate the expression of all the genes transcribed by RNA Pol II. In contrast, gene-specific repression involves the sequence-specific binding of a repressor protein to the promoter region of specific target genes (reviewed in Gaston and Jayaraman, 2003; Payankaulam *et al*., 2010).

1.3.2. Transcriptional regulatory elements

A typical eukaryotic protein-coding gene comprises two distinct families of *cis*-acting regulatory elements: the promoter and distal regulatory elements (Butler and Kadonaga, 2002; Levine and Tjian, 2003). These *cis*-regulatory elements contain a series of short DNA sequence motifs (between 6 and 20 bp) that are specifically recognized and bound by transcription factors to either increase or decrease transcription of gene targets (Maston *et al*., 2006; Georges *et al*., 2010).

Promoters are divided into core and proximal promoters depending on their binding partners and distance from the TSS (Fig. 4) (Kim and Park, 2011). The core promoter encompasses the TSS (defined as +1) and flanking sequences extending about ~35 bp in each direction, to which RNA Pol II and the general transcriptional machinery bind to initiate and direct transcription. In higher eukaryotes, this region is highly diverse and can include multiple regulatory elements that interact with various components of the basal transcriptional machinery, such as the TATA box, the initiator element (Inr), the downstream promoter element (DPE), the downstream core element (DCE), the TFIIB-Recognition Element (BRE) and the motif ten element (MTE) (reviewed in Butler and Kadonaga, 2002; Smale and Kadonaga, 2003; Maston *et al*., 2006). The most familiar core promoter element is the TATA box, an A/T rich sequence, usually located at approximately 25 to 30 nt upstream of the TSS. Its consensus sequence, TATA(A/T)A(A/T)(A/G), is recognized by TBP, a subunit of the TFIID complex, constituting the first step in PIC assembly (reviewed in Smale and Kadonaga, 2003). Although an important nucleation point, PIC assembly and ultimately, transcription initiation does not depend on TATA box (which is absent from many

promoters) or on any single element. In fact, with the exception of the BRE, which is specifically recognized by TFIIB, all the other core promoter elements are TFIID-interaction sites and stabilize PIC assembly. The variability in content and organization shown by core promoters contributes to the regulatory specificity of genes (Maston *et al*., 2006).

The proximal promoter is defined as the region immediately upstream from the core promoter (within <1 kb) that contains DNA sequences (e.g. CCAAT box, octamer module and GC-box) recognized by specific transcription factors (Maston *et al*., 2006; Kim and Park, 2011). The interaction between these regulatory DNA elements and transcription factors stimulates transcription by stabilizing the binding of general transcription factors to nearby core promoters (Kim and Park, 2011). These factors do not always function as classical activators or repressors, instead, they might serve as tethering elements that recruit distal enhancers to the core promoter (Levine and Tjian, 2003).

Distal regulatory elements are often located far away from the genes they control, scattered over distances of more than 100 kb from the TSS, either upstream of the promoter, in a intron or even at the 3´ end of a gene (Levine and Tjian, 2003; Maston *et al*., 2006). These long-range regulatory elements are essential in mediating the complex patterns of gene expression in different cells types (Levine and Tjian, 2003; Heintzman and Ren, 2009). Examples of some of these regulatory elements include enhancers, silencers, insulators and locus control regions (LCR) (reviewed in Noonan and McCallion, 2010).

Enhancers are typically composed of clusters of DNA-binding sites for transcriptional regulators that work cooperatively to enhance transcription, independent of their orientation and distance from the promoter. The spatial organization and orientation of the transcription

binding sites within an enhancer can be critical to its regulatory activity. Silencers are sequence-specific elements that confer a negative, (i.e., silencing or repressing) effect on the transcription of a target gene (Fig. 4) (Lee and Young, 2000; Maston *et al*., 2006).

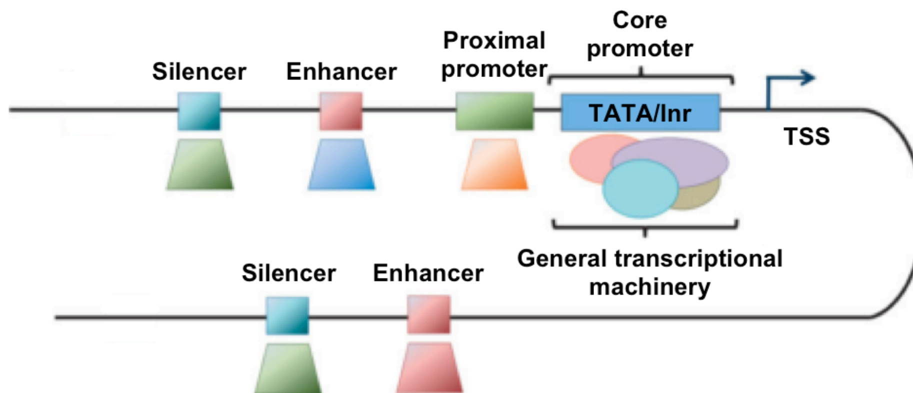

Figure 5: Schematic representation of cis-regulatory elements. The core promoters directly upstream of transcription start site (TSS) bind the general transcriptional machinery. Proximal promoters bind cognate sequence-specific transcription factors (shown as trapezoids). Distal enhancers and silencer elements also provide binding sites for sequence-specific transcription factors, but have opposite regulatory effects on the transcription activity of regulated genes (adapted from Kim and Park, 2011).

1.3.3. The role of chromatin

Chromatin is the state in which DNA is packaged within the cell. The nucleosome is the fundamental unit of chromatin and it is composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped. The core histones are predominantly globular except for their N-terminal tails, which are unstructured. A striking feature of histones, and particularly of their tails, is the large number and

type of modified residues they possess (Kouzarides, 2007; Clapier and Cairns, 2009).

Both histone tails and globular domains are subject to a vast array of post-translational modifications. These modifications include methylation of arginine (R) residues, methylation, acetylation, ubiquitination, ADP-ribosylation, sumoylation of lysines (K) and phosphorylation of serines and threonines. Modifications that are associated with active transcription, such as acetylation of H3 and H4 or di- or trimethylation of H3 K4, are commonly referred to as euchromatin modifications. Modifications that are localized to inactive genes or regions, such as H3 K9me and H3 K27me, are often termed heterochromatin modifications (Li *et al*., 2007).

Modifications may affect higher-order chromatin structure by affecting the contact between different histones in adjacent nucleosomes or the interaction of histones with DNA. Of all the known modifications, acetylation has the most potential to unfold chromatin since it neutralizes the basic charge of the lysine (Kouzarides, 2007). Typically, histone acetylation occurs at multiple lysine residues and is usually carried out by a variety of histone acetyltransferase (HAT) complexes. Distinct patterns of lysine acetylation on histones have been proposed to specify distinct downstream functions such as the regulation of co-expressed genes (Li *et al*., 2007).

1.3.4. Transcription factors

Transcription factors are modular proteins consisting of a number of domains. The three major domains are a DNA-binding domain (DBD), a transactivation domain (TAD), and a dimerization domain (Kim and Park,

2011). In addition, transcription factors typically have a nuclear localization sequence (NLS), and some also have a nuclear export sequence (NES).

The DBD recognizes a specific DNA sequence and positions the transcription factor to the DNA. Transcription factors are typically grouped into families based on the similarities of the DBD structure. Such transcription factor families share a common motif, which is defined as a cluster of amino acid residues that has a characteristic three-dimensional folding pattern and carries out a specific function (Georges *et al*., 2010; Pan *et al*., 2010). As a consequence, it is common that many transcription factors within a family recognize either the same, or very similar, consensus DNA target sequences (Georges *et al*., 2010). Some of the more common and well described families include those containing a basic leucine zipper (bZIP), helix-loop-helix (HLH), Pit-Oct-Unc (POU), poxvirus and zinc finger (POZ), ETS or a forkhead DBD (Kelly and Daniel, 2006; Georges *et al*., 2010).

The TAD is necessary for stimulating the activity of the transcription factor and the dimerization domain for the formation of homodimers or heterodimers (Maston *et al*., 2006).

1.3.4.1. Signal transducers and activators of transcription (STATs)

STATs are a family of latent cytoplasmic proteins that function as signalling transducers between the plasma membrane and the nucleus, and as transcription factors, activating a diverse set of genes (Darnell, 1997; Yu and Jove, 2004). These proteins are activated by a series of extracellular signalling ligands, such as cytokines, growth factors, and hormones (e.g growth hormone – GH, prolactin – PRL), and therefore integrate diverse signalling pathways, controlling crucial biological

processes. Some of these include cell differentiation, proliferation, apoptosis, angiogenesis and immune responses (Horvath, 2000; Levy and Darnell, 2002).

The general model of activation of STAT proteins relies on tyrosine phosphorylation events carried out by tyrosine kinases (TKs), like Janus kinase (JAK) proteins (JAK-STAT signalling pathway), receptor TKs or non-receptor TKs (e.g. SRC, ABL). Signalling initiates with binding of ligands to specific cell-surface receptors, which leads to receptor dimerization and to the activation of associated-TKs (transphosphorylation). These subsequently phosphorylate the receptor on tyrosine residues, providing docking sites for STATs recruitment. Once bound to the receptor complex, STATs are phosphorylated on specific tyrosine residues by the TKs, dimerize (homo or heterodimers) and translocate to the nucleus, where they bind to a consensus site (TTN_5AA) in the promoter of target genes and activate transcription (Bowman *et al*., 2000; Levy and Darnell, 2002; Alvarez and Frank, 2004).

In mammals, the STAT family comprises seven members – STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 – (Darnell, 1997) with a very similar functional domain structure and limited sequence homology, with exception of the STAT5 isoforms (Buitenhuis *et al*., 2004). All STAT proteins contain an N-terminal domain (mediates dimer-dimer interactions), a coiled-coil domain (important in interactions with other proteins), a highly conserved DBD, a Src-homology 2 (SH2) domain, a linker domain (bridges DBD and SH2 domain) and a C-terminal region that contains a TAD, as well as the critical tyrosine residue. The DBD is necessary for direct contact with specific DNA and its sequence determines the binding specificity for each STAT. The SH2 domain (common structural motif among signalling molecules) mediates protein-

protein interactions through direct binding to specific phosphotyrosines, thereby is required for the recruitment of STATs to phosphorylated receptors and for the reciprocal SH2-phosphotyrosine interactions between monomeric STATs to form dimers. The most divergent region between STATs, and thus confering specificity, is the TAD that is essential for transcriptional activation. It also contains the tyrosine residue (e.g. Tyr694 for STAT5A) crucial for STAT activation and consequent, dimerization and nuclear translocation. With exception of STAT2 and STAT6, all STATs have in the TAD a conserved serine residue, whose phosphorylation further regulates STATs activity and contributes to maximal transcriptional activity (reviewed in Calò *et al*., 2003; Paukku and Silvennoinen, 2004; Santos and Costa-Pereira, 2011).

The STAT5 isoforms, STAT5A and STAT5B proteins, which are encoded by two distinct but closely related genes located in *tandem* on human chromosome 17, share over 90% of sequence identity with some differences at their TAD. Both proteins are widely expressed, but exhibit different expression profiles. These proteins share most of their biological functions, but display also non-redundant functions *in vivo* (Buitenhuis *et al*., 2004).

1.3.4.2. BCL-6

BCL-6 was cloned from a translocation in diffuse large B-cell lymphoma (DLBCL). It is expressed in normal germinal centre (GC) B-cells and a subset of GC T-cells and has an essential role in normal antibody responses (Wagner *et al*., 2011). These alterations cause the deregulated expression of the *BCL-6* gene by a mechanism called promoter substitution, that is the juxtaposition of heterologous promoters,

derived from other chromosomes, to the BCL-6 coding domain (Chang *et al*., 1996).

BCL-6 belongs to a subset of transcription factors which all have a similar structure composed of an N-terminal POZ domain and several zinc finger modules at the C-terminus (Dent *et al*., 2002). Its N-terminal POZ domain is able to recruit co-repressor molecules and histone deacetylase and thereby mediate transcriptional repression (Chang *et al*., 1996). An additional property is that the DNA-binding sequence recognized by BCL-6 conforms to the STAT family consensus-binding sequence (Dent *et al*., 1997), and BCL-6 has subsequently been shown to bind with varying affinities to different STAT sites (Harris *et al*., 1999). Thus an additional mechanism of action of BCL-6 might be the modulation of the effects of STATs by competing with differing affinities to different STAT target sequences. Such a role may be particularly important in situations in which STATs are responsible for driving conflicting biological processes, for example proliferation and differentiation (Logarajah *et al*., 2003).

1.4. Objectives

The work described in this thesis had the following objectives:

- Determine the effect of Rac1 signalling on gene expression regulation through the transcriptional repressor BCL-6;
- Characterize whether gene transcription can be modulated by a STAT5/BCL-6 antagonism in colorectal cells;
- Characterize the role of Rac1 signalling in the regulation of STAT5 and BCL-6 responsive target genes.

1.5. References

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Chapter 2

Rac1 signalling modulates BCL-6-mediated repression of gene transcription

Patrícia Barros, Peter Jordan and Paulo Matos

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Author's Note

The work presented here was based on previous experiments in the host laboratory, which had identified several candidate Rac1-interacting molecules involved in signal transduction, in colorectal cell lines. Some of these molecules were tested and analysed during my graduation thesis, of which the transcriptional repressor BCL-6 was chosen as best candidate to integrate my PhD research project.

The author of this thesis declares to have conducted the majority of the described experimental work and contributed to the experimental design, laboratory work and manuscript writing.

2.1. Summary

Rac1 is a member of the Rho family of small GTPases that regulates signalling pathways involved in cell adhesion and migration but also the regulation of gene transcription. Here we describe that the transcriptional repressor BCL-6 is regulated by Rac1 signalling. Transfection of active Rac1 mutants into colorectal DLD-1 cells led to increased expression of a BCL-6-controlled luciferase reporter construct. Conversely, inhibition of endogenous Rac1 activation by the Rac1 inhibitor NSC23766 decreased reporter activity. Moreover, BCL-6 lost its typical localization to nuclear dots upon activation of Rac1 and became predominantly soluble in a non-chromatin bound cell fraction. Rac1 signalling also regulated the expression of endogenous BCL-6-regulated genes, including the p50 precursor NFKB1/p105 and the cell adhesion molecule *CD44*. Interestingly, these effects were not stimulated by the alternative splice variant Rac1b. The mechanism of BCL-6 inhibition does not involve formation of a stable Rac1/BCL-6 complex and is independent of Rac-induced ROS production or JNK activation. We show that PAK1 mediates the inhibition downstream of Rac and can directly phosphorylate BCL-6. Together, these data provide substantial evidence that Rac1 signalling inhibits the transcriptional repressor BCL-6 in colorectal cells and reveal a novel pathway that links Rac1 signalling to the regulation of gene transcription.

2.2. Introduction

The Rho family of small GTPases contains 20 different gene members (Wherlock and Mellor, 2002; Boureux *et al.*, 2007), of which RhoA, Rac1 and Cdc42 have been best characterized (Hall, 1998; Ridley, 2001; Etienne-Manneville and Hall, 2002). These GTPases typically cycle between an inactive, GDP-bound, and an active, GTP-bound state. The transition between these states is controlled by three distinct types of proteins *in vivo*, the guanine nucleotide exchange factors (GEFs), which activate; and the GTPase-activating proteins (GAPs) or the guanine nucleotide dissociation inhibitors (GDIs), which both inactivate GTPases (reviewed in DerMardirossian and Bokoch, 2005; Bos *et al.*, 2007). Once in the GTP-loaded conformation, Rho GTPases become able to interact with downstream effector proteins that initiate further signalling events in the cell. The corresponding cellular responses range from changes in cell morphology to changes in gene expression.

Rac1, in particular, has been documented to stimulate the polymerization of actin filaments leading to the formation of *lamellipodia* and affecting the stability of adherens junctions (Fukata and Kaibuchi, 2001). Rac signalling further activates the protein kinase PAK, the c-Jun N-terminal kinase (JNK) and the production of reactive oxygen species (ROS) (Schuringa *et al.*, 2001). Moreover, recent data have revealed that Rac1 has also distinct roles in the regulation of gene transcription (Benitah *et al.*, 2004). For instance, the stimulation of JNK by Rac signalling can lead to the activation of its target transcription factors c-Jun, ATF, ELK or AP-1. Also, Rac signalling can activate proteins of the STAT family (Schuringa *et al.*, 2001; Park *et al.*, 2004), and the formation of protein complexes between Rac1 and STAT3 or STAT5 were described (Simon

et al., 2000; Tonozuka *et al.*, 2004; Kawashima *et al.*, 2006). Likewise, an active Rac1 mutant amplifies the transcriptional activation mediated by β-catenin and TCF/LEF (Esufali and Bapat, 2004).

A further important transcription factor stimulated by Rac1 is NF-κB. The NF-κB family is composed of five transcription factors that form homodimers or heterodimers with each other, namely RelA, RelB, c-Rel, p50 and p52. Unlike the three Rel proteins, p50 and p52 are produced through proteolytic processing from two inhibitory precursor proteins, NF-κB1/p105 and NF-κB2/p100 respectively. The NF-κB dimers remain transcriptionally inactive as long as associated with an NF-κB inhibitor protein, such as IκBα or the NF-κB2/p100 precursor protein. Signalling from GTP-bound Rac1 activates the IKK protein kinase complex resulting in the phosphorylation of both IκBα and NF-κB2/p100 proteins. In addition, the inhibitory complexes are recruited to sites at the plasma membrane where Rac is activated and brings them into proximity with the SCF ubiquitin ligase complex (Boyer *et al.*, 2004; Matos and Jordan, 2006). This leads to proteolytic degradation of IκBα and subsequent nuclear translocation of the transcriptionally competent RelA/p50 dimer (the canonical NF-κB pathway) but also promotes proteolytic processing of NF-κB2/p100 to p52, with subsequent transcriptional activation of RelB/p52 dimers (Matos and Jordan, 2006). The canonical, IκBα-regulated NF-κB pathway is also stimulated by Rac1b, an alternative splice variant that exists predominantly in the active GTP-bound state in cell lines (Jordan *et al.*, 1999; Fiegen *et al.*, 2004; Singh *et al.*, 2004; Matos and Jordan, 2006). Whereas Rac1b does not activate several classical Rac signalling pathways, including *lamellipodia* formation or the activation of PAK1 or JNK activities, it retains the ability to induce IκBα phosphorylation, nuclear translocation of RelA and

transcriptional stimulation of luciferase reporter constructs containing either a consensus NF-κB binding motif or the native cyclin D1 promoter (Matos *et al.*, 2003; Matos and Jordan, 2006; Esufali *et al.*, 2007).

BCL-6 is a transcriptional repressor (Seyfert *et al.*, 1996) and was identified as one of the most frequently translocated genes in B-cell non Hodgkin's lymphomas (Dalla-Favera *et al.*, 1999; Staudt *et al.*, 1999). BCL-6 contains carboxy-terminal zinc finger modules that bind DNA in a sequence-specific manner, especially the high affinity site TTCCT(A/C)GAA (Chang *et al.*, 1996; Huynh and Bardwell, 1998). The genes repressed by BCL-6 in germinal centre B cells are involved in lymphocyte activation and differentiation, immunoglobulin (Ig) isotype switching, and regulation of inflammation or cell cycle progression (Shaffer *et al.*, 2000; Dent *et al.*, 2002; Niu, 2002). The repressor activity of BCL-6 can be regulated by post-translational modifications. Both acetylation and phosphorylation events were shown to downregulate BCL-6 ability to repress transcription, the former impairing its recruitment of histone deacetylases (HDACs) (Bereshchenko *et al.*, 2002), and the latter leading to its proteasomal degradation (Niu *et al.*, 1998; Phan *et al.*, 2007).

Here we describe a novel link of Rac1 signalling to the regulation of gene transcription. We found that the transcriptional repressor BCL-6 is inhibited in colorectal tumour cells following Rac1 activation. This leads to increased expression of endogenous BCL-6-regulated genes including NFKB1/p105, the p50 precursor, and the cell adhesion molecule *CD44*. The mechanism of BCL-6 inactivation requires PAK1-mediated phosphorylation of BCL-6 downstream of Rac1 and is not triggered by splice variant Rac1b.

2.3. Material and Methods

2.3.1. Cell culture and transfection

DLD-1 colorectal cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) (all reagents from Gibco, Carlsbad, CA, USA) and regularly checked for absence of mycoplasm infection. Cells were transfected at 60 to 80% confluence using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and analysed 16-20 h later. Total amounts of transfected plasmid DNA were 4 μg per 60-mm dish for immunoprecipitation and 2 μg of DNA per 35-mm dish for immunofluorescence, cell fractionation and reporter assays. Transfection efficiency in DLD-1 cells was 50 to 70% as judged microscopically by expression of 2 μg of green fluorescent protein (GFP) expression vector. For RNA interference experiments, DLD-1 cells at 30 to 40% confluence were transfected in 35-mm dishes with 200 pmol of the indicated small interfering RNAs (siRNAs) using LipofectAMINE 2000 (Invitrogen), transfected again after 24 h with expression vectors or reporter constructs, and analysed 24 h later. The pre-designed siRNA oligonucleotides were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) with the following references: αPAK siRNA (sc-29700), γPAK siRNA (sc-36183), BCL-6 siRNA (sc-29791), and a scramble control oligonucleotide (5'-AGG UAG UGU AAU CGC CUU GTT) from Eurofins MWG Operon (Ebersberg, Germany). For drug treatments, cells were incubated for 16 to 20 h with 200 μM Rac inhibitor NSC23766 (Calbiochem, Darmstadt, Germany) or with 25 μM NADPH-oxidase inhibitor diphenyleneiodonium chloride (DPI) (Sigma, St. Louis, MO, USA), or 10 μM PAK inhibitor IPA-3 (Calbiochem).

2.3.2. DNA plasmids and constructs

The following published constructs were received as gifts: pcDNA3-HA-IκBα (A32A36) from M. Karin (University of California, San Diego, CA, USA), pcDNA3-HA-RelB from C. V. Paya (Mayo Clinic, Rochester, MN, USA), SAPKβ-MKK7 from U. Rapp (Würzburg, Germany), PAK1-wt, PAK1-K299R and PAK1-T423E from J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA, USA), the 3x-κB-luc vector (three copies of the Igκ-κB-motif immediately upstream of the β-globin TATA-box) (Lernbecher *et al.*, 1993) from B. Baumann (University of Ulm, Germany) and the 5xBCL-6-vector and the pGL3 control vector (Huynh *et al.*, 2000) from V.J. Bardwell (University of Minnesota, Minneapolis, MN, USA). *Rac1* and *Rac1b* cDNAs as well as their Q61L mutants were subcloned as an EcoRI/BamHI fragment into pcDNA3-Myc, pEGFP (Clontech, Mountain View, CA, USA) and pDsRed-C1 (Clontech) vectors as previously described (Matos *et al.*, 2003; Matos and Jordan, 2006). For their subcloning into pDsRed-C1 (Clontech), the respective pEGFP vectors were cut using the EcoRI/BamHI restriction sites. pEGFP-BCL-6 was generated by PCR amplification of the *BCL-6* cDNA from pmT2T-HA-BCL6, provided by R. Dalla-Favera (Columbia University, New York, NY, USA), using a forward primer (5'-GGT ACC ATG GCC TCG CCG GCT GAC A) and a reverse primer (5'-TCA GCA GGC TTT GGG GAG CT), followed by subcloning into pEGFP-C3 using KpnI and SmaI. All PAK1 constructs were subcloned into pEGFP-C3 vector using HindIII/EcoRI restriction sites. All constructs were confirmed by automated DNA sequencing.

2.3.3. Analysis of transcript expression and semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted from cell lysates with the RNeasy kit (Qiagen, Hilden, Germany) and 1 µg reverse transcribed using random primers (Invitrogen) and Ready-to-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK). The primers (F*, forward; R*, reverse) for the specific amplification of BCL-6 were BCL6-F* (5'- AGA GCC CAT AAA ACG GTC CT) and BCL6-R* (5'-AGT GTC CAC AAC ATG CTC CA); for NFKB1 were p105-F* (5'-CCT GGA TGA CTC TTG GGA AA) and p105-R* (5'-TCA GCC AGC TGT TTC ATG TC); for CD44 were CD44-F* (5'-TCT GTG CAG CAA ACA ACA CA) and CD44-R* (5'-TAG GGT TGC TGG GGT AGA TG); for PAK1 were PAK1-444F* (5'-GTC AGC TGA GGA TTA CAA TTC) and PAK1-661R* (5'-GAG ATG TAG CCA CGT CCC GAG); for PAK2 were PAK2-431F* (5'-CTC CTG AGA AAG ATG GCT TTC) and PAK2-632R* (5'-ACA TGT GAA TCA CCA ACT GGT); for PAK3 were PAK3-437F* (5'-GTG CAC ATG GAT ACA TAG CAG) and PAK3-663R* (5'-TGT GAC CTC TTT ATT TGG TAC); for BAZF were BAZF-e1F* (5'-AGA GCA CAC AAG GCA GTT CTC) and BAZF-e2R* (5'-GTG CAG TGG CTG GAG AGA GG); and for RNA polymerase II (Pol II) were Pol II-F* (5'-GAG CGG GAA TTT GAG CGG ATG C) and Pol II-R* (5'-GAA GGC GTG GGT TGA TGT GGA AGA). Amplification reactions were performed using AmpliTaq polymerase (Perkin-Elmer, Waltham, MA, USA) using the following basic program: 30 s at 94ºC, 30 s at the annealing temperature, and 30 s at 72ºC. The annealing temperature and number of cycles for each PCR were as follows: 58ºC and 30 cycles for BCL-6, 64ºC and 30 cycles for BAZF, 56ºC and 29 cycles for PAK1 and PAK2, 58ºC and 35 cycles for PAK3, 58ºC and

28 cycles for NFKB1, 60ºC and 30 cycles for CD44, and 64ºC and 28 cycles for Pol II. All reactions included an initial denaturation step of 5 min at 94ºC and a final extension step of 10 min at 72ºC. To allow a semi-quantitative analysis of transcript levels, all amplification conditions were experimentally optimised to correspond to the linear amplification phase, using serial dilutions of control cDNAs. The products were separated on 2% agarose gels containing ethidium bromide and band intensities were quantified on digitalised images using ImageJ software (National Institutes of Health – NIH) followed by normalization to Pol II expression levels. No amplification was obtained when RNA was mock reverse transcribed without adding reverse transcriptase.

2.3.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Samples were boiled for 10 min, centrifuged briefly and resolved in 10 to 15% SDS-PAGE mini-gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using a Mini Trans-Blot cell (Bio-Rad; Hercules, CA, USA). Membranes were blocked in Tris-buffered saline (TBS), 0,1% Triton X-100, 5% milk powder, probed using the indicated antibodies, and then incubated with a secondary peroxidase-conjugated antibody (BioRad) followed by chemiluminescence detection. The antibodies used for Western blots were as follows: polyclonals anti-Histone H2B (sc-10808), anti-BCL-6 clone N3 (sc-858) and anti-c-Myc clone A14 (sc-789) from Santa Cruz Biotechnology; monoclonal anti-Rac1 clone 23A8 from Upstate Biotechnologies (#05-389; Charlottesville, Virginia, USA); polyclonal anti-hemagglutinin (anti-HA; H6908) and monoclonal anti-α-tubulin clone B-5-1-2 (as a loading control; T6074) from

Sigma; polyclonal anti-GFP (ab290) and monoclonal anti-PAK1 (ab40795) from Abcam (Cambridge, UK); polyclonal anti-PAK1/2/3 from Cell Signaling Technology (#2604; Danvers, MA, USA); and polyclonals anti-p50 (HM1238) and anti-RelA (HM1240) from Hypromatrix (Worcester, MA, USA). For densitometric analysis, films from at least three independent experiments were digitalized and analysed using ImageJ software (NIH).

2.3.5. Immunoprecipitation

Approximately 2 x 10^6 DLD-1 cells were seeded in 60-mm dishes, transfected as indicated, and assayed 16 to 20 h later. For co-precipitation experiments, cells were washed in cold PBS and lysed on ice in 250 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 1% [v/v] Nonidet P-40 (NP-40), 100 mM NaCl, 10% [v/v] glycerol, 5 mM $MgCl_2$, and a protease inhibitor cocktail [Sigma]). Total lysates were then sonicated on ice (10 pulses of 20 s at 40% power on a Sonics Vibra Cell sonicator) and cleared by centrifugation at 2,500 x *g* for 5 min. An aliquot of 0.1 volume was added to 5x Laemmli sample buffer. The remaining lysate was incubated for 1 h at 4ºC with mouse monoclonal anti-c-Myc clone 9E10 (M5546; Sigma) or anti-GFP (ab1218; Abcam) antibodies at 2 μg ml^{-1}, pre-coupled to protein G-agarose beads (Roche Applied Science, Penzberg, Germany). Beads were then washed five times with an excess of lysis buffer containing 300 mM of NaCl and the precipitated protein complexes were solubilised in 2x Laemmli sample buffer and analysed on Western blots as described above. Immunoprecipitation of protein substrates for *in vitro* kinase assays followed the same methodology, except that cell lysis was performed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl

[pH 7.5], 1% [v/v] NP-40, 150 mM NaCl, 0,5% [w/v] sodium deoxycholate, 0,1% [w/v] SDS, and a protease inhibitor cocktail [Sigma]). All results were confirmed in at least three independent experiments.

2.3.6. Cell fractionation

Nuclear proteins were separated into a soluble pool not retained in the nucleus and into a chromatin-bound insoluble pool according to previously described procedures (Solan *et al.*, 2002). Briefly, cells were washed in cold phosphate-buffered saline (PBS), scraped off and lysed on ice for 10 min in 200 µl of fractionation buffer (50 mM Tris-HCl [pH 7.9], 0.1% [v/v] NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, and a protease inhibitor cocktail [Sigma]). The soluble fraction was collected by centrifuging the lysate at 3,500 × *g* for 5 min and adding the supernatant to 50 µl of 5x Laemmli sample buffer. The pellet containing the insoluble nuclear fraction was washed once in fractionation buffer and then resuspended in 250 µl of 1x Laemmli sample buffer supplemented with 5 mM $MgCl_2$ and 50 U endonuclease (Benzonase, Sigma) to digest nucleic acids. Equal volumes of both fractions were analysed side by side on Western blots. Results were confirmed in at least three independent experiments.

2.3.7. Confocal immunofluorescence microscopy

Cells were grown on glass cover slips (10 by 10 mm), transfected and incubated as indicated above, then washed twice in PBS, immediately fixed with 4% (v/v) formaldehyde in PBS for 30 min at room temperature, and subsequently permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min at room temperature. Myc-tagged PAK (Myc-PAK) was detected

with mouse anti-c-Myc clone 9E10 (Sigma), followed by goat anti-mouse TexasRed (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells were then briefly stained with 0.5 ng/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and washed in PBS, and the cover slips mounted in VectaShield (Vector Laboratories, Burlingame, CA, USA) and sealed with nail polish. Images were recorded with the 405-nm, 488-nm and 532-nm laser lines of a Leica TCS-SPE confocal microscope and processed with Leica and Adobe Photoshop software.

2.3.8. Luciferase reporter assay

Approximately 5 x 10^5 DLD-1 cells were seeded in 35-mm dishes, transfected with 50 ng of the pRL-TK luciferase reporter (for constitutive expression of *Renilla* luciferase as internal control; Promega, Fitchburg, WI, USA) and 1 μg of either standard NF-κB or pGL3-5xBCL-6 or pGL3 control reporter. For experiments titrating individual proteins, 500 to 1,000 ng of the indicated construct was co-transfected, whereas for the co-expression of two proteins the amount of construct was previously adjusted to yield comparable expression levels. At 16 to 20 h post-transfection in the absence or presence of the NADPH oxidase inhibitor DPI (Sigma) or the Rac inhibitor NSC23766 (Calbiochem), cells were lysed, assayed with the Dual-Luciferase reporter assay (Promega) following the manufacturer's instructions, and measured in an Anthos Lucy-2 luminometer. Lysates were assayed in duplicate samples and additional aliquots were analysed by Western blotting to document protein expression levels. All firefly luciferase values were first normalized to the internal control values obtained for *Renilla* luciferase and then plotted as

the increase over the value of untreated or vector control. The values displayed were from at least three independent transfection assays.

2.3.9. *In vitro* protein kinase assays

For *in vitro* protein kinase assays, either 1 µg of a recombinant fragment of human BCL-6 (amino acids 3 to 484 lacking the C-terminal zink finger domains) (sc-4105; Santa Cruz Biotechnology) or the beads containing immunoprecipitated GFP-tagged BCL-6 (GFP-BCL-6) protein were resuspended in 20 µl of kinase reaction buffer (30 mM Tris-HCl [pH 7.5], 10% [v/v] glycerol, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 37.5 mM $MgCl_2$ and 250 µM ATP) and incubated in the presence of 5 µCi [γ-^{32}P] ATP at 30ºC for 60 min with 25, 50, 100, or 200 ng recombinant PAK1 (#0357-0000-1; ProQuinase, Freiburg, Germany). Then, 5x Laemmli sample buffer was added to the reaction mixtures, and proteins were separated by SDS-PAGE and then transferred to PVDF membrane. The membrane was first analysed by autoradiography, followed by Western blotting using the indicated antibodies.

2.4. Results

2.4.1. Rac1 activation leads to an increase in NFKB1/p50 protein levels

Previously we reported that Rac1 signalling stimulates NF-κB transcriptional activity through both the canonical RelA/p50 pathway and the RelB/p52 dependent pathway in colorectal cells (Matos and Jordan, 2006). In the course of these studies, we noticed that the expression of

active Rac1 not only increased NF-κB reporter vector activity but also the protein level of p50, whereas expression of its dimerization partner RelA remained unaffected (Fig. 2.1). Moreover, we observed that p50 levels decreased when activation of endogenous Rac1 was repressed by the inhibitor NSC23766 (Gao *et al.*, 2004).

The p50 subunit is produced through constitutive proteolytic processing of the precursor protein p105, which is transcribed from the *NFKB1* gene. A previous report has shown evidence that the *NFKB1* gene promoter could be stimulated by RelA/p50 itself in hematopoietic cells (Cogswell *et al.*, 1993). In order to test whether the RelA complex is involved in regulating NFKB1/p50 expression in colorectal cells, we first co-transfected DLD-1 cells with expression vectors encoding a constitutively active Rac1-L61 mutant and the non-degradable super-repressor IκBα (A32A36) (DiDonato *et al.*, 1996), which inhibits RelA/p50 activation. We found that the presence of the super-repressor significantly inhibited the Rac1-mediated activation of the NF-κB transcriptional reporter, but the increase in p50 levels was still detected (Fig. 2.1). This increase in p50 was also observed when the Rac1-mediated production of ROS, an upstream event of RelA/p50 activation, was inhibited by the NADPH oxidase inhibitor DPI.

2.4.2. Rac1 regulates NFKB1 expression by releasing BCL-6-mediated transcriptional repression

A previous report has demonstrated that the *NFKB1* promoter contains binding sites for the transcription factor BCL-6 (Li *et al.*, 2005), a

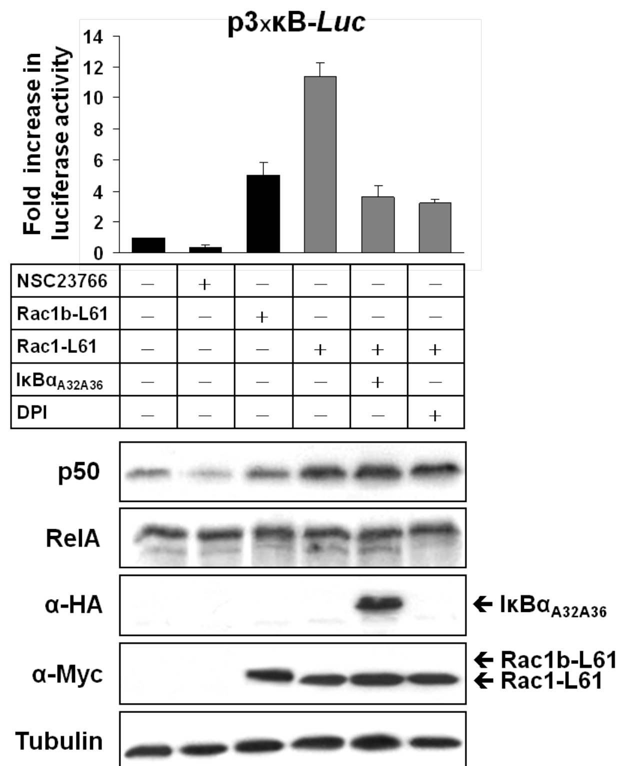

Figure 2.1. Rac1 activation modulates NFKB1/p50 protein levels. DLD-1 colorectal cells were transfected or treated with drug as indicated in the figure (+, transfected or treated with drug; –, not transfected or treated with drug) and lysed 24 h later. In one lysate aliquot, the luciferase activity of the co-transfected NF-κB reporter plasmid was measured, whereas in another aliquot, the indicated protein levels were determined by Western blotting. Note that the presence of an active Rac1 mutant was particularly efficient in stimulating reporter gene activity and in increasing p50 protein levels, while the amount of RelA remained unchanged (tubulin levels served as a loading control). Whereas reporter gene transcription was strongly inhibited by DPI (inhibitor of NADPH oxidase and ROS formation) or by the super-repressor IκBα (A32A36), both treatments had no effect on the Rac1-L61-stimulated p50 increase. (NSC23766 is an inhibitor of endogenous Rac1 activation).

repressor identified in B-cell lymphoma (Seyfert *et al.*, 1996; Dalla-Favera *et al.*, 1999; Staudt *et al.*, 1999). In addition, a highly related repressor protein, BAZF/BLC6b, has been identified (Sakashita *et al.*, 2002), which binds the same promoter sites as BCL-6. To analyse the contribution of

both factors to NFKB1 expression, we first determined by RT-PCR whether endogenous *BCL-6* and *BAZF* transcripts were expressed in three colorectal cell lines as well as in the B-cell precursor leukemia 697 cell line and erythroleukemia HEL cells as positive controls. We found endogenous *BCL-6* transcript expression in the three colorectal and the erythroleukemia cell line, whereas *BAZF* transcript was expressed only in HEL cells (Fig. 2.2A). These data identified BCL-6 as a candidate regulator of NFKB1/p50 expression in colorectal cells, prompting us to transfect cells with increasing amounts of an expression vector encoding the BCL-6 protein. Intriguingly, the endogenous *NFKB1* transcript expression (Fig. 2.2B), as well as the corresponding NFKB1/p50 protein levels (Fig. 2.2C), were clearly inhibited by the expression of BCL-6. In contrast, expression of active Rac1 led to increased expression of both the *NFKB1* transcript (Fig. 2.2B) and NFKB1/p50 protein (Fig. 2.2C). Conversely, when the endogenous Rac1 activation in DLD-1 cells was impaired by treating cells with the Rac inhibitor NSC23766, expression of *NFKB1* transcript and NFKB1/p50 protein was inhibited (Fig. 2.2B and 2.2C). These data indicated that Rac1 signalling could modulate *NFKB1* gene expression via the transcriptional repressor BCL-6.

In order to test whether Rac1 activation can regulate the transcriptional activity of BCL-6, we utilized a previously described BCL-6-controlled reporter gene (Huynh *et al.*, 2000). When this reporter was co-expressed with BCL-6, a clear repression of transcriptional activity was observed (Fig. 2.3A). Repression was also evident when activation of endogenous Rac1 was inhibited with NSC23766. In contrast, transcriptional activity of the reporter was clearly promoted by siRNA-mediated depletion of endogenous BCL-6 or upon co-transfection with Rac1-L61, in a dose-dependent manner (Fig. 2.3A). These data provide

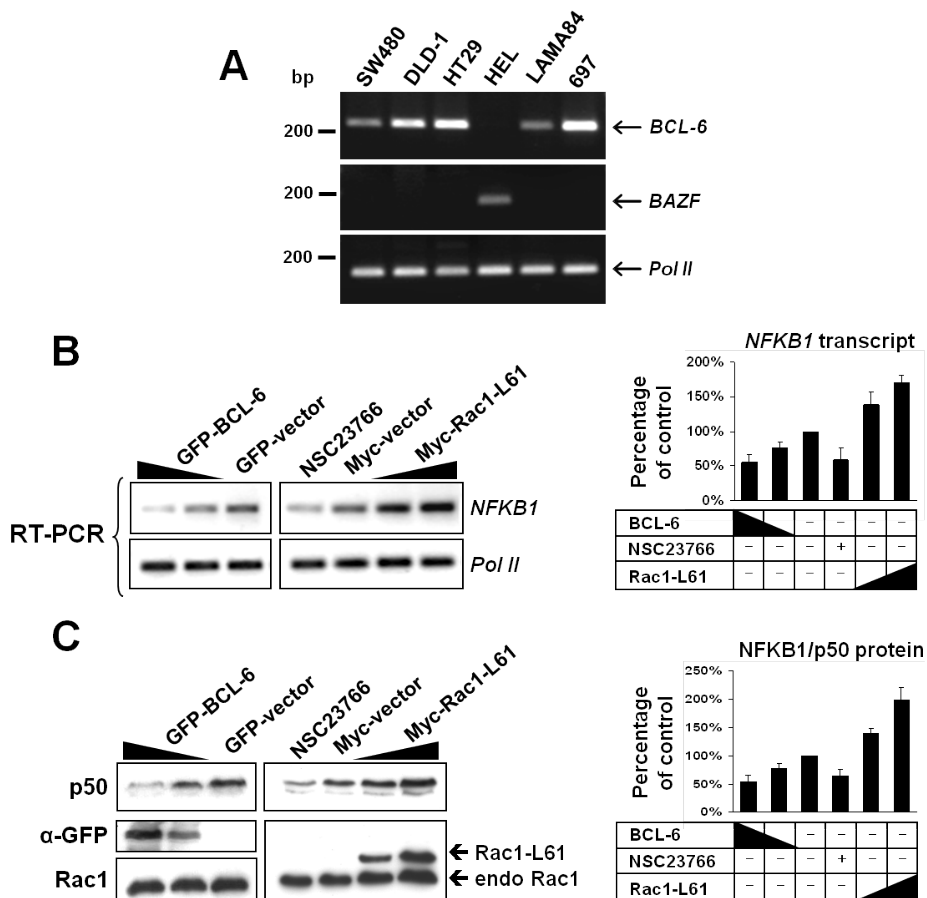

Figure 2.2. Rac1 modulates NFKB1 expression via BCL-6. (A) The expression of endogenous BCL-6 and the highly related repressor protein BAZF/BLC6b was tested by RT-PCR in three colorectal cell lines and three hematopoietic cell lines, as indicated. The amplification of RNA polymerase II (Pol II) served as internal control. **(B and C)** Role of BCL-6 overexpression or modulation of Rac1 signalling in NFKB1/p50 expression. DLD-1 cells were transfected with either GFP control vector versus increasing amounts (indicated by the height of the black triangle) of GFP-BCL-6, or with Myc control vector versus increasing amounts of Myc-Rac1-L61, or mock transfected and treated with the Rac1 inhibitor NSC23766. Cells were lysed following 24 h to isolate either total RNA or whole protein. **(B, left panel)** *NFKB1* or control *Pol II* transcripts were amplified by semi-quantitative RT-PCR, and (right panel) band intensities were quantified from digital images by densitometry. **(C, left panel)** Western blot showing NFKB1/p50 protein levels as well as levels of transfected GFP-BCL-6 or Myc-Rac1-L61. **(B and C, right panels)** Detection of endogenous Rac1 served as a loading control, and band intensities were quantified by densitometry. Symbols: +, transfected or treated with drug; –, not transfected or treated with drug. α-GFP, anti-GFP antibody.

substantial evidence that Rac1 signalling regulates gene expression via BCL-6. Interestingly, we observed that the splice variant Rac1b could not significantly affect the BCL-6-controlled reporter gene (Fig. 2.3A).

We next asked whether another endogenous BCL-6 target gene, the cell adhesion molecule *CD44* (Shaffer *et al.*, 2000), was modulated by Rac1 activation in DLD-1 cells. We observed that ectopic expression of BCL-6 or inhibition of endogenous Rac1 activity led to decreased *CD44* transcript expression, whereas transfection of Rac1-L61 promoted an increase (Fig. 2.3B). Together, our results strongly indicate that Rac1 activation releases BCL-6 repression from target genes including *NFKB1*, *CD44* and a BCL-6-specific luciferase reporter.

2.4.5. Active Rac1 induces nuclear redistribution and chromatin release of BCL-6

In order to obtain mechanistic insights into how Rac1 activation would influence BCL-6 activity, we first studied its effect on the subcellular localization of BCL-6 using immunofluorescence microscopy and cell fractionation. As shown in Figure 2.4A, the expression of BCL-6 alone revealed a strictly nuclear localization in DLD-1 cells with the typical concentration of BCL-6 in numerous nuclear dots that has previously been described (Cattoretti *et al.*, 1995; Huynh *et al.*, 2000). In the presence of Rac1-L61, BCL-6 lost accumulation in nuclear dots and appeared diffuse in the nucleoplasm. In contrast, splice variant Rac1b-L61 had little effect on the accumulation of BCL-6 in dots in the nucleus (Fig. 2.4A). This is in agreement with the poor stimulation of the BCL-6-controlled reporter gene that we observed for Rac1b-L61 (Fig. 2.3A).

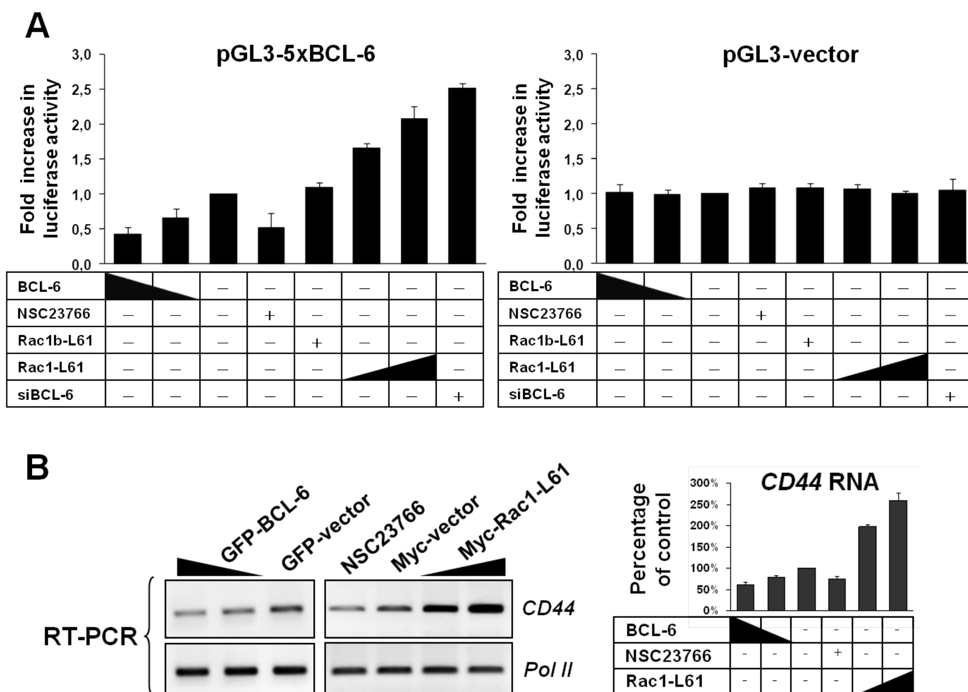

Figure 2.3. Rac1 releases transcriptional repression by BCL-6. (A) DLD-1 cells were transfected with a transcriptional luciferase reporter vector under the control of five consensus BCL-6 binding motifs (Huynh *et al.*, 2000) or the respective empty pGL3 control vector. Cells were co-transfected with the indicated expression vectors and siRNAs or mock transfected and treated with the Rac1 inhibitor NSC23766. The increasing or decreasing amount of vector is indicated by the height of the black triangle. **(B)** Effect of BCL-6 or Rac1 signalling on the endogenous BCL-6 target gene *CD44*. (*Left panel*) Cells were treated as described in the legend to Figure 2.2, *CD44* or control *Pol II* transcripts were amplified by semi-quantitative RT-PCR and band intensities were quantified from digital images by densitometry (*right panel*). Symbols: +, transfected or treated with drug; –, not transfected or treated with drug. siBCL-6, BCL-6-specific siRNA.

To test whether these differences in localization would represent altered chromatin binding, we applied a previously described cell fractionation protocol (Kazansky *et al.*, 1999; Dhordain *et al.*, 2000; Solan *et al.*, 2002), which separates transcription factors into a soluble pool that is extracted from the nucleus and into a chromatin-bound pool that remains insoluble. Under these experimental conditions, the expression of

BCL-6 alone revealed the majority of the protein in the insoluble chromatin-bound fraction (Fig. 2.4B). This is compatible with its role as a transcriptional repressor and corroborates the inhibition of *NFKB1* and *CD44* gene expression observed in Figures 2.2B and 2.3B. When we determined the fractionation of BCL-6 in cells co-expressing an active mutant of the splice variant Rac1b, only a very small increase in the soluble fraction was observed. In contrast, co-expression of activated Rac1 led to a remarkable transition of BCL-6 from the chromatin-bound insoluble fraction into the soluble pool (Fig. 2.4B). In these experiments, the total amount of BCL-6 protein apparently remained unaffected.

Altogether, these data demonstrate that upon activation of Rac1, the transcription factor BCL-6 becomes relocalized within the nucleus, is no longer retained in the chromatin-bound fraction and loses its activity to repress target genes.

2.4.6. Modulation of BCL-6 by Rac1 signalling requires PAK1

For further insights into the effect of Rac1 on BCL-6 activity, we tested the interaction of Rac1 and BCL-6 by co-immunoprecipitation. Whereas Rac1-L61 co-precipitated with RelB, in agreement with previously described data (Matos and Jordan, 2006), we found no evidence for the formation of a stable complex between BCL-6 and active Rac1 under the same experimental conditions (Fig. 2.5). The lack of interaction between Rac1 and BCL-6 suggested that Rac1 affects BCL-6 activity indirectly through a downstream signalling pathway.

The generation of ROS through the stimulation of NADPH oxidase activity is a Rac function conserved in immune and epithelial cells. Moreover, ROS are known to modulate the activity of several transcription

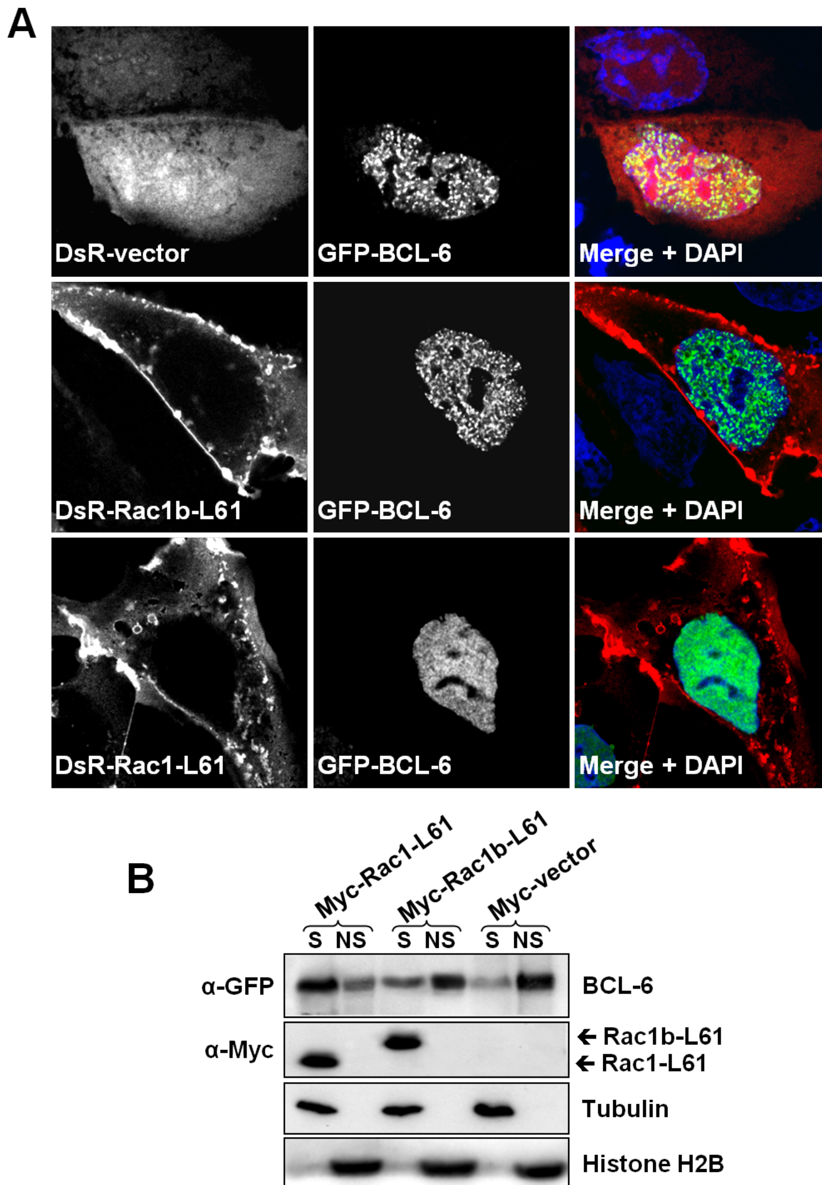

Figure 2.4. Active Rac1 affects subnuclear location and chromatin binding of BCL-6. (A) DLD-1 cells were co-transfected with GFP-BCL-6 and either DsRed empty vector, DsRed-Rac1-L61 or DsRed-Rac1b-L61 as indicated. Cells were fixed after 24 h, nuclei were counterstained with DAPI, and fluorescent signals were recorded by confocal microscopy. **(B)** Presence of BCL-6 in a soluble or chromatin-bound form. DLD-1 cells were co-transfected with GFP-BCL-6 and the indicated Myc-tagged vectors and lysed after 24 h so that a soluble (S) and a non-soluble (NS), chromatin-bound fraction was obtained. Western blot analysis

of these fractions is shown. Histone 2B was detected as a marker for insoluble chromatin-bound proteins, and β-tubulin was detected as a marker for soluble factor. α-GFP, anti-GFP antibody; α-Myc, anti-Myc antibody.

factors (Wu, 2006), including NF-κB downstream of Rac1 and Rac1b (Matos and Jordan, 2006). We therefore treated Rac1-L61-expressing cells with DPI, a cell-permeable inhibitor of the NADPH oxidase widely used to block the generation of ROS (Sulciner *et al.*, 1996; Sundaresan *et al.*, 1996; Bonizzi *et al.*, 1999; Matos and Jordan, 2006). We found that, although this treatment clearly inhibited activation of an NF-κB-driven luciferase reporter (Fig. 2.1), it had no effect on the increase in the BCL-6-driven reporter activity (Fig. 2.6A).

In order to determine whether the observed modulation of BCL-6 repression was Rac1 specific, we compared the effects of activated RhoA, Rac1, and Cdc42. We found that active Cdc42 also produced a moderate but significant stimulation of the BCL-6 reporter (Fig. 2.6A).

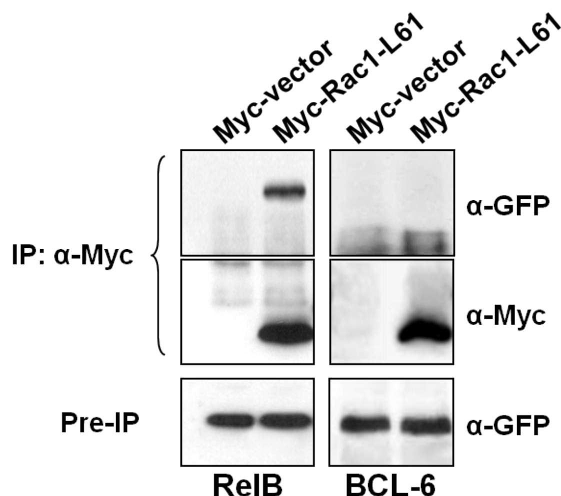

Figure 2.5. Active Rac1 and BCL-6 are not found in a protein complex. DLD-1 cells expressing Myc-control vector or Myc-Rac1-L61 were co-transfected with either GFP-BCL-6 or GFP-RelB. Whereas RelB co-immunoprecipitated with Myc-Rac1-L61, confirming previously described data (Matos and Jordan, 2006), no such complex was detected between Rac1-L61 and BCL-6. IP: α-Myc, immunoprecipitation with anti-Myc antibody; Pre-IP, pre-immunoprecipitation.

Stimulation of the protein kinases PAK and JNK are two classical downstream pathways that are stimulated by Rac1 and Cdc42, but not by RhoA or by the Rac1b splice variant (Matos *et al.*, 2003; Singh *et al.*, 2004), both of which failed to inhibit BCL-6 activity (Fig. 2.6A). Thus, the activity of the BCL-6 luciferase reporter was analysed in cells transfected with previously described constitutively active mutants of JNK (SAPKβ-MKK7) (Rennefahrt *et al.*, 2002) and PAK1 (PAK1-T423E) (Sells *et al.*, 1997). As shown in Fig. 2.6A, active JNK had no effect, whereas the expression of constitutively active PAK1 significantly stimulated transcription from the BCL-6 reporter. We further determined whether the catalytic activity of PAK1 was involved in the observed BCL-6 reporter stimulation. The reporter vector was co-transfected with active Rac1-L61 in the presence of a dominant-negative, kinase-dead PAK1 mutant (PAK1-K299R) (Sells *et al.*, 1997). These experiments revealed a clear reduction in Rac1-mediated transcriptional stimulation (Fig. 2.6A), suggesting that the Rac1-L61-stimulated transcription from the BCL-6 reporter required Rac1-induced PAK activation.

GTP-bound Rac1 can activate PAK1 (α-PAK), PAK2 (γ-PAK), and PAK3 (β-PAK). Thus, we determined which endogenous PAK isoform could be mediating the observed effects downstream of active Rac1 in DLD-1 cells. Using RT-PCR, we found that only *PAK1* and *PAK2* transcripts were expressed in colorectal cells, whereas *PAK3* transcript was detected in a glioblastoma cell line (Fig. 2.6B). In order to directly compare the expression levels of PAK1 and PAK2, colorectal cell lysates were analysed by Western blotting using an anti-PAK1/2/3 antibody. We found that PAK1 was by far the most prominent isoform expressed (Fig. 2.6C). We then determined whether the Rac1-L61 stimulated transcription from the BCL-6 reporter required endogenous PAK1. Expression of

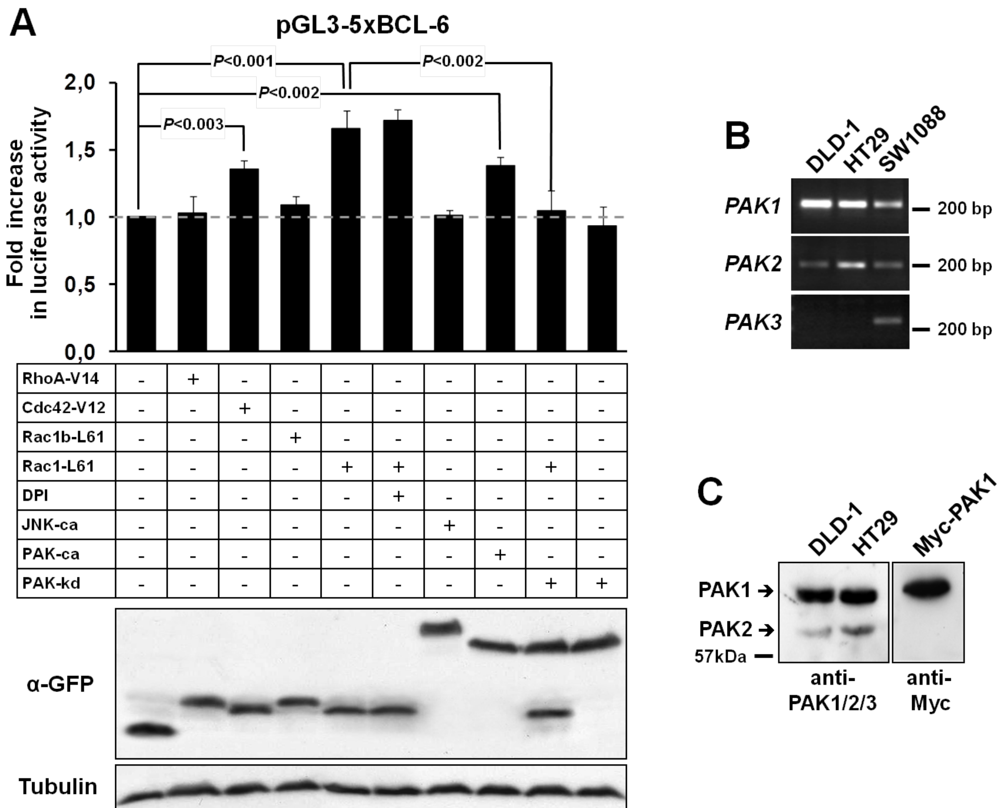

Figure 2.6. PAK1 acts downstream of Rac1 in the release of transcriptional repression by BCL-6. (A) DLD-1 cells were transfected with the transcriptional BCL-6 luciferase reporter vector and one of the indicated GFP-tagged expression vectors encoding either activated small GTPase mutants or protein kinase mutants. Luciferase activity was determined in cell lysates and expression of transfected proteins was documented by Western blotting. A graph with the observed changes in luciferase activity relative to GFP empty vector-transfected control cells (*top panel*) and immunoblots with the expression levels of the GFP-tagged proteins (*middle panel*) and β-tubulin as a loading control (*bottom panel*) are shown. The migration of molecular weight markers is indicated. Note that transcriptional repression by BCL-6 was released in the presence of Rac1-L61, Cdc42-V12 and a constitutively active (ca) PAK1 mutant, whereas a kinase-dead (kd) PAK1 prevented the Rac1-L61 mediated increase in luciferase activity. **(B)** RT-PCR analysis to determine the expression of PAK1, PAK2, and PAK3 in DLD-1 and HT29 colorectal cells compared to SW1088 glioblastoma cells. **(C)** Western blot analysis to directly compare the expression levels of PAK1 and PAK2 in DLD-1 or HT29 cells using an anti-PAK1/2/3 antibody. Note that PAK1 is the most prominent isoform expressed. Symbols: +, transfected or treated with drug; –, not transfected or treated with drug.

endogenous PAK1 or endogenous PAK2 was depleted by transfection of cells with specific siRNAs. As shown in Fig. 2.7A, these oligonucleotides specifically depleted either PAK1 or PAK2, however, only the depletion of PAK1 affected both the endogenous and the Rac1-L61-stimulated 5xBCL-6 activation (Fig. 2.7B). In addition, prior incubation of DLD-1 cells with IPA-3, a specific inhibitor that prevents activation of group I PAKs by allosteric targeting of their autoregulatory domain, blocked the effect of Rac1-L61 on the 5xBCL-6 reporter (Fig. 2.7B). In these experiments, no detectable changes in the total amount of BCL-6 protein were observed.

Altogether, these data indicate that PAK1 is a critical link between Rac1 activation and transcriptional repression by BCL-6.

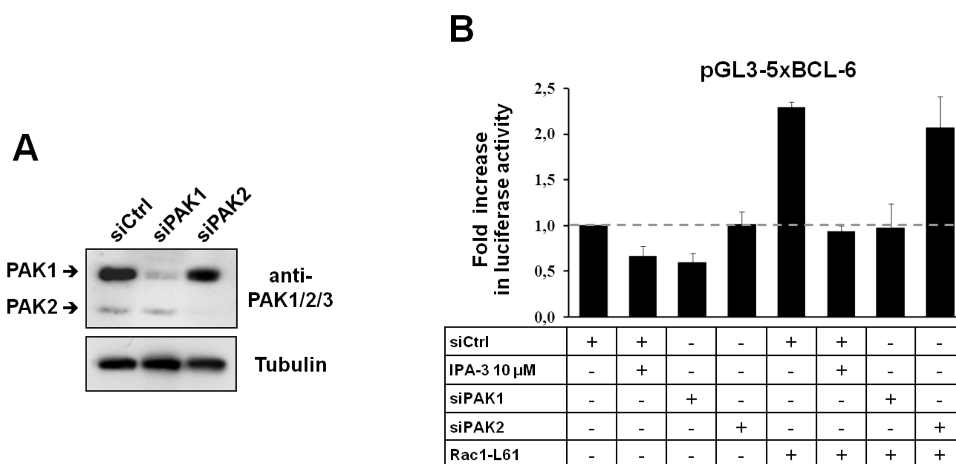

Figure 2.7. Interference with PAK1 by depletion or inhibitor treatment blocks Rac1-mediated activation of BCL-6. (A) Western blot showing the efficiency and specificity of PAK1- or PAK2-specific siRNAs transfected into DLD-1 cells. Detection of β-tubulin served as a loading control. **(B)** DLD-1 cells were transfected with the indicated siRNAs and 24 h later transfected again with the 5xBCL-6 transcriptional luciferase reporter vector in the presence (+) or absence (–) of Rac1-L61. When indicated, cells were incubated with 10 μM PAK inhibitor IPA-3. Symbols: +, transfected or treated with drug; –, not transfected or treated with drug. Ctrl, control.

2.4.7. PAK1 binds to and phosphorylates BCL-6

Since PAK1 overexpression stimulated the BCL-6 reporter, we used immunofluorescence microscopy to test whether PAK1 could also affect the nuclear redistribution of BCL-6 observed in the presence of active Rac1. As shown in Fig. 2.8A, overexpression of the kinase-dead Myc-PAK1-K299R mutant apparently enhanced the dot-like localization pattern of BCL-6 in the nucleus, whereas overexpression of a kinase-competent PAK1 redistributed BCL-6 to a more diffuse nucleoplasmic pattern (Fig. 2.8B). In addition, the expression of PAK1 decreased the amount of BCL-6 remaining in the insoluble chromatin-bound cell fraction, whereas kinase-dead PAK1 did not affect chromatin-binding (Fig. 2.8C). PAK1 also clearly localized to the nucleus. In fact, we observed a correlation between the expression level of BCL-6 and the recruitment of PAK1 from the cytoplasm into the nucleus. In particular, a pixel intensity analysis in confocal images revealed that cells with an equivalent overall level of ectopic PAK1 expression (Fig. 2.8D, top graph) differed in their nuclear PAK1 signal (Fig. 2.8D, bottom graph), depending on the expression level of BCL-6 (Fig. 2.8D, middle graph).

The generation of pixel overlap maps from the confocal images allowed the calculation of Pearson's correlation values, which suggested colocalization between nuclear PAK1 and BCL-6 (data not shown). We thus analysed whether a PAK1/BCL-6 complex could be isolated by co-immunoprecipitation from DLD-1 colorectal cells. Using the previously described co-precipitation of Rac1-L61 with PAK1 (Matos *et al.*, 2003) as a positive control, we demonstrate that PAK1 can form a stable complex with BCL-6 (Fig. 2.8E).

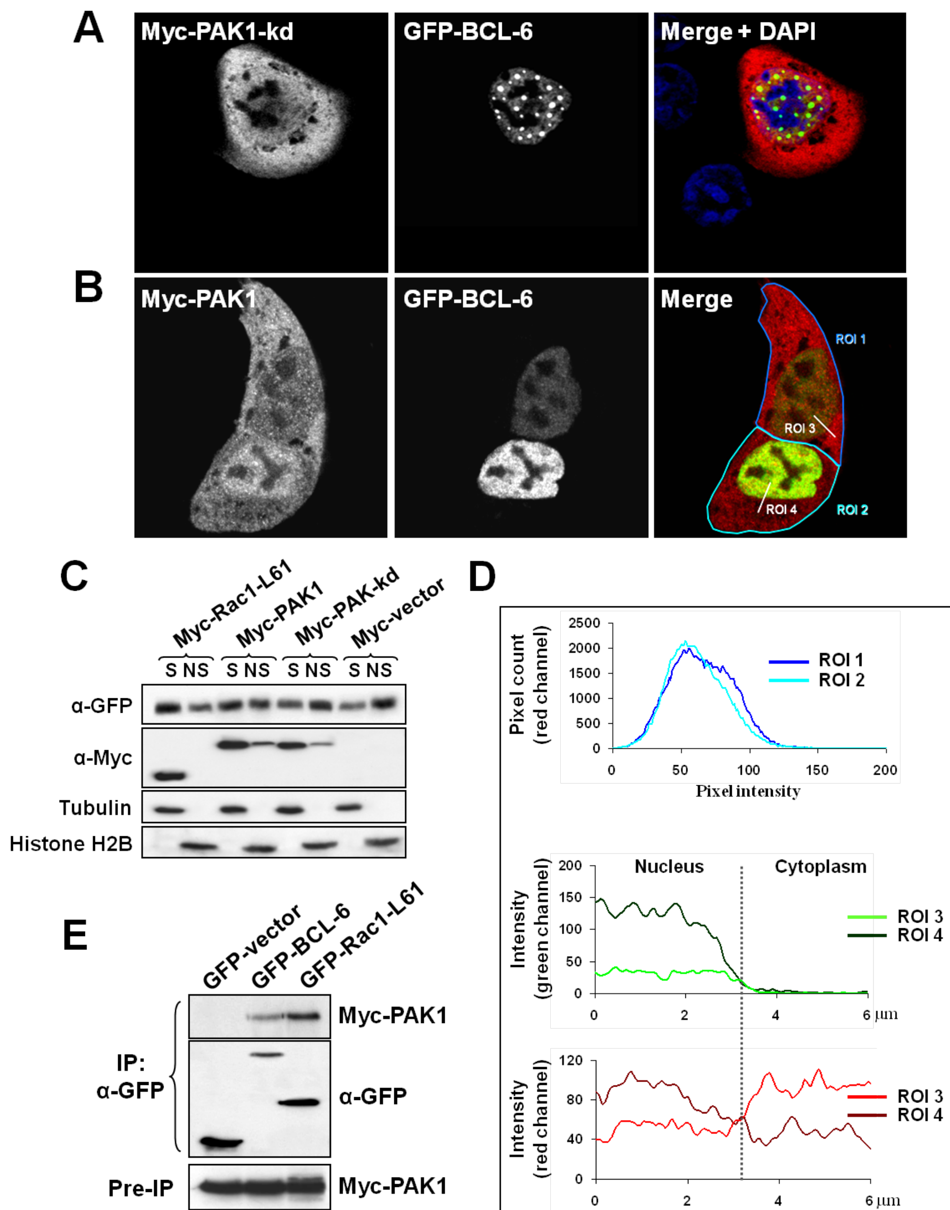

Figure 2.8. Effect of PAK1 on nuclear BCL-6. (A and B) DLD-1 cells were co-transfected with GFP-BCL-6 and the kinase-dead Myc-PAK1-K299R mutant **(A)** or with wild-type Myc-PAK1 **(B)**. The cells were fixed after 20 h and analysed by confocal microscopy. Note in panel A, the increase in nuclear dot localization of BCL-6 in the presence of dominant-negative PAK1 but the diffuse nucleoplasmic pattern with wild-type PAK1 in panel B. ROI 1, region of interest 1.

(C) Presence of BCL-6 in the chromatin-bound fraction. DLD-1 cells were co-transfected with GFP-BCL-6 and the indicated Myc-tagged vectors and separated into a soluble (S) and a non-soluble (NS) chromatin-bound fraction, as described in the legend to Fig. 2.4B. **(D)** The intensity of the overall fluorescent PAK1 signal in the two representative cells shown in panel B, with different amounts of BCL-6 expression, was determined (*top panel*, compare regions of interest ROI 1 and ROI 2) and found to be equivalent. Then the intensities of the nuclear versus cytoplasmic PAK1 and BCL-6 signals were compared along the axes indicted as ROI 3 and ROI 4. Note that the distribution of PAK1 signal between the nucleus and the cytoplasm (ROI 3 and ROI 4 in the red channel [*bottom panel*] correlates with the signal intensity of BCL-6 in the nucleus (ROI 3 and ROI 4 in green channel [*middle panel*]). **(E)** BCL-6 and PAK1 co-immunoprecipitate. DLD-1 cells were co-transfected with Myc-PAK1 and either GFP control vector or GFP-BCL-6 or GFP-Rac1-L61 as a positive control. Cells were lysed after 24 h, extracts were incubated with anti-GFP antibodies (α-GFP) and the presence of co-precipitated Myc-PAK1 was analysed (*top panel*). Successful precipitation of GFP-tagged proteins (*middle panel*) as well as equal expression of Myc-PAK1 in total cell extracts (Pre-IP, bottom panel) is also shown. α-Myc, anti-Myc antibody.

Since BCL-6 can be phosphorylated by mitogen-activated protein kinases (MAPKs) (Niu *et al.*, 1998), we asked whether it could be a direct substrate for PAK1. Using an *in vitro* phosphorylation assay, we added increasing amounts of full-length, recombinant PAK1 to a recombinant 484-amino acid fragment of the BCL-6 protein. Under these conditions, a concomitant increase of BCL-6 phosphorylation was observed (Fig. 2.9A, top panel), which generated electrophoretic band-shifts that were confirmed by Western blot analysis with an anti-BCL-6 serum (Fig. 2.9A, middle panel). These data suggest the presence of multiple phosphorylation sites for PAK1 in the BCL-6 fragment.

To confirm that PAK1 also phosphorylates the full-length protein, GFP-BCL-6 was immunoprecipitated from DLD-1 cells using stringent RIPA buffer conditions to avoid background phosphorylation events and then incubated *in vitro* with recombinant PAK1. As shown in Fig. 2.9B, full-length GFP-BCL-6 became clearly phosphorylated by PAK1, although the higher molecular weight of the GFP-tagged protein did not allow the

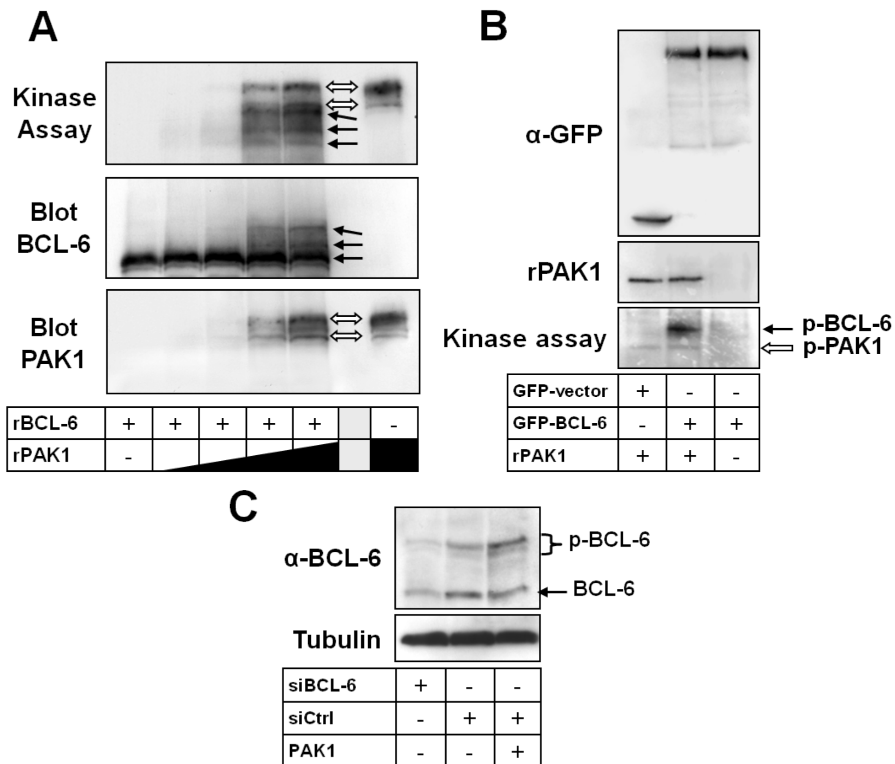

Figure 2.9. PAK1 phosphorylates BCL-6. (A) A recombinant N-terminal BCL-6 fragment (rBCL-6) and increasing amounts of recombinant PAK1 (rPAK1) were incubated in an *in vitro* protein kinase assay before proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were exposed to X-ray films (*top panel*), followed by sequential immunoblot detection of BCL-6 (*middle panel*) and PAK1 (*bottom panel*). Note the shifts in electrophoretic migration of the phosphorylated rBCL-6 as well as of autophosphorylated PAK1 bands. The presence (+) or absence (–) of rBCL-6 and the increasing amount of rPAK1 is indicated by the height of the black triangle or black rectangle. **(B)** GFP control vector or full-length GFP-BCL-6 were transfected into DLD-1 cells, immunoprecipitated with anti-GFP antibodies (α-GFP) using RIPA buffer and then incubated *in vitro* in the presence (+) or absence (–) of 200 ng rPAK1. Western blots to document successful protein precipitation (*top panel*) and the presence of rPAK1 (*middle panel*) are shown. The corresponding autoradiograph shows phosphorylation of GFP-BCL-6 by PAK1 as well as PAK1 autophosphorylation (*bottom panel*). **(C)** Detection of endogenous BCL-6 by Western blotting in DLD-1 cells transfected with either BCL-6-specific siRNAs (siBCL-6) or control siRNA (siCtrl) in the presence or absence of Myc-PAK1. Two specific bands were detected, and the shift in electrophoretic migration upon transfection of PAK1 indicates that the top BCL-6 band is endogenous phospho-BCL-6 (p-BCL-6) (bars show migration of 75- and 100-kDa markers).

detection of possible band shifts. We then analysed whole-cell lysates and detected two BCL-6 protein bands of about 80 and 120 kDa by Western blotting, both of which became specifically depleted upon transfection of cells with BCL-6 siRNAs (Fig. 2.9C). The higher-molecular-mass band was clearly increased upon transfection of cells with PAK1, indicating a phosphorylation-dependent band shift of the endogenous BCL-6 protein.

2.5. Discussion

The main finding in this work is that Rac1 regulates the transcription factor BCL-6 via PAK1 and counteracts the repression of its target genes.

The BCL-6 transcriptional repressor is one of the most frequently translocated genes in B-cell non-Hodgkin's lymphomas (Dalla-Favera *et al.*, 1999; Staudt *et al.*, 1999). *BCL-6* translocations do not alter the *BCL-6* coding sequence but associate the gene with other promoter region, such as the IgH enhancer, and this deregulates BCL-6 expression. Moreover, the activity of BCL-6 can be modulated through post-translational modifications. For instance, acetylation of lysine 379 downregulates its ability to repress transcription, probably due to impaired recruitment of histone deacetylases (Bereshchenko *et al.*, 2002), while phosphorylation by MAPKs in B cells was shown to target BCL-6 for rapid degradation by the ubiquitin-proteasome pathway (Niu *et al.*, 1998; Phan *et al.*, 2007).

More recently, evidence has accumulated that BCL-6 is also expressed in non-hematopoietic cells. Its expression was detected in olfactory sensory neurons (Otaki *et al.*, 2005), in healthy skin and epidermal neoplasms (Kanazawa *et al.*, 1997), in uroepithelial cells (Lin *et al.*, 2003; Huang *et al.*, 2007) and in epithelial cells of the mammary gland

(Logarajah *et al.*, 2003). BCL-6 expression has also been detected in HeLa cells (Allman *et al.*, 1996), and in this study, we document for the first time its expression in colorectal cells.

Here we found that Rac1 signalling affects the subnuclear localization and transcriptional repressor activity of BCL-6 and demonstrated these effects using four different approaches. First, a reporter construct expressing the luciferase gene under the control of five BCL-6 binding sites immediately upstream of the simian virus 40 (SV40) promoter (Huynh *et al.*, 2000) was used and shown to become repressed in cells co-transfected with BCL-6, as expected. Repression of reporter activity was further observed when endogenous Rac1 activation was diminished by treating cells with the Rac1-specific inhibitor NSC23766. In contrast, expression of an active Rac1 mutant led to increased luciferase activity. These data clearly indicate a modulation of BCL-6 in response to Rac1 signalling. Second, the expression of two previously described endogenous BCL-6 target genes, *NFKB1* (Li *et al.*, 2005) and *CD44* (Shaffer *et al.*, 2000), was analysed. As was observed with the BCL-6 reporter, overexpression of BCL-6 and inhibition of endogenous Rac1 activation by NSC23766 decreased expression of these genes. In contrast, transfection of activated Rac1 increased their expression up to 2- and 2.6-fold, respectively. Third, we determined the subnuclear distribution of BCL-6, which has previously been reported to accumulate in characteristic nuclear foci (Cattoretti *et al.*, 1995; Huynh *et al.*, 2000). In the presence of active Rac1, a clear redistribution of BCL-6 from these nuclear foci to a more diffuse, homogenous nucleoplasmic localization was observed (Fig. 2.4A). Fourth, our cell fractionation studies corroborate these results by showing the transition of BCL-6 from an insoluble, chromatin-bound form into a soluble form in the presence of active Rac1

(Fig. 2.4B). Together, these data provide substantial evidence that BCL-6-mediated gene repression is negatively regulated by Rac1 signalling.

The mechanism of how Rac1 affects BCL-6 activity apparently does not involve formation of a stable complex between BCL-6 and active Rac1, because both proteins did not co-immunoprecipitate (Fig. 2.5). Although one cannot disregard the possibility of a transient interaction occurring between the two proteins, these data suggest that BCL-6 rather responds to a signalling pathway downstream of Rac1.

Rac signalling activates the production of ROS via NADPH oxidases (Schuringa *et al.*, 2001), and ROS are known to modulate the activity of several transcription factors such as AP-1, ETS, Smad, Snail and NF-κB (Wu, 2006). However, we demonstrate that Rac1-induced production of NADPH oxidases had no effect on BCL-6 activity. Overexpression of activated Cdc42 also induced a weak but significant stimulation of the BCL-6 reporter, and Cdc42 shares the downstream effectors PAK and JNK with Rac1 (Hall, 2005). Because BCL-6 can be downregulated by phosphorylation in B cells (Niu *et al.*, 1998; Phan *et al.*, 2007), we tested the effect of constitutively active kinase mutants on BCL-6 activity.

We found that the activation of PAK was required for the observed changes in BCL-6 activity. PAK1 was found to be the predominantly expressed PAK isoform in the colorectal cells studied, and its overexpression mimics the effect of active Rac1 on nuclear distribution, chromatin-binding, or transcriptional activity of BCL-6. Moreover, interfering with PAK1 function by siRNA-mediated depletion, cell treatment with inhibitor IPA-3, or expression of a dominant-negative PAK1 mutant strongly inhibited the effect of Rac1-L61 on the BCL-6 reporter. In all these experiments, no detectable changes in the total amount of BCL-6

protein were observed. PAK1 could be isolated in a protein complex with BCL-6 and was able to phosphorylate BCL-6 *in vitro* (Fig. 2.9). PAK1 was further recruited to the cell nucleus in BCL-6-overexpressing cells and colocalized with BCL-6 in the nucleoplasm. Together, these data identify PAK1 as the critical mediator between Rac1 activation and BCL-6 downregulation.

PAK1 phosphorylation has been found to modulate various transcriptional regulators with respect to their transcriptional activity, subnuclear location and nuclear import or export (Kumar *et al.*, 2006). Our results therefore reveal that the regulation of BCL-6 repressor activity by PAK1 constitutes yet another pathway through which this kinase exerts its control over specific transcriptional events. In addition, the identification of PAK1 as the link between Rac1 and BCL-6 is in agreement with our observation that splicing variant Rac1b, which was previously shown unable to stimulate PAK1 activation (Matos *et al.*, 2003; Singh *et al.*, 2004), had no significant effect on the BCL-6 transcriptional reporter activity, on the subnuclear localization of BCL-6, and on its transition into a soluble nuclear fraction.

One of the physiological target genes for BCL-6 repression is *NFKB1* encoding the p105 precursor protein for the NF-κB member p50. In response to Rac1 signalling, we found increased levels of *p105* transcripts and of p50 protein, which is generated from p105 via a constitutive proteolytic pathway (Karin *et al.*, 2002; Moorthy *et al.*, 2006). p50 can dimerize with various Rel partner proteins and the resulting protein complexes can either activate or repress transcription (Fujita *et al.*, 1993; Baer *et al.*, 1998; Zhong *et al.*, 2002; Driessler *et al.*, 2004; Wessells *et al.*, 2004; Guan *et al.*, 2005). Therefore, the physiological effect of increasing p50 levels in response to Rac1 activation is not readily

apparent. We speculate that a short-term effect will be an increased availability of transcriptionally competent Rel protein/p50 dimers that make any NF-κB stimulation more robust. On a longer term, however, increasing p50 levels may favor the formation of p50/p50 homodimers that can act as repressors and downregulate the NF-κB response, similar to what has been described during the inflammatory response (Baer *et al.*, 1998; Driessler *et al.*, 2004).

Another endogenous BCL-6 target gene that is expressed in colon is *CD44* (Shaffer *et al.*, 2000). The CD44 family is a family of cell-surface glycoproteins involved in cell-matrix adhesion and growth factor presentation and was shown to influence cell growth, survival and differentiation. Members of the CD44 family have been implicated in the progression and metastasis of tumours (Ponta *et al.*, 2003), including colorectal tumours (Wielenga *et al.*, 1993; Herrlich *et al.*, 1995; Gotley *et al.*, 1996; Ropponen *et al.*, 1998; Wielenga *et al.*, 1999). Our data therefore suggest that a deregulation of Rac1 signalling may contribute to the altered CD44 expression described in colorectal tumours.

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Chapter 3

Rac1 signalling modulates a STAT5/BCL-6 transcriptional switch on cell-cycle-associated target gene promoters

Patrícia Barros, Eric W.-F. Lam, Peter Jordan
and Paulo Matos

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Author's Note

The work described in the previous chapter raised additional questions that were addressed in this chapter.

This study included a collaboration with Prof. Eric Lam's lab, where the thesis author had the opportunity to stay for a short period to learn from their expertise and experience in the use of the chromatin immunoprecipitation technique.

The author of this thesis declares to have conducted the majority of the described experimental work and contributed to the experimental design and manuscript writing.

3.1. Summary

Gene expression depends on binding of transcriptional regulators to gene promoters, a process controlled by signalling pathways. The transcriptional repressor BCL-6 downregulates genes involved in cell-cycle progression and becomes inactivated following phosphorylation by the Rac1 GTPase-activated protein kinase PAK1. Interestingly, the DNA motifs recognized by BCL-6 and STAT5 are similar. Because STAT5 stimulation in epithelial cells can also be triggered by Rac1 signalling, we asked whether both factors have opposing roles in transcriptional regulation and whether Rac1 signalling may coordinate a transcription factor switch. We used chromatin immunoprecipitation to show that active Rac1 promotes release of the repressor BCL-6 while increasing binding of STAT5 to a BCL-6-regulated reporter gene. We further show in colorectal cell lines that the endogenous activation status of the Rac1/PAK1 pathway correlated with the phosphorylation status of BCL-6 and STAT5. Three cellular genes (cyclin D2, $p15^{INK4B}$, small ubiquitin-like modifier 1) were identified to be inversely regulated by BCL-6 and STAT5 and responded to Rac1 signalling with increased expression and corresponding changes in promoter occupancy. Together, our data show that Rac1 signalling controls a group of target genes that are repressed by BCL-6 and activated by STAT5, providing novel insights into the modulation of gene transcription by GTPase signalling.

3.2. Introduction

A crucial process in gene expression is the initiation of gene transcription. Before ribonucleic acid (RNA) polymerase II can transcribe the coding information of a given gene into RNA, it generally needs to be recruited to the respective gene promoter by specific transcription factors. These factors recognize conserved short DNA sequence motifs in the promoter but usually only bind to them following transcription factor activation and chromatin remodelling. Consequently, transcriptional regulation is frequently preceded by cellular signalling events. For example, activation of growth factor receptors at the plasma membrane stimulates the Ras/RAF/extracellular signal-regulated kinase (ERK) pathway, and activated ERK translocates into the nucleus where it phosphorylates transcription factors such as ETS-like transcription factor 1 (ELK-1) and Myc, enabling them to bind and activate target gene promoters (Plotnikov *et al.*, 2011). A different strategy is used by activated cytokine receptors, which stimulate tyrosine phosphorylation of the signal transducers and activators of transcription (STAT) family of transcription factors at the plasma membrane and these activated factors then translocate into the nucleus to activate their target genes (Horvath, 2000).

Another signalling molecule activated downstream of membrane receptors is the small guanosine triphosphatase (GTPase) Rac1, initially discovered for its ability to stimulate the polymerization of actin filaments and cell migration (Hall, 1998). In addition, Rac1 has distinct roles in the regulation of gene transcription (Benitah *et al.*, 2004). For instance, the stimulation of c-Jun N-terminal kinase (JNK) by Rac signalling leads to the phosphorylation and subsequent activation of the transcription factors c-Jun, ELK, activating transcription factor (ATF) or activator protein 1

(AP-1). A further transcription factor stimulated by Rac1 signalling is nuclear factor kappa-light-chain-gene-enhancer of activated B cells (NF-κB) and involves the phosphorylation and proteolytic degradation of the cytoplasmic inhibitor proteins IκBα and NF-κB2/p100 (Boyer *et al.*, 2004; Matos and Jordan, 2006).

Some STAT factors were also reported to be regulated by Rac1. They form a family of seven transcription factors, are found in the cytoplasm under basal conditions and enter the nucleus following their activation by tyrosine phosphorylation (Horvath, 2000). STAT3 binds directly to active Rac1, possibly targeting STAT3 to tyrosine kinase signalling complexes (Simon *et al.*, 2000). In addition, Rac1 and a GTPase-activating protein, MgcRacGAP, bind directly to phosphorylated STAT3 and STAT5A, promoting their nuclear translocation and activity (Tonozuka *et al.*, 2004; Kawashima *et al.*, 2006).

Previously, we reported a novel link between Rac1 signalling and transcriptional regulation. Rac1 activation leads to p21-activated kinase 1 (PAK1)-mediated phosphorylation of the transcriptional repressor B-cell lymphoma (BCL)-6 in colorectal tumour cells and inactivates its repressor function (Barros *et al.*, 2009). *BCL-6* was initially identified as a repressor gene translocated in B-cell non-Hodgkin's lymphomas (Seyfert *et al.*, 1996; Staudt *et al.*, 1999; Dent *et al.*, 2002). Later, BCL-6 expression has also been detected in non-hematopoietic tissues, including skeletal muscle (Bajalica-Lagercrantz *et al.*, 1998), uroepithelial cells (Lin *et al.*, 2003; Huang *et al.*, 2007), olfactory sensory neurons (Otaki *et al.*, 2005), skin (Kanazawa *et al.*, 1997), epithelial cells of the mammary gland (Logarajah *et al.*, 2003) and HeLa cells (Allman *et al.*, 1996). BCL-6 contains carboxy-terminal zinc finger modules that bind DNA in a sequence-specific manner (Chang *et al.*, 1996; Huynh *et al.*, 2000). The

genes repressed by BCL-6 are best studied in germinal centre B cells and are involved in lymphocyte activation and terminal differentiation, including cell-cycle regulation (Dalla-Favera *et al.*, 1999; Staudt *et al.*, 1999; Shaffer *et al.*, 2000; Niu, 2002).

Interestingly, the DNA motifs recognized by BCL-6 are highly homologous to the core binding sequence TTCNNNGAA of STAT factors STAT5 (Dent *et al.*, 1997; Horvath, 2000). This raised the hypothesis that both factors may have opposing roles in the transcriptional regulation of some target genes. Here, we used chromatin immunoprecipitation (ChIP) to show that active Rac1 promotes release of the repressor BCL-6 from promoters together with increased binding of STAT5. We also identify three endogenous target genes involved in cell-cycle control that were inversely regulated by BCL-6 and STAT5 and responded to Rac1 signalling with a transcription factor switch.

3.3. Material and Methods

3.3.1. Cell culture and transfection

DLD-1 and SW480 colorectal cells were maintained in Dulbecco's minimal essential medium (DMEM) and HT29 cells were kept in Roswell Park Memorial Institute (RPMI) medium, both supplemented with 10% (v/v) foetal bovine serum (FBS) (all reagents from Gibco, Carlsbad, CA, USA) and regularly checked for absence of mycoplasm infection. Cells were transfected as previously described (Barros *et al.*, 2009) using a 1:2 proportion (μg/μl) of DNA:LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) and total amounts of transfected plasmid DNA of 12 μg per 100-mm dish for ChIP assays, 4 μg per 60-mm dish for

immunoprecipitation and pull-down assays and 2 µg per 35-mm dish for cell fractionation, reporter assays, immunofluorescence and transcript expression analysis in case of DLD-1 and SW480 cells, but twice the amount for HT29 cells. When required, the amount of DNA was adjusted with empty vector. Optimal transfection efficiencies were 60 to 80% in DLD-1 or SW480 cells and 40 to 60% in HT29, as judged microscopically by expression of green fluorescent protein (GFP)-tagged vector and cells analysed 16-20 h later.

For RNA interference experiments, cells were transfected at 20 to 40% confluence in 35-mm dishes with either 200 pmol (DLD-1) or 400 pmol (HT29) of the indicated siRNAs using LipofectAMINE 2000 (Invitrogen) and analysed 48 h later. The small interfering RNA (siRNA) oligos against BCL-6 (sc-29791), STAT5A (sc-29495) and PAK1 (sc-29700) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and a scramble control oligonucleotide (5'-AGG UAG UGU AAU CGC CUU GTT) from Eurofins MWG Operon (Ebersberg, Germany).

3.3.2. DNA plasmids and constructs

The following published constructs were received as gifts: PAK1-wt, kinase-dead dominant-negative PAK1-K299R and constitutively active PAK1-T423E mutants from J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA, USA), and the 5xBCL-6-vector and the pGL3 control vector (Huynh *et al.*, 2000) from V.J. Bardwell (University of Minnesota, Minneapolis, MN, USA). *Rac1* cDNA and their Q61L and T17N mutants were subcloned as an EcoRI/BamHI fragment into pcDNA3-Myc, pEGFP (Clontech, Mountain View, CA, USA) and pDsRed-C1 (Clontech) vectors as previously described (Matos *et al.*, 2003; Matos and Jordan, 2006;

Barros *et al.*, 2009). All pEGFP-PAK1 constructs and pEGFP-BCL-6 were previously described (Barros *et al.*, 2009). STrEP-tagged BCL-6 was generated by subcloning a BamHI/XhoI fragment from pcDNA3-BCL-6 into vector pEXPR-IBA105 (IBA, Göttingen, Germany). pEGFP-STAT5A was generated by polymerase chain reaction (PCR) amplification of the *STAT5A* cDNA from pMX-STAT5A (gift from B. Groner, University of Frankfurt, Germany) using a forward primer (5'-ATG GCG GGC TGG ATT CAG G) and a reverse primer (5'-ATC TCA GGA CAG GGA GCT TCT) and subcloned into pEGFP-C2 using EcoRI restriction sites. All constructs were confirmed by automated DNA sequencing.

3.3.3. Analysis of transcript expression by quantitative reverse transcription-PCR

Total RNA was extracted from cell lysates with the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and 1 µg reverse transcribed using random primers (Invitrogen) and Ready-to-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK). *CCND2*, *CDKN2B* and small ubiquitin-like modifier 1 (*SUMO1*) transcript levels were determined by quantitative real-time PCR (qPCR) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the primers and PCR conditions summarized in Supplementary Table S3.1. Each cDNA sample was diluted 5-fold to guarantee accurate pipetting and 5 µl added to each real-time reaction together with 200 nM primers and SYBR Green Master Mix (Applied Biosystems). Data were analysed with the 7000 SDS 1.1 RQ Software (ΔΔCT method, Applied Biosystems) (Matos *et al.*, 2008) using mock transfections as reference samples. For comparison of gene expression between cell lines a pool of

cDNAs mixed at equal parts from the three cell lines was used as reference.

Semi-quantitative reverse transcription PCR (RT-PCR) was used to estimate siRNA-mediated knockdown of BCL-6 and STAT5A expression. RNA polymerase II (as earlier; Pol II) was amplified as a control gene and two serial dilutions of scramble siRNA sample served to assure semi-quantitative PCR conditions and estimate knockdown efficiency.

3.3.4. PCR array analysis

The Human Cell-Cycle PCR array (PAHS-020, Qiagen, Hilden, Germany) was used according to manufacturer's instructions. An RNA pool from three independent siRNA experiments performed in DLD-1 or HT29 cells was reverse transcribed, then added to a SYBR Green/Rox qPCR Master Mix (PA-012, Qiagen), distributed into the 96-well array plate and measured by qPCR as described above. The quantitative analysis was done on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the following cycling conditions: 10 min at 95ºC and 40 cycles at 95ºC for 15 s and 60ºC for 60 s. Two PCR arrays were used for each experimental condition. Data analysis was performed using the Excel-based tool provided by the manufacturer.

3.3.5. Identification of putative BCL-6/STAT5 binding sites

A 2500 bp of the genomic sequence immediately upstream the annotated transcription initiation sites for *CCND2*, *CDKN2B* and *SUMO1* genes were used to search *in silico* for putative binding sites recognized

simultaneously by BCL-6 and STAT5A. Several algorithms were employed (http://www.gene-regulation.com/; http://www.biobase-international.com/; http://www.genomatix.de) using the score values obtained for the previously described BCL-6/STAT5 site in *CCND2* (Fernández de Mattos *et al.*, 2004) as a reference for parameter adjustment and best putative site selection.

3.3.6. Chromatin immunoprecipitation (ChIP)

When indicated, DLD-1, SW480 or HT29 cells were transfected with expression vectors and assayed 16 h later. ChIP was performed as previously described (Fernández de Mattos *et al.*, 2004). Briefly, approximately 10 x 10^6 cells per ChIP were cross-linked with 1% formaldehyde for 10 min at 37ºC, washed twice in cold phosphate-buffered saline (PBS), scraped off and lysed sequentially in Buffer I (0.25% [v/v] Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES-KOH [pH 6.5]), Buffer II (200 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES-KOH [pH 6.5]) and finally ressuspended in Sonication buffer (50 mM Tris-HCl [pH 8.1], 5 mM EDTA, 150 mM NaCl, 0.5% [v/v] Triton X-100, 0.5% [v/v] SDS), all supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Lysates were then sonicated to obtain chromatin fragments between 200 and 500 bp length (40% power on a Sonics Vibra Cell sonicator). Cleared samples were diluted 5 times with Buffer D (50 mM Tris-HCl [pH 8.1], 5 mM EDTA, 150 mM NaCl, 0.5% [v/v] Triton X-100, protease inhibitor cocktail [Sigma]) and incubated overnight at 4ºC with either anti-BCL-6 clone N3 (sc-858; Santa Cruz Biotechnology), anti-STAT5A (#13-3600; Invitrogen) or control anti-rabbit immunoglobulin G (IgG) (P0448; DakoCytomation, Glostrup, Denmark)

antibodies, preserving 1/10 lysate volume as input control. Protein G-conjugated magnetic beads (Invitrogen) were then added for 1 h at 4ºC. Beads were thoroughly washed with Wash buffer (20 mM Tris-HCl [pH 8.1], 150 mM NaCl [pH 8.1], 2 mM EDTA, 1% [v/v] Triton-X-100, 0.1% [v/v] SDS) and with TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH8.0]). Co-precipitated DNA was extracted with 1% [v/v] SDS and 0.1 M $NaHCO_3$ solution and purified with the QIAquick PCR purification kit (Qiagen) after cross-link reversion. Input control samples were subjected to the same treatment. The selected putative STAT5A/BCL-6 binding regions were amplified from ChIP samples with the primers and conditions described in Supplementary Table S3.1. As a specificity control, a genomic fragment between intron 8 and intron 10 of the MutY homolog (*MUTYH*) gene (Accession number NG_008189) was amplified. Products were separated on 2% agarose gels containing ethidium bromide. Two serial dilutions of the "input DNA" control were co-amplified to guarantee semi-quantitative PCR conditions and allow product quantity extrapolation from band intensities analysed on digital images using ImageJ software (National Institutes of Health – NIH), which were then normalized to the control sample.

3.3.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Samples were prepared and detected as described (Matos *et al.*, 2003; Barros *et al.*, 2009). The antibodies used for Western blots were as follows: polyclonals anti-c-Myc clone A14 (sc-789), anti-Histone H2B (sc-10808) and anti-BCL-6 clone N3 (sc-858) from Santa Cruz Biotechnology; monoclonal anti-GFP from Roche (#11814460001;

Penzberg, Germany); monoclonal anti-PAK1 from Abcam (ab40795; Cambridge, UK); monoclonal anti-Rac1 clone 23A8 from Upstate Biotechnologies (#05-389; Charlottesville, Virginia, USA); polyclonals anti-phospho-PAK1 (Ser199/204)/PAK2 (Ser192/197) (#2605) and anti-phospho-STAT5A (Tyr694) (#9351) from Cell Signalling Technology (Danvers, MA, USA); monoclonal anti-STAT5A from Invitrogen (#13-3600) and monoclonal anti-α-tubulin clone B-5-1-2 (as loading control; T6074) from Sigma. For densitometric analysis, films from at least three independent experiments were digitalized and analysed using ImageJ software (NIH).

3.3.8. Active Rac pull-down assays and immunoprecipitation

Approximately 2 x 10^6 DLD-1 cells were seeded in 60-mm dishes, transfected as indicated, and assayed 16 to 20 h later. Cells were washed in cold PBS and lysed on ice in 250 μl of non-denaturing lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1% [v/v] Nonidet P-40 (NP-40), 10% [v/v] glycerol, 5 mM $MgCl_2$, and a protease inhibitor cocktail [Sigma]). For Rac pull down assay, total lysates were cleared by centrifugation at 2,500 x *g* for 5 min and 0.1 volume was added to 5x Laemmli sample buffer. The remaining lysate was incubated for 1 h at 4°C with a biotinylated CRIB-domain peptide pre-coupled to streptavidin-agarose beads (Sigma) as previously described (Matos and Jordan, 2006). Beads were washed three times with excess lysis buffer and the precipitated protein complexes were solubilised in 2x Laemmli sample buffer. Total lysates and precipitates were then analysed by Western blot as described above. The co-immunoprecipitation procedure (Barros *et al.*, 2009) was identical, with exception that total lysates were sonicated on ice (10 pulses

of 20 s at 40% power on a Sonics Vibra Cell sonicator) before incubation with streptavidin-agarose beads (Sigma), that were washed five times with an excess of lysis buffer containing 300 mM of NaCl. Precipitates and sonicated lysates were also analysed by Western blot.

All results were confirmed in at least three independent experiments.

3.3.9. Cell fractionation

Proteins were separated into a soluble pool not retained in the nucleus and into a chromatin-bound insoluble pool according to previously described procedures (Solan *et al.*, 2002; Barros *et al.*, 2009). Briefly, cells were washed in cold PBS, scraped off and lysed on ice in fractionation buffer (50 mM Tris-HCl [pH 7.9], 0.1% [v/v] NP40, 1.5 mM $MgCl_2$, 10 mM KCl and a protease inhibitor cocktail [Sigma]). The soluble fraction was collected by centrifugation and adding the supernatant to 5x Laemmli sample buffer. The pellet containing the insoluble nuclear fraction was washed once in fractionation buffer and then resuspended in 1x Laemmli sample buffer supplemented with 5 mM $MgCl_2$ and 50 U endonuclease (Benzonase, Sigma) to digest nucleic acids. Equal volumes of both fractions were analysed side by side on Western blots. Results were confirmed in at least three independent experiments.

3.3.10. Luciferase reporter assay

The use of the pGL3-5xBCL-6 reporter vector in DLD-1 cells was previously described (Barros *et al.*, 2009). Briefly, cells were co-

transfected with pRL-TK luciferase reporter (internal control; Promega, Fitchburg, WI, USA), pGL3-5xBCL-6 or pGL3 control reporters, and the indicated expression constructs. After 16 to 20 h cells were lysed and assayed with the Dual Luciferase Reporter Assay (Promega) and measured in an Anthos Lucy-2 Luminometer. Lysates were assayed in duplicates and additional aliquots analysed by Western Blot to document protein expression levels. Normalized luciferase values were plotted as fold-increase over the value of control treatments and correspond to at least three independent transfection assays.

3.3.11. Confocal immunofluorescence microscopy

Experiments were performed as previously described (Barros *et al.*, 2009). Cells were grown on glass cover slips (10 by 10 mm), transfected and incubated as indicated above, then washed twice in PBS, immediately fixed with 4% (v/v) formaldehyde in PBS for 30 min at room temperature, and subsequently permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min at room temperature. Cells were then briefly stained with 0.5 ng/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and washed in PBS, and the cover slips mounted in VectaShield (Vector Laboratories, Burlingame, CA, USA) and sealed with nail polish. Images were recorded with a Leica TCS-SPE confocal microscope and processed with Leica and Adobe Photoshop software.

3.3.12. Statistical analysis

Statistical significance of the differences between treated and control samples was analysed using two tailed Student's *t*-tests and indicated in the figures by an asterisk (*) whenever $p < 0.05$.

3.4. Results

3.4.1. Rac1 signalling promotes transcription by repressing BCL-6 and stimulating STAT5

Recently, we used a BCL-6 reporter gene construct (Fig. 3.1A) in which five repeats of a BCL-6 recognition motif control luciferase expression and found that Rac1 signalling acts as an upstream regulator of BCL-6 in colorectal DLD-1 cells (Barros *et al.*, 2009). When this reporter was transfected into DLD-1 cells together with GFP-tagged BCL-6, a further repression was observed, whereas depletion of endogenous BCL-6 expression by RNA interference led to transcriptional activation (Fig. 3.1B). In the course of these studies, we noticed that the expression of active Rac1-L61 had a stronger stimulatory effect on reporter gene transcription than a constitutively active PAK1-T423E mutant (Fig. 3.1B), although PAK1 is activated downstream of Rac1 and was shown to phosphorylate BCL-6 (Barros *et al.*, 2009). We thus reasoned that Rac1 may activate additional PAK1-independent pathways that affect reporter gene activation. One candidate pathway was activation of STAT5 because STAT5 was reported to recognize BCL-6 binding motifs in some cellular genes, including cyclin D2 or prolactin (Shaffer *et al.*, 2000; Tang *et al.*, 2002; Fernández de Mattos *et al.*, 2004; Meyer *et al.*, 2009; Tran *et al.*, 2010), and because it formed a complex with active Rac1 promoting STAT5 nuclear import and transcriptional activation (Kawashima *et al.*, 2006).

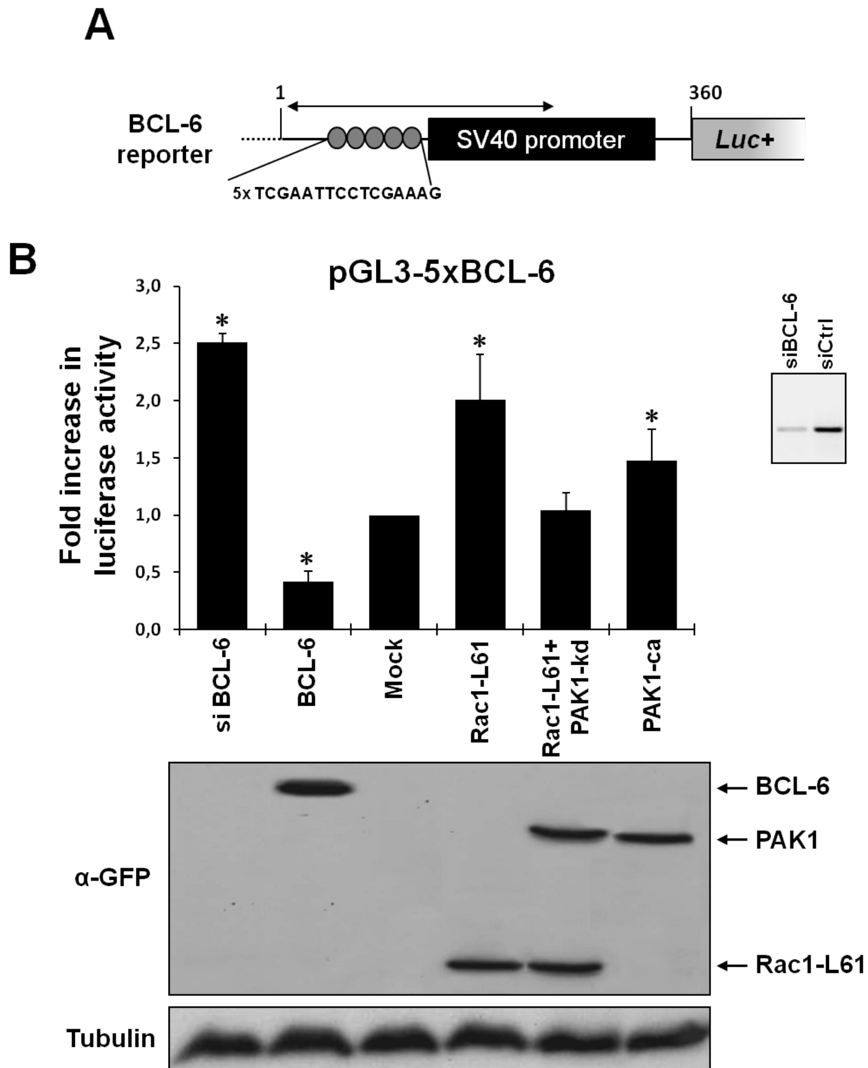

Figure 3.1. Rac1 signalling promotes transcription by repressing BCL-6 and stimulating STAT5. (A) Schematic representation of the transcriptional luciferase reporter vector under the control of five consensus BCL-6 binding motifs. **(B)** DLD-1 cells were co-transfected with the reporter vector and the indicated expression vectors or siRNAs. Cells were lysed 24 h later and luciferase activity was measured and graphically displayed, $^{*}P<0.05$. The Western blot below the graph shows the levels of transfected GFP-tagged BCL-6, Rac1-L61, PAK1 kinase-dead (kd) or PAK1 constitutively active (ca) mutants. Detection of endogenous α-tubulin served as loading control. The small insert beside the graph shows a Western blot of endogenous BCL-6 to document the efficiency of its siRNA-mediated depletion.

To test whether active Rac1 could promote nuclear translocation of STAT5 in DLD-1 cells, we first applied a cell fractionation protocol, which separates transcription factors into a soluble pool that is extracted from the nucleus and a chromatin-bound pool that remains insoluble (Barros *et al.*, 2009). Under control conditions, STAT5 was detected in the soluble fraction (Fig. 3.2A), whereas in cells co-expressing an active Rac1-L61 mutant a notable transition of STAT5 into the chromatin-bound insoluble fraction was observed (Fig. 3.2A). Second, we visualised the effect of active Rac1 on STAT5 by fluorescence microscopy in cells co-transfected with DsRed-Rac1-L61 and GFP-STAT5A. As shown in Figure 3.2B, a clear transition of STAT5 into the nucleus was observed.

To confirm whether STAT5 was able to activate the BCL-6 reporter gene under these conditions, both constructs were co-transfected into DLD-1 cells and increased luciferase transcription was measured (Fig. 3.3A). We then co-expressed STAT5 and constitutively active PAK1 to test whether their combined transcriptional activation would mimic that induced by Rac1-L61. As shown in Figure 3.3A, simultaneous stimulation of PAK1 and STAT5 could indeed account for the complete stimulatory effect induced by Rac1 signalling.

3.4.2. Rac1 signalling switches promoter occupancy from BCL-6 to STAT5

These results suggested that Rac1 signalling activates two independent pathways of transcriptional regulation that target the same reporter gene. To obtain further support for this conclusion, we determined the occupancy of the reporter gene promoter by either BCL-6 or STAT5 under the various experimental conditions. For this, DLD-1 cells were

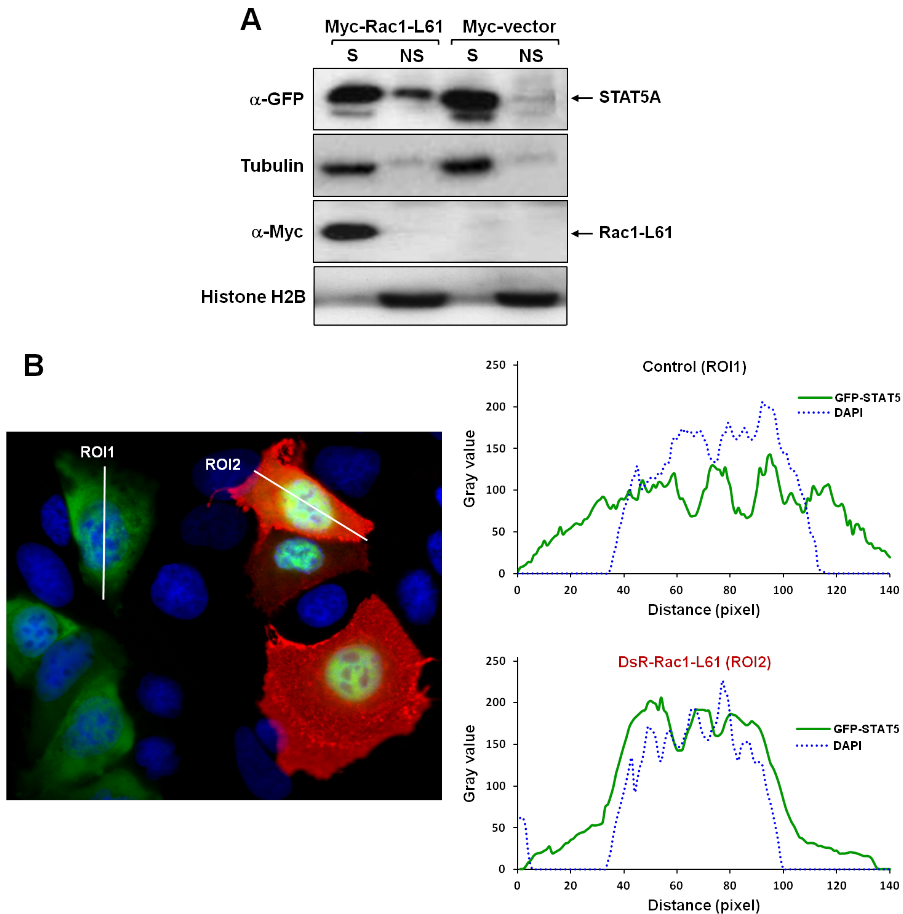

Figure 3.2. Rac1 signalling affects chromatin binding and subnuclear location of STAT5. (A) DLD-1 cells were transfected with Myc-Rac1-L61 or control empty vector and 24 h later analysed by Western blot for the subcellular distribution of STAT5 between a soluble (S) and a chromatin-bound non-soluble (NS) fraction (detection of α-tubulin and histone H2B served as controls). Note that active Rac1 promotes retention of STAT5 in the non-soluble chromatin fraction. **(B)** Subcellular localization of STAT5 determined by confocal fluorescence microscopy in DLD-1 cells co-transfected with DsRed-Rac1-L61 and GFP-STAT5A. The overlay image of the DAPI, GFP and DsRed channels is shown. A microscopic field was chosen that contained side by side untransfected cells (blue nuclei), cells that transfected only with GFP-STAT5A (green cells) and cells that co-transfected with both GFP-STAT5A and DsRed-Rac1-L61 (red cells). Note the nuclear STAT5 signal in Rac1-expressing red cells. In addition, two plots are given showing the DAPI and GFP signal intensities measured along the indicated regions of interest (ROI, white lines). The signal intensity of GFP did not increase across the nuclear DAPI region when cells expressed only GFP-STAT5A (green cells, ROI 1), whereas nuclear GFP signal clearly increased when cells co-expressed active Rac1 (red cells, ROI 2), confirming nuclear translocation of GFP-STAT5A.

co-transfected with the BCL-6 reporter gene and either control vector or active Rac1-L61 or active PAK1-T423E and the presence of either transcription factor at the reporter gene promoter was analysed by ChIP. As shown in Figure 3.3B, BCL-6 was the predominantly bound factor in control cells, however, upon expression of active Rac1 BCL-6 binding was reduced and STAT5 became the predominantly bound factor at the promoter. To exclude that the observed changes in promoter occupancy were the result of epitope masking (due to an interaction of BCL-6 and STAT5 at the promoter that could interfere with recognition by their specific ChIP antibodies), co-precipitation studies were carried out (Supplementary Fig. S3.1). No evidence was found for complex formation between the two transcription factors, indicating that changes in promoter occupancy reflected changes in bound proteins. In case of active PAK1, BCL-6 was also partially reduced, and this is in agreement with our previous data that PAK1 phosphorylates BCL-6 and promotes its release from chromatin and loss of repressor activity. However, in contrast to active Rac1, PAK1 was unable to invert the promoter occupancy from BCL-6 to STAT5. Together, these data support the conclusion that Rac1 signalling activates two independent pathways to promote a switch in promoter occupancy from BCL-6 to STAT5.

3.4.3. Correlation of Rac1 signalling and activation of BCL-6 or STAT5 in different cell lines

To understand the physiological relevance of the observed transcriptional switching at the reporter gene, we first characterized the endogenous activity levels of Rac1, PAK1, STAT5 and BCL-6 in three different colorectal cell lines using Western blot analysis. As shown in

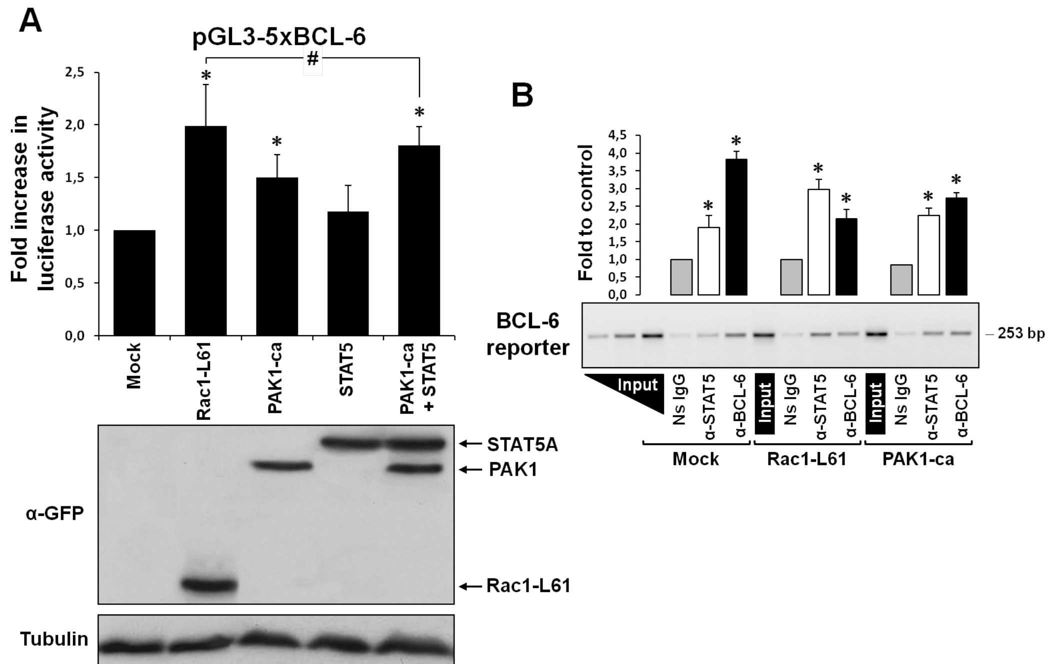

Figure 3.3. Rac1 signalling switches promoter occupancy from BCL-6 to STAT5A. (A) DLD-1 cells were co-transfected with the reporter and the indicated expression vectors, as described for Figure 3.1B. Note that STAT5 activates the BCL-6 luciferase reporter and, when combined with PAK1, reaches the stimulation levels normally induced by active Rac1, **P*<0.05 and #*P*>0.05. **(B)** ChIP of the reporter vector with anti (α-)-BCL-6, α-STAT5 or a non-specific antibody (Ns IgG) from lysates of DLD-1 cells transfected with the indicated expression vectors. A representative semi-quantitative PCR of the precipitated promoter fragment quantities with a graphical representation of the respective band intensities quantified by densitometry from digital images obtained in three independent transfection experiments, **P*<0.05, is shown. Two serial dilutions of input DNA were co-amplified to guarantee semi-quantitative PCR conditions and allow product quantity extrapolation from band intensities.

Figure 3.4, SW480 cells revealed the strongest endogenous Rac1 activation level, followed by HT29 and DLD-1 cells. Curiously, SW480 cell lost PAK1 expression, whereas in HT29 and DLD-1 cells, active Rac1 was proportional to active PAK1, as well as to the levels of phospho-BCL-6 and phospho-STAT5. Interestingly, SW480 cells expressed BCL-6 as well as STAT5 but lacked any significant activation by phosphorylation. This

suggested that repression by BCL-6 should be predominant in these cells, indicating their usefulness as a negative control for the transcriptional switch to STAT5 in subsequent experiments.

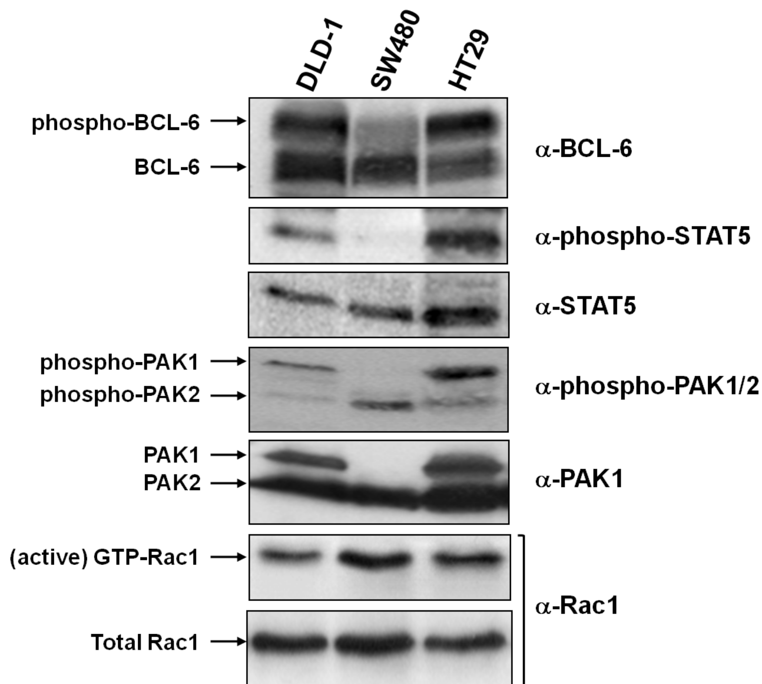

Figure 3.4. Correlation of Rac1 signalling and activation of PAK1, BCL-6 or STAT5 in different cell lines. Equivalent lysate quantities of DLD-1, SW480 and HT29 colorectal cells were separated by gel electrophoresis and analysed by Western blot using the indicated antibodies to compare protein levels. The active Rac1 fraction was obtained by CRIB-pull down assays, as described (Matos and Jordan, 2006).

3.4.4. Identification of endogenous genes inversely regulated by BCL-6 and STAT5

As a next step to identify physiological targets of the observed transcriptional switching, an array of 84 cell-cycle-related genes was tested for opposite effects of BCL-6 and STAT5 on gene expression. For

this, the two cell lines that showed endogenous BCL-6 and STAT5 activation, DLD-1 and HT29, were independently transfected with siRNAs targeting either BCL-6 or STAT5 (Supplementary Fig. S3.2). qPCR analysis of the resulting gene expression levels identified three genes that were affected in opposite sense by the downregulation of either BCL-6 or STAT5, namely cyclin D2 (*CCND2*), cyclin-dependent kinase inhibitor $p15^{INK4B}$ (*CDKN2B*) and small ubiquitin-like modifier 1 (*SUMO1*) (Fig. 3.5A).

To determine the respective promoter occupancies in these three genes, overlapping binding motifs for BCL-6 and STAT5 were identified using a Transfac® database-based algorithm (see Methods section and Fig. 3.5B) and used to design ChIP experiments in the three aforementioned cell lines. It was found that both factors were bound to the three identified promoters regions, albeit to different extent; however, not to a control genomic fragment. In SW480 cells (that express no PAK1 and have little phospho-BCL-6; Fig. 3.4) the predominant factor that was bound to all three promoters was BCL-6, whereas STAT5 was close to background levels (Fig. 3.6A, grey bars). In contrast, HT29 cells contained more STAT5 bound to these promoters than BCL-6 (Fig. 3.6A, white bars), in agreement with their higher endogenous levels of active PAK1, phospho-BCL-6 and phospho-STAT5 (cf. Fig. 3.4). In DLD-1 cells (Fig. 3.6A, black bars) comparable promoter binding levels were detected for both factors (except for the *CCND2* promoter that had more BCL-6 bound). Again, this is in good agreement with the observation described in Figure 3.4 that endogenous levels of active PAK1, phospho-BCL-6 and phospho-STAT5 in DLD-1 were lower than in HT29 but higher than in SW480 cells.

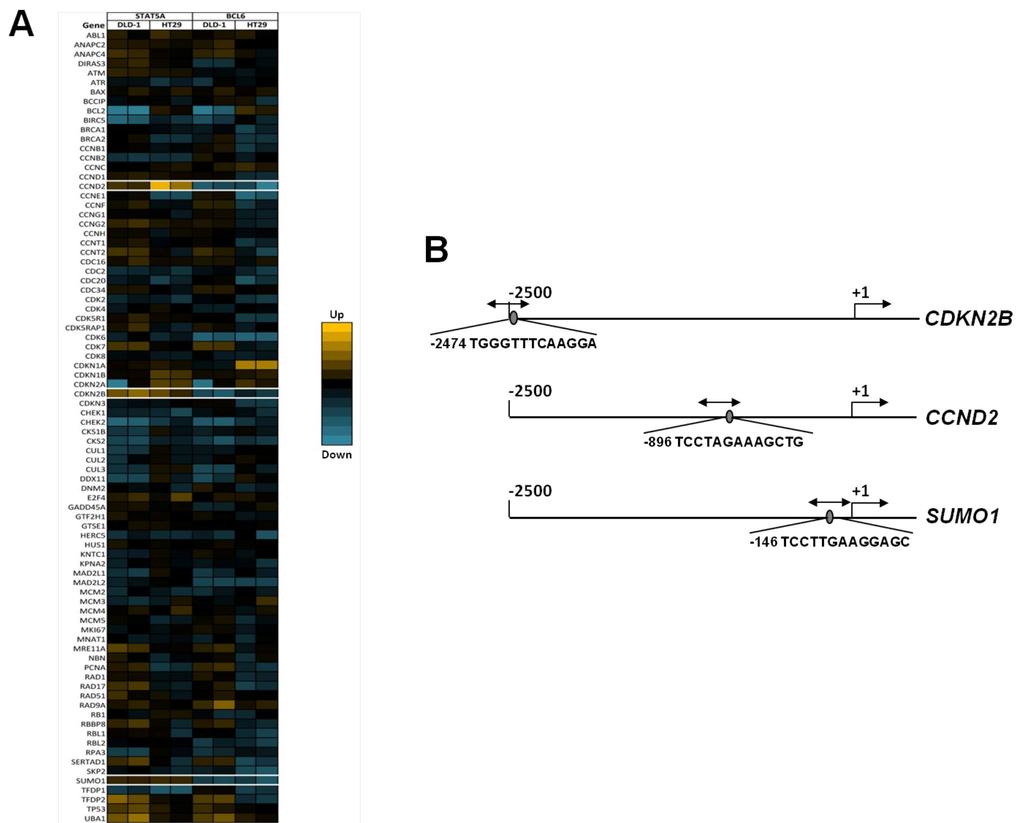

Figure 3.5. Identification of endogenous genes inversely regulated by BCL-6 and STAT5. (A) DLD-1 and HT29 cells were transfected with either BCL-6 or STAT5-specific siRNA oligonucleotides and lysed following 48 h for RNA extraction (Supplementary Fig. S3.2). A heat map display of the gene expression analysis of a cell-cycle PCR array probed with RNA samples obtained from BCL-6 or STAT5-depleted DLD-1 and HT29 cells, is shown. Of the 84 genes on the array, three were identified to be regulated by BCL-6 and STAT5 in opposite sense (white boxes). **(B)** Schematic representation of the promoter regions of the three endogenous genes inversely regulated by BCL-6 and STAT5 showing the selected best putative motifs, with equivalent predicted binding scores for both factors.

Next, these data on the promoter occupancies of the *CCND2*, *CDKN2B* and *SUMO1* genes were matched to the corresponding gene expression levels, validated by qPCR using independently designed PCR primers (Fig. 3.6B). HT29 cells that had less BCL-6 repressor bound than

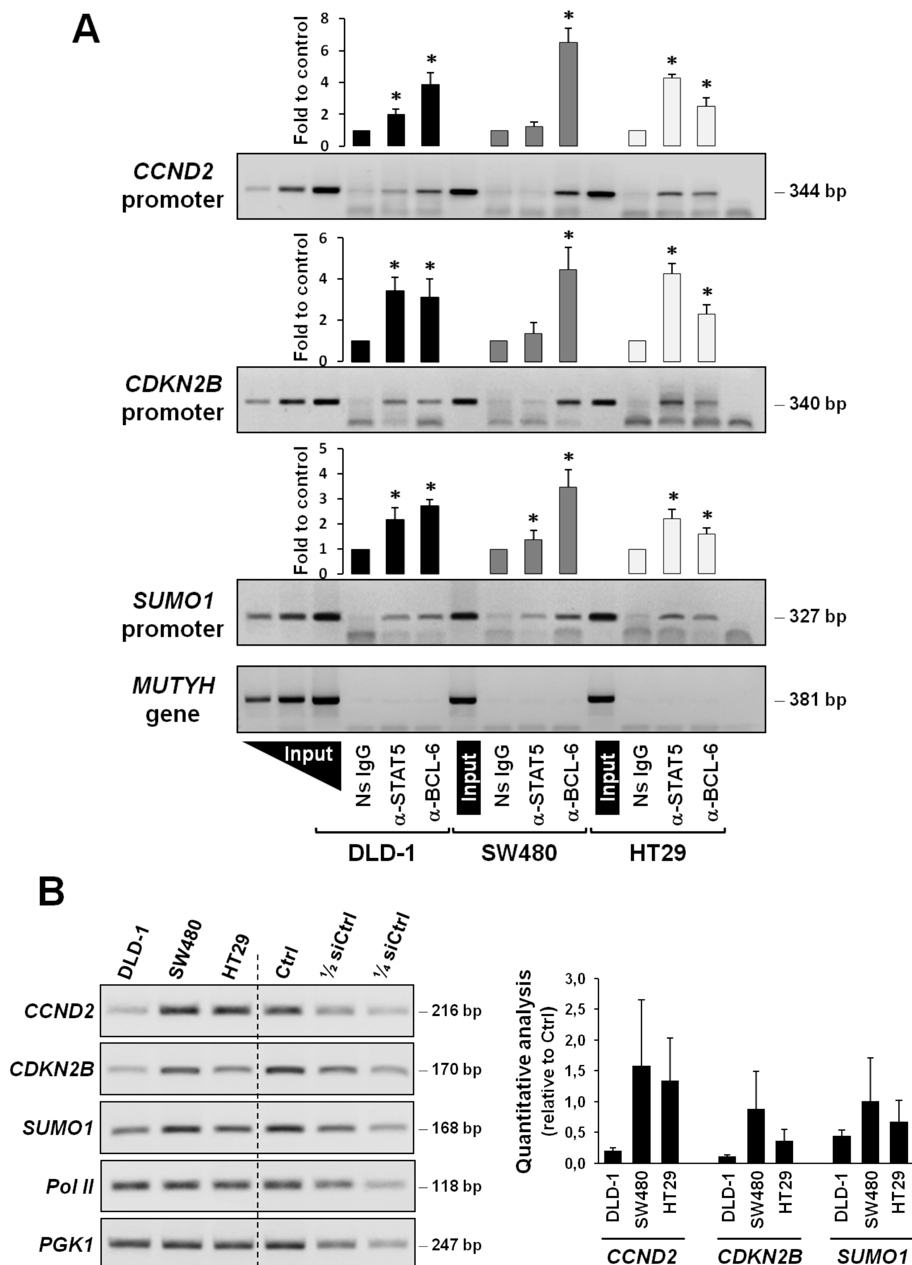

Figure 3.6. Regulation of expression of the *CCND2*, *CDKN2B* and *SUMO1* genes. (A) Promoter occupancies with BCL-6 and STAT5 at the *CCND2*, *CDKN2B* and *SUMO1* gene promoters was determined by ChIP with the indicated antibodies using lysates of the three indicated cell lines (see legend to Fig. 3.3B for details). A representative semi-qPCR of the precipitated promoter

fragments and a graphical representation of the respective band intensities, *P<0.05, are shown. A control genomic fragment from the *MUTYH* gene was amplified to confirm the specificity of the precipitated target gene promoters. Note that BCL-6 binds predominantly in the PAK1-lacking SW480 cells and whereas a switch to STAT5 occurs in HT29 cells with active Rac1/PAK1 signalling. **(B)** Gene expression data corresponding to the ChIP analysis of the three genes in the three cell lines. Left panel shows representative semi-quantitative RT-PCRs, whereas graph at the right shows the result of qPCR analysis of cDNAs collected from the three cell lines at three different splitting times. Genes encoding RNA polymerase II (*Pol II*) and the glycolytic enzyme phosphoglycerate kinase 1 (*PGK1*) were amplified as control housekeeping genes and a pool of cDNAs mixed at equal parts from the three cell lines was used as reference for qPCR. Serial dilutions served to assure semi-quantitative conditions in the conventional RT-PCR reactions.

DLD-1 cells also revealed higher expression levels for all three genes. Surprisingly, SW480 cells also expressed all three genes considerably, although BCL-6 was predominantly bound in these cells, indicating they use different mechanisms to activate these cell-cycle regulating genes.

3.4.5. Rac1 signalling controls reciprocal roles of BCL-6 and STAT5 in target gene expression

As final evidence that the transcription factor switch is physiologically meaningful, the promoter occupancies at the three genes were determined and compared with changes in their respective expression levels following activation or inhibition of Rac1 signalling in the three different cell lines. For this, cells were transfected with vectors encoding either dominant-negative or wild-type PAK1, or dominant-negative or active Rac1, or with siRNA oligonucleotides directed against endogenous PAK1 (depletion documented in Fig. S3.2B). The three genes revealed equivalent results, which are represented in Figure 3.7 for the *SUMO1* gene by displaying the levels of promoter-bound BCL-6 or STAT5 alongside the respective target gene transcript levels and

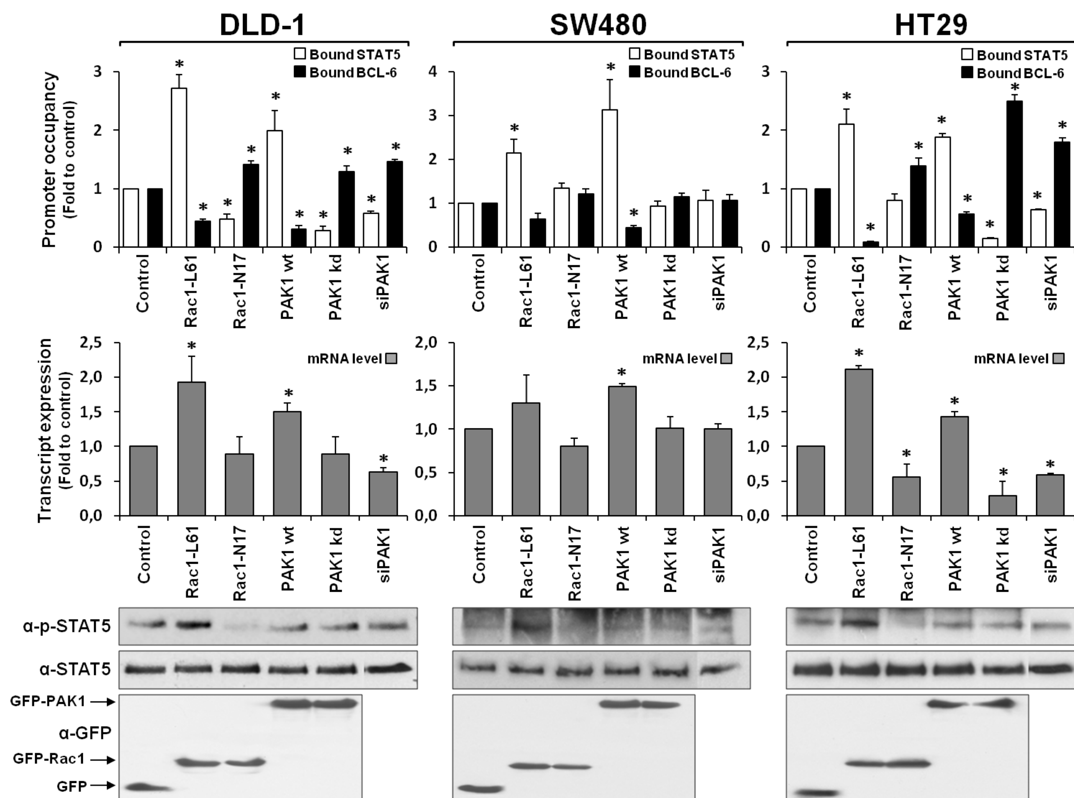

Figure 3.7. Rac1 signalling controls target gene expression by inverting promoter occupancy with either BCL-6 or STAT5. The representative analysis of the *SUMO1* gene is shown in the indicated three colorectal cell lines following their transfection with constructs that either activate or inhibit Rac1 signalling. Top panels show the graphical display of promoter occupancy by ChIP using either anti-BCL-6 (black columns) or anti-STAT5 (white columns) and middle panels the respective gene expression levels (grey columns) (see legend to Fig. 3.6 for further details), *$P<0.05$. Bottom panels show Western blot analysis of the cell lysates demonstrating the expression levels of the transfected GFP, GFP-Rac1 or GFP-PAK1 constructs, as well as the resulting phosphorylation status of endogenous STAT5. Note that in SW480, which lack endogenous PAK1, depletion of endogenous PAK1 by siRNAs transfection (documented in Fig. S3.2B) or expression of dominant-negative PAK1 has no effect on promoter-bound BCL-6, whereas re-expression of PAK1 leads to loss of BCL-6 from the *SUMO1* promoter and an increase in gene expression. In the other two cell lines, inhibition of Rac1 or PAK1 are clearly correlated with more BCL-6 bound and less gene expression, while activation of Rac1 or PAK1 promoted STAT5 binding to the promoters and increased transcription.

protein expression levels. Comparable data for the *CCND2* and *CDKN2B* genes are shown in Figure 3.8. When PAK1 was transfected into SW480 cells, which lack endogenous PAK1, a loss of BCL-6 from the promoter of all three genes was induced (Fig. 3.7, top panel; Fig. 3.8, top panels), which slightly increased their expression levels. By contrast, the depletion of endogenous PAK1 had no effect on promoter occupancy or gene expression (Fig. 3.7, middle panel; Fig. 3.8, bottom panels), a result in agreement with the fact that no endogenous PAK1 is expressed in SW480 cells. When SW480 cells were transfected with active Rac1, a small increase in STAT5 phosphorylation and binding to the promoter was observed, however, the overall effect on gene expression was negligible because the lack of PAK1 compromised BCL-6 removal from the promoter. These data confirm our previous assumption that SW480 cells represent a negative control and cannot respond to Rac1 signalling with the transcriptional switch between BCL-6 and STAT5.

In contrast, HT29 and DLD-1 cells both switched BCL-6 and STAT5 at the three gene promoters upon transfection with active Rac1 (Fig. 3.7, top panel; Fig. 3.8, top panels), accompanied by a clear increase in STAT5 phosphorylation (Fig. 3.7, bottom panel) and in gene expression (Fig. 3.7, middle panel; Fig. 3.8, bottom panels). Upon transfection of these cells with PAK1, BCL-6 was lost from the three gene promoters and expression increased slightly, however, no significant increase in STAT5 phosphorylation occurred. In the presence of PAK1-specific siRNAs (as well as a dominant-negative PAK1 mutant), BCL-6 promoter occupancy increased and expression of the three genes was inhibited.

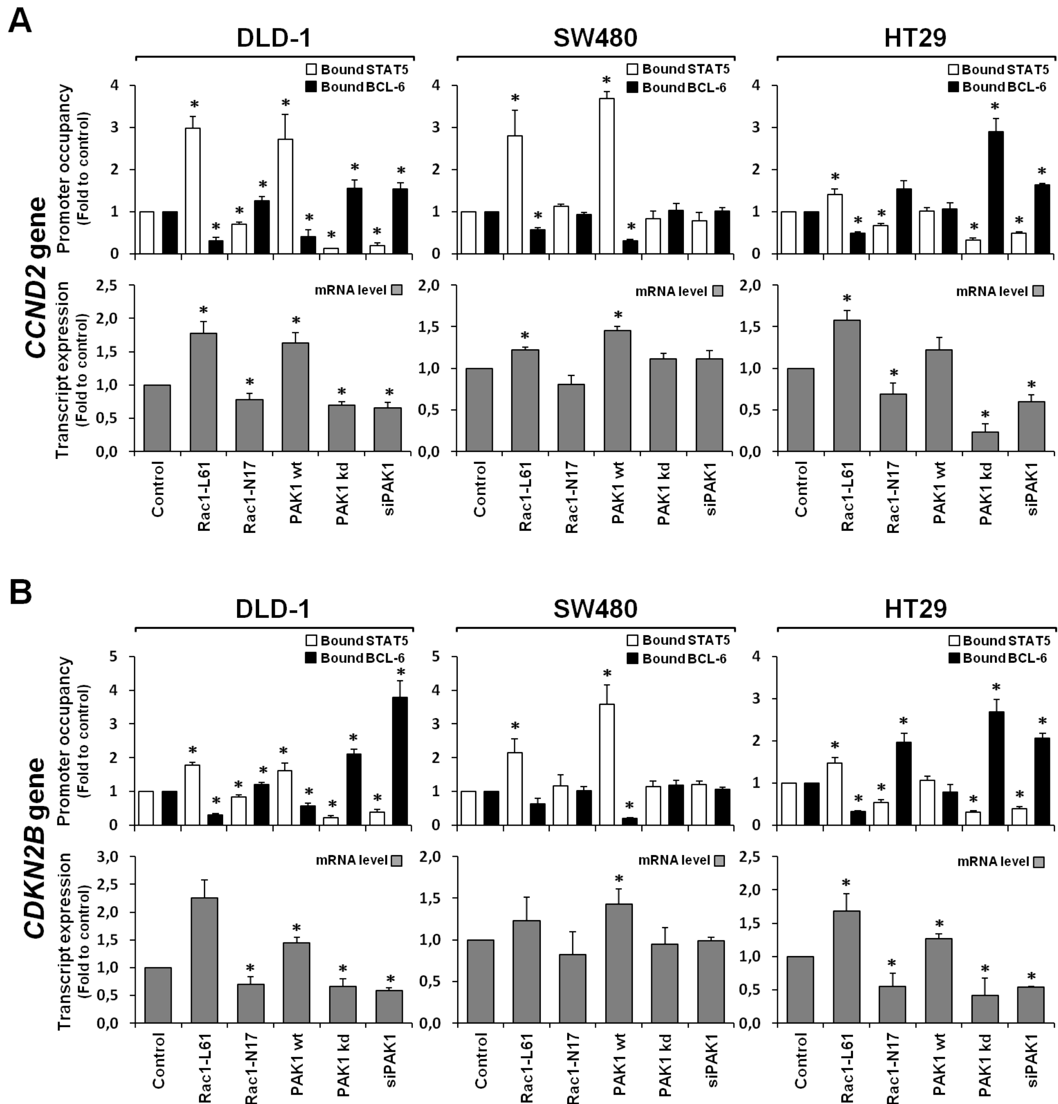

Figure 3.8. Rac1 signalling controls *CCND2* and *CDKN2B* gene expression by inverting promoter occupancy with either BCL-6 or STAT5. The representative analysis of the *CCND2* **(A)** and *CDKN2B* **(B)** genes is shown in the indicated three colorectal cell lines following their transfection with constructs that either activate or inhibit Rac1 signalling. **(A and B)** Top panels show the graphical display of promoter occupancy by ChIP using either anti-BCL-6 (black columns) or anti-STAT5 (white columns) and bottom panels the respective gene expression levels (grey columns), *$P<0.05$ (see legend to Fig. 3.7 for further details).

Altogether, these data provide evidence for the model proposed in Figure 3.9, showing that Rac1 signalling has a dual effect on transcriptional regulation of the *CCND2*, *CDKN2B* and *SUMO1* genes.

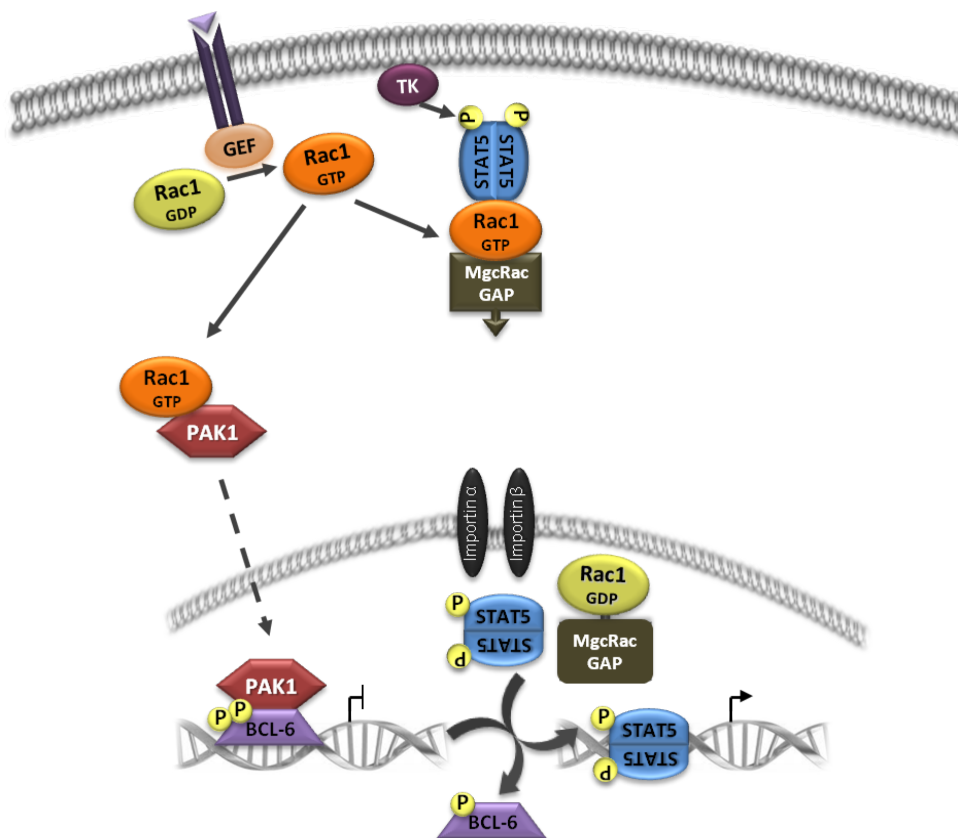

Figure 3.9. Proposed model for the role of Rac1 signalling in the observed transcriptional switch. On receptor activation, guanine nucleotide exchange factors (GEF) promote GTP binding of Rac1 that stimulates two independent pathways. Active Rac1 binds and activates protein kinase PAK1 that migrates into the nucleus and phosphorylates chromatin-bound BCL-6, leading to its inactivation and loss of promoter occupancy. In parallel, a protein complex is formed between active Rac1, MgcRacGAP and STAT5, promoting phosphorylation by a tyrosine kinase (TK) and translocation into the nucleus. Here, MgcRacGAP stimulates GTP hydrolysis by Rac1 and phospho-STAT5 is released and activates gene transcription following binding to the vacant promoter sites previously repressed by BCL-6.

First, Rac1 activates PAK1 that phosphorylates BCL-6 leading to its removal from the target gene promoter and a concomitant increase in gene expression. In parallel, Rac1 activates phosphorylation and nuclear translocation of STAT5, which binds to the same sequence motif in the gene promoter that is recognized by BCL-6 and further increases gene expression.

3.5. Discussion

The main finding in this work is that Rac1 signalling activates gene transcription by inducing a switch from repressor BCL-6 to activator STAT5 at the promoter of certain cellular target genes in colorectal cells.

Although BCL-6 is best known as a regulator of B lymphocyte growth and differentiation, it is also expressed in epithelial tissues including skin (Kanazawa *et al.*, 1997), the mammary gland (Logarajah *et al.*, 2003), HeLa cells (Allman *et al.*, 1996) and colorectal cells (Barros *et al.*, 2009). Similarly, STATs were described as integral parts of cytokine signalling pathways in hematopoietic cells (Horvath, 2000), but meanwhile their role in epithelial cancers has been well documented (Calò *et al.*, 2003). In particular, aberrant activation of STAT5 was found in prostate (Li *et al.*, 2005) and colorectal cancer (Xiong *et al.*, 2009). In these cases, the activation of STAT5 can be mediated by Rac1 signalling, either through the production of reactive oxygen species downstream of G protein-coupled receptor (GPCR) stimulation, leading to activation of the tyrosine kinase JAK (Pelletier *et al.*, 2003) and/or through complex formation with MgcRacGAP promoting nuclear import of phospho-STAT5 (Kawashima *et al.*, 2006). Indeed, our study in the colorectal cell lines confirmed that

activated Rac1 led to increased phosphorylation of STAT5 (Fig. 3.7) and an increase in chromatin-bound nuclear STAT5 (Fig. 3.2A).

Previous reports have suggested that STAT5 and BCL-6 could bind in a mutually exclusive manner to the same sequence motif in the promoters of certain target genes (Shaffer *et al.*, 2000; Tang *et al.*, 2002; Fernández de Mattos *et al.*, 2004; Meyer *et al.*, 2009; Tran *et al.*, 2010). Our data clearly support these studies and show, side by side, that the switch in promoter occupancy between BCL-6 and STAT5 correlates directly with changes in gene expression of either a BCL-6-controlled luciferase reporter vector or of three endogenous gene promoters. More importantly, we show for the first time that this switch is regulated by Rac1 signalling and occurs in colorectal tumour cells. Several pieces of evidence contributed to these data. First, ChIP assays revealed that BCL-6 and STAT5 were bound to the identified gene promoters in the three colorectal cell lines. Second, the endogenous activation status of Rac1, PAK1, and phosphorylated BCL-6 or STAT5 correlated well with promoter occupancies in the cell lines, without detectable changes in the total amount of STAT5 or BCL-6 proteins. Third, experimental activation of Rac1 promoted STAT5 phosphorylation and accumulation in the chromatin-bound nuclear fraction. Fourth, the transcript expression levels of the three endogenous genes mirrored their promoter occupancies and responded to activation or inhibition of upstream Rac1 or PAK1 signalling.

As described earlier, the three colorectal cell lines studied differed in their endogenous activation levels of Rac1 signalling and the resulting inhibition of BCL-6 or stimulation of STAT5. SW480 cells apparently lost PAK1 expression and therefore are unable to phosphorylate BCL-6, except when transfected with ectopic PAK1 (Fig. 3.7 and Fig. 3.8). Unexpectedly, these cells still revealed significant expression of the

CCND2, *CDKN2B* and *SUMO1* genes, which we identified as inversely regulated target genes for BCL-6 and STAT5. This experimental observation indicates that other mechanisms for transcriptional activation of *CCND2*, *CDKN2B* and *SUMO1* exist and were used by these cells. Because the control of gene expression involves combinatorial patterns of transcription factor binding, the inhibitory effect of BCL-6 was most likely overcome in SW480 cells by other transcription factors that respond to different signalling inputs. For example, the ability of Myc to induced CCND2 as well as CDKN2B expression has been reported (Bouchard *et al.*, 1999; Staller *et al.*, 2001) and SW480 cells carry an oncogenic mutation in the *KRAS* gene (Matos *et al.*, 2008), a strong activator of several signalling pathways.

In contrast, HT29 and DLD-1 cells shared the same regulatory pattern of BCL-6 inhibition and STAT5 activation, differing only in the extent of BCL-6 inhibition, which was more pronounced in HT29 cells. However, on transfection of active Rac1 or PAK1 mutants, the resulting transcriptional stimulation became almost identical in both cell lines. The same was true for the strong inhibitory effect after depletion of endogenous PAK1 by RNA interference or transfection of a dominant-negative PAK1 mutant, whereas SW480 cells did not respond to either treatment. Together, these data provide substantial evidence that Rac1 signalling promotes a switch at the targeted promoters with a release of BCL-6 and enhanced binding of STAT5 to the same site.

Of the 84 cell-cycle related genes analysed, three (3.6%) were clearly identified as inversely regulated by BCL-6 and STAT5. *CCND2* encodes cyclin D2, which functions as a regulatory subunit of CDK4 or CDK6 required for cell-cycle G1/S transition. CCND2 overexpression has

been reported in colorectal tumours and cell lines (Mermelshtein *et al.*, 2005; Liu *et al.*, 2010).

The *SUMO1* gene encodes a small ubiquitin-like protein that can be covalently attached to proteins as a monomer or a lysine-linked polymer. Unlike ubiquitin, sumoylation is not involved in proteolytic degradation of the attached protein but rather modulates nuclear transport or transcriptional regulation (Gareau and Lima, 2010).

CDKN2B encodes the cyclin-dependent protein kinase inhibitor protein p15 encoded by the INK4b locus, which can form a complex with CDK4 or CDK6, and prevent their activation by cyclin D. Although CCND2 and SUMO1 overexpression are consistent with the pro-proliferative role usually associated with increased Rac1 signalling, the role of CDKN2B during colorectal cancer progression remains unclear. Intriguingly, the expression of p15 was also found significantly increased in higher grade prostate carcinomas (Zhang *et al.*, 2006), indicating that alternative mechanisms may exist to inactivate its inhibitor function.

Although the particular functional consequences require further clarification, our findings provide a mechanistic model for how Rac1 signalling promotes switching between transcription factors (see Fig. 3.9). Beyond the rapid regulation by Rac1 signalling, the described interplay between STAT5 and BCL-6 is likely also modulated at the long term because STAT5 was found to act as a transcriptional repressor on the *BCL-6* gene itself (Walker *et al.*, 2007). In addition, STAT5 has been described to act as a transcriptional repressor on other genes (Luo and Yu-Lee, 1997; Nelson *et al.*, 2004). This underlines the requirement for a genome-wide study to understand which genes are activated or repressed by BCL-6 or STAT5 alone, and which genes are regulated reciprocally by the switch between both factors that is described in this manuscript.

These differences could reside in the sequence motifs of the corresponding promoters or be mediated by the binding of additional protein factors. Our data are thus a contribution to uncover how Rac1 signalling shapes gene expression and how the deregulation of Rac1 activity that is observed for example in cancer (Sahai and Marshall, 2002) promotes cell proliferation.

3.6. Acknowledgements

We thank J. Chernoff (Fox Chase Cancer Center, Philadelphia), R. Dalla-Favera (Columbia University), V.J. Bardwell (University of Minnesota) and B. Groner (University of Frankfurt) for generously providing plasmids. Julie Millour is acknowledged for her support in setting up the ChIP technique. This work was supported by the Fundação para a Ciência e Tecnologia, Portugal through grants PPCDT/SAU-OBS/57660/2004 to P.J., PTDC/SAU-GMG/119586/2010 to P.M., PEst-OE/BIA/UI4046/2011 to the BioFig research unit, fellowship BD 29789/2006 to P.B. and contract Ciência2007 to P.M.. The work was further supported by an EMBO fellowship ASTF 425.00-2009 to P.B..

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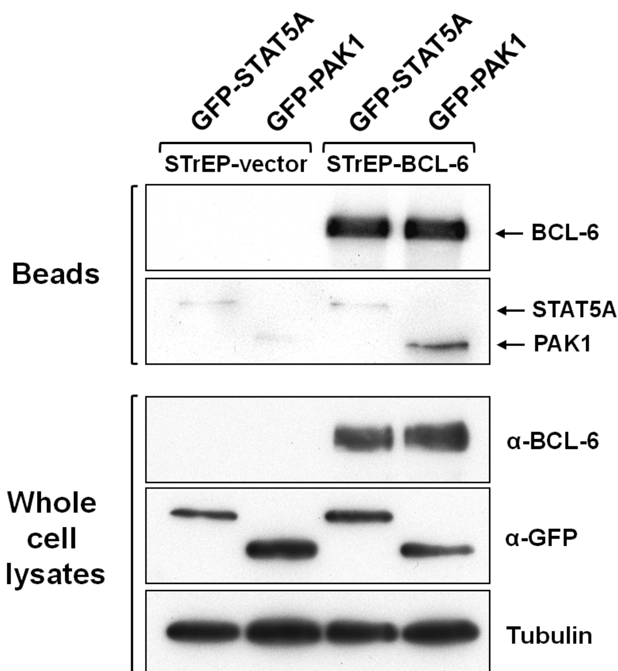
http://www.biobase-international.com/

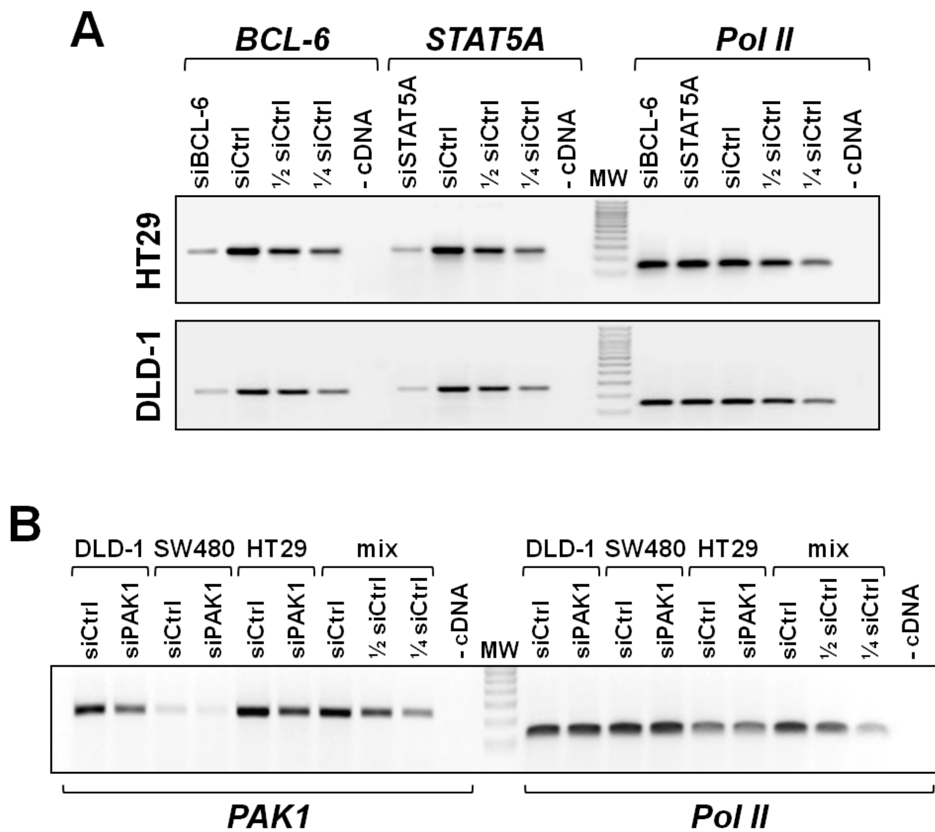
http://www.gene-regulation.com/

http://www.genomatix.de

3.8. Supplementary Data



Supplementary Figure S3.1. Analysis of protein complex formation between the transcription factors STAT5A and BCL-6. DLD-1 cells were co-transfected with STrEP-tagged BCL-6 or STrEP empty vector and either pEGFP-STAT5A or pEGFP-PAK1 (IP positive control; see Barros *et al.*, 2009). After 16 h of expression cells were lysed in non-denaturing lysis buffer and extracts incubated with streptavidin-agarose (Sigma) to precipitate BCL-6. Beads were washed 5x with an excess of lysis buffer and the precipitated protein complexes analysed by Western blot as indicated. Note that the experimental conditions allowed the detection of the complex formed between BCL-6 and PAK1; however, no interaction was observed between BCL-6 and STAT5A. Lack of interaction between both factors assures that the changes in promoter occupancy observed in Figures 3.3B, 3.7 and 3.8 reflect true switching between BCL-6 and STAT5 rather than an interaction between both proteins at the promoter that could interfere with recognition by their specific ChIP antibodies.



Supplementary Figure S3.2. Semi-quantitative RT-PCR to document the efficiency of the indicated gene depletions. Cells were transfected as indicated and lysed following 48 h for RNA extraction. Semi-quantitative RT-PCR was used to document the efficiency of the respective gene depletions. RNA polymerase II (*Pol II*) was amplified as a control gene and two serial dilutions of scramble siRNA (siCtrl) served to assure semi-quantitative PCR conditions and estimate knockdown efficiency. MW= 100 bp molecular weight marker. **(A)** DLD-1 and HT29 cells were transfected with either scramble control (siCtrl), or BCL-6 (siBCL-6) or STAT5A-specific (siSTAT5A) siRNA oligonucleotides. Note the levels of specific depletion of BCL-6 and STAT5A (lanes 1 and 6, respectively). **(B)** DLD-1, SW480 and HT29 cells were transfected with either control or PAK1 (siPAK1)-specific oligos. Serial dilutions of a mixture (mix) of the three cell lysates following transfection with scramble siRNA (siCtrl) served to estimate knockdown efficiency specific PAK1 depletion is evident in lanes 2 and 6.

Supplementary Table S3.1. List of primers and PCR conditions. (F* – forward, R* – reverse; Ta – Annealing temperature)

Primer name	Sequence	Ta ºC	Cycles
Primers used to clone STAT5A cDNA			
STAT5A-F*	5'ATGGCGGGCTGGATTCAGG	62	35
STAT5A-R*	5'ATCTCAGGACAGGGAGCTTCT		
Primers used for qPCR amplification of gene transcripts (10 min at 95ºC, then 40 cycles of 15 sec at 95ºC and 30 sec at 60ºC)			
CCND2-F*	5'GCCATCTGTGGGCTCCAGCA	60	40
CCND2-R*	5'AGGGGTGCTGGCTTGGTCCA		
CDKN2B-F*	5'CTGCGGAATGCGCGAGGAG	60	40
CDKN2B-R*	5'TCATGACCTGGATCGCGCG		
SUMO1-F*	5'AAGTGACGCGAGGCGTAGCG	60	40
SUMO1-R*	5'AGGTTTTGCCTCCTGGTCAGACA		
PGK1-F*	5'CAGTTTGGAGCTCCTGGAAG	60	40
PGK1-R*	5'CACTGCACCCTGGATTTGCA		
Pol II-F*	5'CGCAATGAGCAGAACGGCGC	60	40
Pol II-R*	5'TCTGCATGGCACGGGGCAAG		
Primers used for promoter amplification following ChIP (5 min at 94ºC, 30 s at 94ºC, 30 s at Ta, 15 s at 72ºC and 5 min at 72ºC)			
BCL-6 reporter			
pGL3-ChIP-F*	5'CGAGCTCTTACGCGTGCTA	62	27
pGL3-ChIP-R*	5'CCTCGGCCTCTGCATAAATA		
CCND2 promoter			
CCND2-ChIP-F*	5'GTATCTCCTGTAAAGACAGCC	62	35
CCND2-ChIP-R*	5'CCTGCATCTGCTGACAAGC		
CDKN2B promoter			
CDKN2B-ChIP-F*	5'GATGCCACTCATTCCCTTCTAC	62	35
CDKN2B-ChIP-R*	5'ATGGCTCACCTCACAGCACACC		
SUMO1 promoter			
SUMO1-ChIP-F*	5'TCAGTCGTCAGAGACGCGCAA	62	35
SUMO1-ChIP-9R*	5'ACAACACCGCGGCTGCAGTAA		
Negative control			
MUTYH-ChIP-F*	5'GGGACTGACGGGTGATCTCT	62	35
MUTYH-ChIP-R*	5'AGAGGGGCCAAAGAGTTAGC		
Primers used for semi-quantitative transcript amplification (5 min at 94ºC, 30 s at 94ºC, 30 s at Ta, 30 s at 72ºC and 5 min at 72ºC)			
BCL-6-F*	5'AGAGCCCATAAAACGGTCCT	62	30
BCL-6-R*	5'AGTGTCCACAACATGCTCCA		
STAT5A-F*	5'GCCATTGACTTGGACAATCC	62	30
STAT5A-R*	5'AGCTGCAATTGTTGGCTTCT		
PAK1-F*	5'GTCAGCTGAGGATTACAATTC	56	28
PAK1-R*	5'GAGATGTAGCCACGTCCCGAG		

Chapter 4

Final Conclusions and Future Perspectives

The Rac1/PAK1 kinase signalling axis has been implicated in the transduction of extracellular signals regulating a diverse array of cellular processes that include cell proliferation, cell survival, cytoskeleton remodelling and gene expression (reviewed in Bokoch, 2003; Bosco *et al*., 2009). Not surprisingly, given their critical roles in cell regulation, the expression and activity of Rac1 and PAK1 have been often found deregulated in several human pathologies, including cancer (reviewed in Bosco *et al*., 2009; Dummler *et al*., 2009; Ong *et al*., 2011). Their association with tumorigenesis, in particular, has placed these molecules in the spotlight as potential therapeutic targets for several cancer types. In fact, a strong effort has been made in recent years towards the development of specific and selective Rac and PAK inhibitors with pharmacological application (Gao *et al*., 2004; Désiré *et al*., 2005; Deacon *et al*., 2008; Crawford *et al*., 2012). Some of these molecules have proven to be specific and quite useful for research purposes (see Chapter 2 and 3) and a few are currently progressing to clinical development (Crawford *et al*., 2012).

Indeed, in the past decade, we have assisted to a remarkable revolution in the field of cancer therapy with the introduction of molecular targeted therapy and the widespread development of specific inhibitors to signalling molecules. This progress in cancer therapeutic drugs was mainly motivated by the concept of “oncogene addiction” that postulates that some cancers apparently depend on one or a few oncogenic proteins for the maintenance of the malignant phenotype, despite the probable accumulation of multiple alterations that contributed to the tumorigenic process. Numerous studies have supported this concept and shown that in certain tumours, inactivation of a single oncogene was sufficient to inhibit the growth and survival of cancer cells. So far, the clinical success

of this approach was mainly achieved by drugs that target oncogenic protein kinases. One hallmark example is the inhibitor imatinib developed against the BCR/ABL fusion protein that characterizes chronic myeloid leukemia and caused complete remission in many patients. However, in many cases drug resistance occurred and required new treatment with modified inhibitors (Weinstein and Joe, 2008; McCormick, 2011; Settleman, 2012). Other examples are the inhibitors of the Ras/RAF/MEK/ERK signalling pathway, which revealed a problem with targeting such pleiotropic factors, due to the high potential for systemic adverse side effects (Mitwally, 2007; Winther and Olsen, 2011; Lee *et al*., 2012) and occurrence of drug resistance (Poulikakos and Solit, 2011; Trujillo, 2011).

In effect, while the concept of increasing treatment specificity by directing it to a specific oncogenic target protein emerged as very promising, few cases have been successful in the clinic (Levitzki and Klein, 2010; Logue and Morrison, 2012). One of the main problems is that cancer cells respond to chronic drug treatment by either selecting mutant clones that are resistant or by adapting their signalling circuitry, taking advantage of pathway redundancy and routes of feedback and cross-talk to maintain their function. A new paradigm is now emerging that emphasizes the need to confront a complicated disease such as cancer from a signalling network perspective, taking into account how effector molecules and signalling pathways interconnect and adapt (Logue and Morrison, 2012). One recent example is the so-called BRAF paradox. The Ras/RAF/MEK/ERK signalling pathway is frequently deregulated in cancer, as a result of activating mutations in the *BRAF* and *RAS* genes. Oncogenic BRAF mutations generally bypass the need for Ras binding and promote constitutive BRAF activation and thus of ERK (Hatzivassiliou

et al., 2010; Heidorn *et al*., 2010; Poulikakos and Solit, 2011). The most frequent BRAF mutant (BRAF-V600E) is found in 50% of malignant melanomas as well as in many colorectal and thyroid cancers (Dhomen and Marais, 2007; Cantwell-Dorris *et al*., 2011) and occurs in a mutually exclusive manner with Ras mutations (Heidorn *et al*., 2010). Intriguingly, the use of a specific BRAF inhibitor showed, in some patients, a contradictory increase in ERK signalling and tumour progression. Later, it was found that the action of this inhibitor depended on the cellular context. Thus, in tumours with BRAF-V600E mutation the inhibitor effectively blocks ERK activation. On the contrary, in tumours harbouring oncogenic Ras, it was found that BRAF-wt and CRAF form a dimer localized with Ras at the membrane and that binding of the inhibitor to BRAF stabilized dimer formation and enhanced CRAF activation, which stimulated ERK signalling and tumour cell proliferation (Hatzivassiliou *et al*., 2010; Heidorn *et al*., 2010; Poulikakos and Solit, 2011). An accumulating body of clinical trial-associated evidence has demonstrated the importance of understanding the detailed regulatory mechanisms involved in signal transduction in order to design the best possible, tumour-tailored therapeutic strategy. Several studies reported that the combined use of multiple drugs, targeting different key players in pertinent signalling pathways can overcome the redundancy and increase treatment effectiveness, reducing the effective drug dosage and therapy duration, lowering toxicity and preventing acquired resistance (Fitzgerald *et al*., 2006; Logue and Morrison, 2012).

Such issues may also condition the use of Rac1 or PAK1-based therapies and hence the end term purpose of the work here presented. Our results provide new data on how the deregulation of Rac1 and PAK1 signalling can contribute to the tumorigenic process in colorectal cancers.

A novel pathway was described in which Rac1 inhibits the activity of the transcriptional repressor BCL-6 through PAK1-mediated phosphorylation. This inhibition was shown to be physiological meaningful, affecting the expression levels of BCL-6 target genes, like *NFKB1* and *CD44* (see Chapter 2). Moreover, Rac1 was shown to activate gene transcription by inducing a switch from repressor BCL-6 to activator STAT5 at the promoter of cell-cycle-associated target genes. *CCND2*, *CDKN2B* and *SUMO1* were thus identified as inversely regulated by BCL-6 and STAT5 and shown to respond to Rac1 and PAK1 signalling with an increase in their transcript levels (see Chapter 3). Moreover, this was the first time that this STAT5/BCL-6 transcriptional switch was demonstrated in epithelial cells and, more importantly, that this switch is under the control of Rac1 signalling (see Chapter 3). Recently, another example of transcription factor switching at a shared promoter binding site was described (Wozniak *et al*., 2012).

Further studies are now required to clarify the impact and significance of the novel mechanism characterized in this work for tumorigenesis. Is this mechanism specific for colorectal cells, or does it also condition gene expression in other cell types? What is the full spectrum of target genes modulated by this switch? What other cellular functions, besides cell cycle regulation, are modulated by this pathway? Can these be targeted along with Rac1 and PAK1 in cancer to avoid redundancy and resistance? Can the target genes of this pathway help us to generate better tumour profiles? Can they be used as new tumour biomarkers, and guide choices for combinatorial drug therapies, targeting these and other signalling pathways and molecules?

Work is ongoing in the host research group to perform a genome-wide study to identify the target genes of the Rac1/PAK1/BCL-6 and

Rac1/STAT5 pathways. This study will involve the use of the ChIP-Seq technique that combines the traditional ChIP with deep sequencing. In this way, the DNA fragments bound by either STAT5 or BCL-6 are enriched and then systematically sequenced, providing sequence readouts that will be aligned to the human genome to identify the candidate target genes and statistically relevant protein binding sites (Dowell, 2010; Kim and Park, 2011). With a list of target genes the research options increase, and several of the questions above can start to be addressed. It is currently clear that patient profiling will be critical for determining the most suitable targets and subsequent lines of a combinatorial treatment and that monitoring of tumours at the signalling level during treatment will be required to achieve a durable response. The ultimate goal of this study is that the novel signalling pathway and the transcriptional switch characterized in this thesis can, in the future, provide useful tools for this. Moreover, we anticipate that once characterized, some or several of the pathway target genes can be used in combination with Rac/PAK and, possibly, other pathway inhibitors to impose a series of signalling blocks that cannot be overcome, thus effectively eradicating the targeted cancer.

In conclusion, we believe this thesis work contributes with new insights on how deregulation of Rac1 signalling affects the cell's transcriptome and opens new directions for research and therapeutic intervention.

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