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Research Article

Rac1b Supports Ligand-Independent Androgen Receptor Activation in Prostate Cancer Progression

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ABSTRACT

Prostate cancer represents one of the leading causes of morbidity and mortality of men worldwide. In precision medicine, tumors are screened for specific genetic alterations known as predictive markers for targeted therapy. In androgen-independent prostate cancer cells and in tissue samples of a prostate cancer patient treated with Goserelin, we identified the self-activating splice variant Rac1b. Importantly, the expression of Rac1b was sufficient to induce AR-dependent gene synthesis. We hypothesized that Rac1b antagonizes androgen depletion induced cancer cell death by blocking pro-apoptotic signalling pathways. In line with that selective knockdown of Rac1b or inhibition of Rac-dependent signalling pathways re-induced apoptosis in androgen-independent prostate cancer cells suggesting Rac1b inhibition as a potential novel therapeutic add on strategy against prostate cancer.

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Introduction

Prostate cancer (PCa) is considered the second leading cause of cancer deaths of men in the western hemisphere [1]. Androgen deprivation therapy (ADT) represents a well-established pharmacological strategy to prevent tumor growth by promoting apoptosis in tumor cells. A major clinical challenge to this approach is posed by frequent development of androgen-independent castration-resistant prostate cancer (CRPC), which is furthermore associated with increased morbidity and poor prognosis. To mirror the progression of human prostate tumors before and after androgen withdrawal, the androgen-controlled human PCa cell line LNCaP and its androgen-independent sub-line C4-2 serve as an effective model system to analyse the basis of androgen independence [2-5].

Small GTP-binding proteins of the Rho-family (Rho GTPases) have emerged as critical factors in driving tumorigenesis [6, 7]. They act as molecular switches cycling between a GTP-bound active and a GDP-

bound inactive state. Rho GTPases govern diverse signaling pathways ranging from migration to apoptosis and are overexpressed in several human tumor entities [8]. One member of this family is Rac1b, a splice product of the RAC1 gene. It contains an in-frame insertion of exon 3b (coding for 19 amino acids), which leads to a fast cycling, activated isoform of the Rac protein [9]. Emerging data from a wide variety of cancer types suggest a crucial function of Rac1b in cancer progression. It contributes to cellular transformation in lung, intestinal and thyroid cancer recently reviewed in [10]. However, the present understanding of Rac1b in PCa is still lacking.

Rho GTPases are targeted by a large subset of bacterial protein toxins, which are used as cell biological tools to manipulate Rho protein functions selectively. While most of these toxins inactivate the GTPases, other toxins constitutively activate Rho proteins [11-13]. Cytotoxic Necrotizing Factors activate Rho proteins by deamidation [14, 15]. Amongst them, *Yersinia pseudotuberculosis* CNFY which preferentially deamidates and activates RhoA, B, and C *in vivo* [16, 17]. Recently, we showed that specific activation of RhoA, B, and C leads to apoptosis of

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the human PCa cell line LNCaP. In contrast, activation of RhoA, B, and C was not sufficient to induce cell death of the androgen-independent cell line C4-2 [18].

Here we show that active Rac1b expression is upregulated in order to suppress RhoA-dependent apoptosis. Moreover, Rac1b blocks cell death induced by androgen withdrawal through the expression of androgen-regulated genes. Selective knockdown of Rac1b or inhibition of Rac-dependent pathways re-induced apoptosis. Importantly, we verified the presence of Rac1b in tissue specimen of a PCa patient.

Materials and Methods

I Cell Lines

Cells of the human PCa cell lines LNCaP and C4-2 were obtained from Dr. Philipp Wolf, Urology, University Medical Center Freiburg. Cell line authentication occurred by short tandem repeat analysis (cell line service CLS (Order number AB160620 and AB 150617)). Cells were checked negative for mycoplasma monthly by commercial means (GATC). Cells were grown in RPMI 1640 medium (Invitrogen) with 15% FCS in the presence of penicillin (100 U/ml) and streptomycin (100 mg/l) at 37°C in a humidified atmosphere of 5% CO₂.

Generation of Rac expressing cell lines: Full-length cDNA of human Rac, Rac1b or Rac-G12V, respectively, were cloned into the retroviral pMIG vector (MSCV IRES GFP). Retroviruses were produced in HEK Phoenix cells maintained in DMEM with 10% FCS. LNCaP cells were transduced with a mixture consisting of virus and RPMI (1:1) by spin-infection with 4 µg/ml polybrene. Cells were centrifuged at 37°C at 2500 rpm for 25 min. They were incubated with the virus for an additional 5 h. Subsequently, the mixture was replaced with fresh media for recovery overnight. The transduction procedure was repeated twice. After 7 days, GFP positive cells were sorted using FACS Aria Fusion or MoFlo Astrios and cultured.

II Preparation of CNFY

For toxin purification, BL21 *E. coli* strains, carrying pGEX-CNFY or the plasmid with the catalytically inactive mutant pGEX-CNFY-C866S were grown in LB-medium, respectively. At OD 0.6, protein synthesis was induced by the addition of 0.1 mM IPTG. Cells were collected by centrifugation and lysed by sonication in lysis buffer (20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonylfluoride (PMSF) and 5 mM dithiothreitol (DTT)). The toxins were purified as GST-fusion proteins by affinity chromatography with glutathione-Sepharose (GE Healthcare, Freiburg, Germany). Loaded beads were washed five times with lysis buffer (without PMSF) at 4°C. The GST-CNF fusion proteins were eluted from the beads by glutathione (10 mM glutathione and 50 mM Tris-HCl (pH 7.4)) twice for 10 min at room temperature.

III Analysis of AR-Dependent Promotor Activation

Activation of the AR was measured in a reporter luciferase assay. Cells were seeded in 96-well plates and transfected with 200 ng/well reporter plasmids pPSA-Luc for AR activation and pRL-TK (Promega) for

measuring transfection efficiency [19]. After 5 h, the medium was changed, and cells were either treated with inhibitors and/or stimulated with the toxin. 24 h after transfection, cells were lysed, and luciferase assays were performed following the manufacturer's protocol (dual-luciferase reporter assay kit, Promega). For inhibition of androgen signalling, LNCaP cells were incubated in culture medium without FCS, supplemented with charcoal-stripped FCS (Sigma Aldrich), or cultivated in normal culture medium in the presence of Enzalutamide (20 µM, Selleck Chemicals) or Abiraterone (10 µM, Selleck Chemicals).

IV Antibodies

Antibody	Host Species	Dilution	Manufacturer
Glycerinaldehyde-3-Phosphat-Dehydrogenase (GAPDH)	Mouse IgG, monoclonal	1 : 20 000	Millipore
Anti-rabbit IgG, peroxidase-coupled	goat Ig, polyclonal	1 : 3000	Rockland/BioMol GmbH
Anti-mouse IgG, peroxidase-coupled	donkey Ig, polyclonal	1 : 3000	Rockland/BioMol GmbH
Rac1	mouse IgG, monoclonal	1 : 3000	Millipore
Rac1B	rabbit Ig, polyclonal	1 : 5000	Millipore
PARP1	rabbit Ig, polyclonal	1 : 5000	Cell Signaling
Caspase 3	rabbit Ig, polyclonal	1:1000	Cell Signalling
Cleaved Caspase-3 (Asp175)	rabbit Ig, polyclonal	1:1000	Cell signalling

V Apoptosis

We used the "ApoOne Homogeneous Caspase-3/7 Assay" (Promega), which is based on the cleavage of the pro-fluorescent caspase consensus substrate Z-DEVD-R110. The cleavage-derived fluorescence is proportional to the number of active caspases 3 and 7. The assay was performed following the manufacturer's instructions. Shortly, cells were seeded in 96-well-plates, cultivated overnight, and treated with the toxins as indicated. The apoptosis-inducing reagent staurosporine (1 µM) was used as the positive control. The Apo-ONE-Caspase-3/7-reagent was added, and the plates were incubated in the dark. Fluorescence was measured in a multi-well plate reader at 499 nm (emission 521 nm).

Poly (ADP-ribose) polymerase (PARP) cleavage by caspase-3 is a hallmark of apoptosis. For the measurement of PARP cleavage cells were seeded in 6 well plates and intoxicated with 1 nM CNFY for different time periods. Inactive toxin mutant was used as a negative control. After washing with ice-cold PBS, cells were incubated with 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM MgCl₂, 10% (v/v) glycerol, 1% (v/v) Igepal and 4% (v/v) protease inhibitor solution (Roche Diagnostics, 1 tablet dissolved in 2 ml water) for 5 min, scraped and centrifuged for 30 min at 14.000 rpm at 4°C. Cell lysates (50 µg) were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% (w/v) non-fat dried milk in TTBS for 60 min, membranes were incubated with rabbit anti-PARP IgG (New England

Biolabs) at 4°C overnight and washed. Goat anti-rabbit-HRP (Biotrend) was used as secondary antibody (30 min, room temperature). Finally, blots were developed using enhanced chemiluminescence.

VI Migration Assay

Cells were plated in sterile culture-insert 24 well plates (ibidi) at 40.000 cells/well and allowed to form a subconfluent cell monolayer. Wounds were scratched by removing the silicon insert in each well. Wound healing capacity was visualized from 0 to 48 h by Lionheart™ FX automated microscope (Biotec, Germany).

VII Colony Formation Assay

To measure the rate of colony formation ability, cells were spread onto six-well plates at 500 cells well and cultured for 11 days at 37°C and 5% CO₂. Following the incubation period, colonies were fixed and stained with 6% glutaraldehyde and 0,5% crystal violet. Images were captured using Amersham Imager 600 and analysed with ImageJ. Experiments were repeated in triplicates.

VIII PAK Pull-Down

The Rac-binding region, encoding the Cdc42 and Rac binding CRIB-domain (amino acids 56-272) of PAK (PAK pull-down) was expressed as GST-fusion protein in *E. coli* BL21. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 10% v/v glycerol, 0,5% v/v Triton X-100 and 1 mM DTT). GST-coupled PAK-CRIB was purified by affinity chromatography with glutathione-Sepharose. Loaded beads were washed three times with lysis buffer and once with buffer A (10% glycerol, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% NP-40, 2 mM MgCl₂, and 0.5 mM PMSF).

Cells were lysed in buffer A and the lysates were cleared by centrifugation. A fraction of the cleared lysates (50 µg of total protein) was analysed by western blotting (input). Total lysate (1 mg) was incubated with protein-loaded beads for 1 h at 4°C by head-over-head rotation. After incubation, beads were washed once with buffer A. Samples were boiled in Laemmli buffer and separated by SDS-PAGE. Rac1 was analysed by western blotting with a specific antibody.

IX Patient and Tissue Specimens

The primary diagnosis of PCa occurred at an age of 59 years in 06/2013. The initial PSA was 16 ng/ml. Prostate biopsy was performed in 06/2013 and revealed intraductal PCa Gleason 10. At that time, based on histological findings, an intraprostatic urothelial carcinoma could not be excluded for differential diagnosis. A second prostate biopsy was performed subsequently. In the second biopsy, a dedifferentiated PCa was diagnosed. Although not recommended in national or international guidelines in 11/2013, a neoadjuvant antihormonal therapy was started by application of a 3-month formulation of goserelin acetate (Zoladex® 10.8-mg depot). The decline of PSA was 3 ng/ml in 01/2014. Tumor Staging was performed by Choline-PET/CT (Nuklearmedizin Uniklinik Freiburg) in 02/2014. A choline-positive multilocal PCa was apparent; furthermore, bilateral multiple choline-positive lymph nodes in the parailiacal region were evident. No clear evidence of distant metastases.

Finally, in 03/2014, a radical prostatectomy with extended lymphadenectomy was performed in terms of multimodal therapy concept. Definitive histology was the following: pT4 (urinary bladder), pN1 (12/26), pM1(LYM), L1, V0, Pn1, R1 (seminal vesicle, ductus deferens) Gleason score 4+4=8, Grading: G3. The use of the tissue was approved by the ethics statement.

X RNA Extraction

RNA extraction from cells or cryo-frozen tissue was performed with the RNeasy Mini Kit (Qiagen, Hilden) according to the manufacturer. In order to prevent RNase contamination, gloves and working area were cleaned with RNaseKiller prior to the RNA extraction. DNase digestion was performed with RNase-free DNase I in order to avoid DNA contamination. RNA was eluted with 35 µL of RNase-free water. The concentration of RNA was determined with a NanoDrop 2000 spectrophotometer.

XI Reverse Transcription/ cDNA Synthesis

Up to 1 µg of total RNA was reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) as per the manufacturers' instruction. The reaction was carried out at 42°C with random hexamers and oligo dTs. The reaction was stopped by heating to 95°C for 5 min. After reverse transcription, cDNA was diluted 1:10 with TE buffer and stored at -20°C.

XII Reverse Transcription Quantitative PCR (qRT-PCR)

Gene expression was determined using qRT-PCR. Each cDNA sample was amplified with SYBR Green, using the GoTaq® qPCR Master-Mix (Promega) and the Eppendorf Mastercycler® ep realplex (Eppendorf). The reaction conditions consisted of 2 µl cDNA, 5 µl GoTaq® qPCR MasterMix, and 0.2 µM primers in a final volume of 10 µl. The primer pairs are listed below. Each primer pair was tested for specificity and the absence of primer dimers with DNA electrophoresis or melt-curve analyses. Each cycle consisted of denaturation at 95°C for 15 s, annealing at 50-55°C, and elongation at 60°C for 45 s. The experiment was carried out three times for each data point. Data analyses were performed with LinRegPCR 2012 (Ruijter *et al.*, 2009). S29 was used as the reference gene. The reference gene normalized levels of expression were calculated as: Relative Expression = N0 (target gene)/N0 (reference gene). Fold change was calculated as follows: fold change = relative expression (target gene)/relative expression (control gene).

Gene	sense	antisense
Rac1b	atgcaggccatcaagtgtgtg	gagggttatctctaccgtacg
Rac1	ctgatcagttacacaaccaatgc	cattggcagaataattgtcaaaaga
29s rRNA	agctgtagcgtatgggtctg	aaggggacatacatcaagcagt
Rac1-Intron1	ttcagggtaccaatgtgtatg	
Rac1-Exon1/Exon2	gggagacggagctgttagg	
Rac1 Exon2		ggcaagttttacctacagc
GFP	aagctgaccctgaagttcatctgc	ctttagttagccgtcgtccttgaa

XIII Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA (www.graphpad.com). Normally distributed data were assessed using unpaired two-tailed Student's t-test or One-way ANOVA with Dunnett's post test. Data that did not follow a Gaussian distribution were tested for statistical significance with a two-tailed Mann-Whitney test or Kruskal-Wallis test. Data are evaluated from at least three independent experiments, as indicated in the figure legends. P-values <0.05 were considered as statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Results

As reported previously, activation of RhoA by the bacterial toxin CNFY results in apoptosis of androgen dependent PCa cells (LNCaP). This was not the case for hormone-independent cells (C4-2), in which we identified more active Rac with higher stability as compared to LNCaP cells. Activated Rac seems to be fundamental for the resistance of androgen-independent cells towards RhoA-induced apoptosis [18].

I Identification of Activated Rac in CNFY-Selected Cells

Treatment of C4-2 cells with the RhoA activator CNFY induced cell death in only 10% of cells. Our hypothesis posits that surviving cells should express activated Rac. To examine this, we purified mRNA of CNFY-surviving cells, generated cDNA, and amplified Rac transcripts by PCR. Amplified Rac cDNA was then cloned into a Topo-blunt vector and amplified in *E. coli* TG1 cells. Single-cell sequencing of generated clones revealed the presence of Rac1b (about 15-20% of clones). Using specified primers (see methods) allowed quantification of Rac1 and Rac1b mRNA in LNCaP and C4-2 cells by qRT PCR (Figure 1).

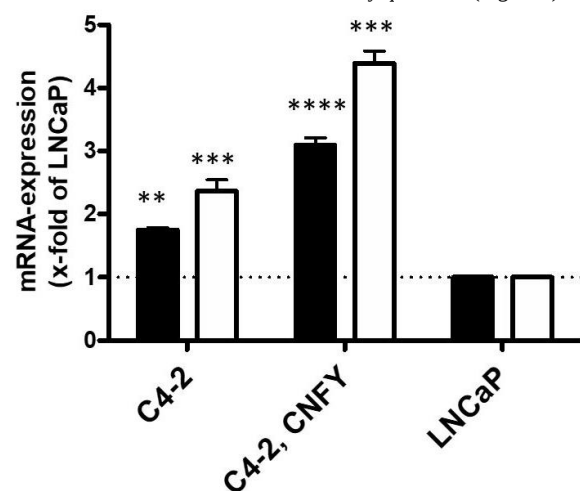


Figure 1: Induction of Rac and Rac1b expression by RhoA activation. A) Quantification of Rac and Rac1b mRNA: LNCaP and C4-2 cells were treated for 24 h with CNFY (1nM) or left untreated as indicated, respectively. Total Rac mRNA (black bars) and Rac1b mRNA (white bars) were analysed by qPCR. Data from 4 independent experiments were normalized to the respective mRNA detected in LNCaP cells (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Total Rac mRNA, as well as Rac1b mRNA, were twice as abundant in C4-2 cells related to LNCaP cells. Incubating C4-2 cells with CNFY further increased the mRNA differential to 4-fold compared to untreated LNCaP cells. LNCaP cells incubated with CNFY became apoptotic and could not be measured. Our data indicate that CNFY stimulated Rac1b generation by positive selection of Rac1b expressing cells.

II Rac1b Blocks Induction of Apoptosis in LNCaP Cells

To analyse whether Rac1b is sufficient to block toxin-induced apoptosis, we generated a stable LNCaP cell line expressing Rac1b or dominant active Rac(G12V) as a positive control, respectively (expression control Figure S1). In LNCaP cells, PARP-cleavage (Figure 2a) and caspase 3/7 activity (Figure 2b) were strongly stimulated upon incubation with CNFY, whereas apoptosis was not induced in C4-2 cells treated with the toxin. Interestingly, stable expression of Rac1b in LNCaP cells (L-Rac1b) completely abolished CNFY-induced apoptosis; also, the expression of Rac(G12V) (cell line named L-G12V) significantly reduced caspase activity and PARP-cleavage. Our data indicate that expression of active Rac is sufficient to inhibit CNFY-induced apoptosis. To exclude the role of unspecific changes mediated by the viral transduction itself, we performed knockdown of Rac1b by siRNA in L-Rac1b cells and screened for re-induction of apoptosis. To this end, we studied PARP cleavage in the absence and presence of CNFY. The successful Rac1b knockdown was verified by western blotting (Figure 2c). According to the function of Rac1b, knockdown of Rac1b in the LNCaP clone stably expressing Rac1b recovered CNFY-induced apoptosis (Figure 2d).

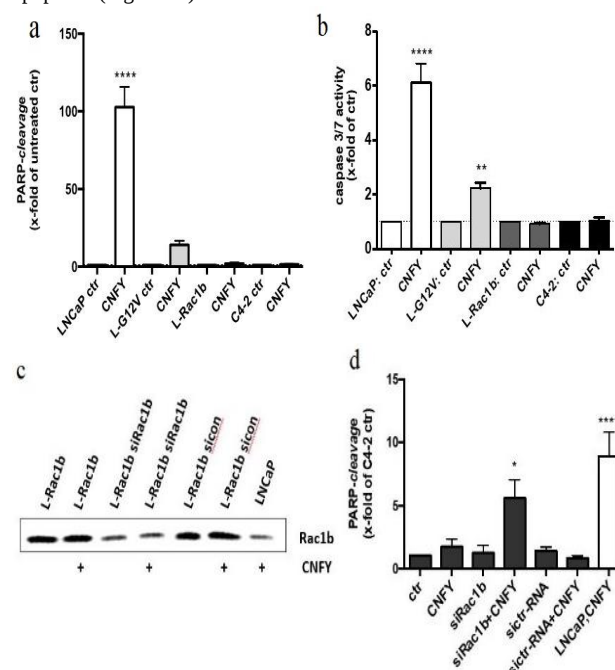


Figure 2: Rac1b blocks induction of apoptosis. LNCaP cells (white bars), C4-2 cells (black bars) and LNCaP cells stably expressing Rac1b (L-Rac1b, light grey bars) or Rac(G12V) (L-Rac(G12V), dark grey bars), respectively were treated with CNFY (1nM) for 72 h (a & b) or 24h (c & d) or left untreated (ctr) as indicated. a) Caspase 3/7 activity was analysed by APO-One and normalized to the untreated controls. Shown are results of three independent experiments as mean plus

standard deviation as x-fold of the untreated control. Data are derived from three independent experiments (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). **b)** Analysis of PARP cleavage: Quantification of more than three western blots showing poly (ADP-ribose) polymerase (PARP)-cleavage, which indicates caspase activity. Following toxin treatment, cells were lysed, and the lysates analysed for PARP (116 kDa) and cleaved PARP (89 kDa) by western blotting. Data are shown as x-fold of untreated control, mean plus standard deviation. **c)** L-Rac1b cells were transfected with siRNA against Rac1b or control siRNA, respectively. Cells were left untreated or incubated with CNFY (1nM) for 24 h. Western blot showing knockdown of Rac1b: Rac1b was enriched by a pulldown with beads-loaded Rac effector PAK and analysed by western blotting. Shown is a typical experiment out of three. **d)** Knockdown of Rac1b in L-Rac1b re-induces apoptosis. Analysis of PARP cleavage in cells treated as described in c: Shown is the quantification of more than three western blots showing poly (ADP-ribose) polymerase (PARP)-cleavage. Data are shown as x-fold of untreated L-Rac1b control, mean plus standard deviation (** $p < 0.01$, *** $p < 0.001$).

III Rac1b Stimulates Migration and Influences the Colony Formation Capacity

In other cancer entities, Rac1b has been shown to stimulate migration and invasion processes. Therefore, we analysed whether Rac expression may influence migration and capacity for colony formation in PCA relevant cell lines.

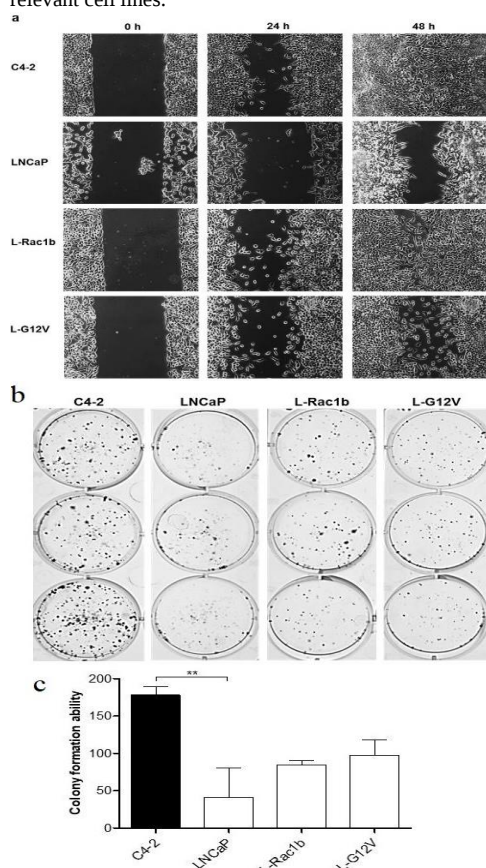


Figure 3: Rac1b increases migration and colony formation of LNCaP cells. Migration and colony formation capacity of LNCaP cells (white

bars), C4-2 cells (black bars) and LNCaP cells stably expressing Rac1b (L-Rac1b, light grey bars) or Rac(G12V) (L-Rac(G12V), dark grey bars), respectively were analysed. **a)** Cells were plated in sterile 24well plates (ibidi) 40.000 cells/well and grown until a monolayer has formed. Following scratching, wound healing capacity was visualized from 0 to 48 h by an automated microscope. Shown is one representative experiment out of two. **b) & c)** 500 cells/well were seeded into a 6 well plate and grown for 11 days, fixed and stained. Images were captured using Amersham Imager 600 and analysed with ImageJ. Shown is one representative experiment (b) and quantification (c) of stained colonies. Data represent three independent experiments as mean plus standard deviation (** $p < 0.01$).

As shown in (Figure 3a), LNCaP cells do not form a monolayer but grow in clusters. Expression of Rac1b or Rac(G12V) changes cell morphology as well as gap closure properties of LNCaP cells, making them more similar to C4-2 cells. However, C4-2 cell migration still outpaced L-Rac1b and L-Rac(G12V) in the gap closure assays performed. To analyse colony formation, cells were seeded in 6-well plates and grown for 10 days. Colonies were fixed and stained. Similar to the gap closure assay, expression of Rac1b or Rac(G12V) slightly but not significantly enhanced the colony formation capacity of LNCaP cells. Colony formation was much stronger in C4-2 cells (Figures 3b & 3c). Our data indicate that active Rac is sufficient to induce migration and colony formation of LNCaP cells, partly bridging the gap with androgen-independent C4-2 cells.

IV Active Rac Induces AR-Dependent Gene Expression by PAK Activation

C4-2 cells can be distinguished from LNCaP cells by their ability to survive and to proliferate in the absence of androgen. Consequently, we asked whether active Rac would be sufficient to induce AR-induced gene expression and performed reporter gene analysis. Expression of the prostate-specific antigen (PSA) is directly stimulated by the hormone-bound androgen receptor. To this end, we studied the expression of luciferase under the control of the PSA promoter in a dual luciferase assay. As expected, luciferase expression was tenfold higher in C4-2 cells compared to LNCaP cells, indicating stronger activation of the PSA promoter.

However, in L-Rac1b cells, luciferase expression was nearly as strong as in C4-2 cells (Figure 4a). Expression and localization (cytosol/nucleus) of the androgen receptor was not different between the cell lines (Figure 4b). This indicates a stronger intrinsic activation of the PSA promotor in C4-2 cells. AR-dependent promotor activation was also stimulated in LNCaP cells, expressing the dominant active mutant Rac(G12V). The data show that Rac1b is sufficient to activate signalling pathways inducing AR-regulated gene synthesis. Moreover, inhibition of Rac activity by the specific Rac inhibitor EHT1864 reduced AR-dependent gene expression in C4-2 cells (Figure 4c). To further analyse downstream signalling, we applied a specific inhibitor of the Rac effector PAK (IPA3). As shown in (Figures 4c & 4d), treatment of C4-2 and LNCaP cells with IPA3 significantly reduced intrinsic and CNFY-triggered stimulation of the PSA promotor in both cell lines, indicating that PAK is involved in Rac-induced stimulation of AR-dependent gene expression.

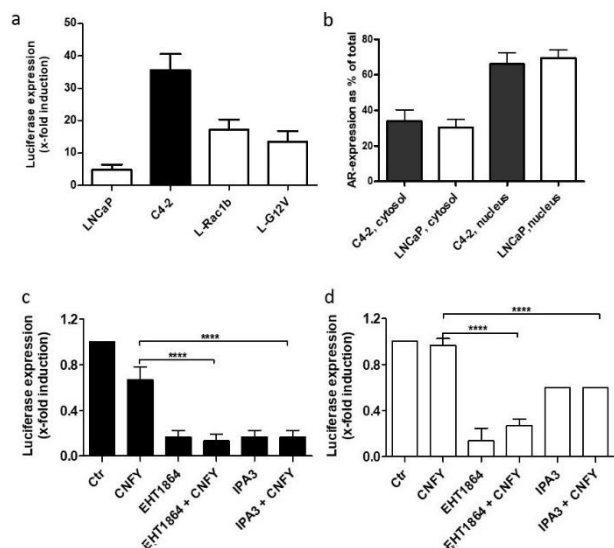


Figure 4: Transcriptional activity of Androgen receptor is dependent on Rac activity. LNCaP cells (white), C4-2 cells (black) and LNCaP cells stably expressing Rac variants (grey), were transfected with pRL-TK to allow normalization and in addition with the reporter plasmid pPSA-Luc which encodes for firefly luciferase under the control of the PSA promotor. **a)** Luciferase assay was performed for 18 h to measure the different transcriptional activity of the androgen receptor in C4-2, LNCaP, LNCaP-Rac1b and L-Rac(G12V). Data shown represent three independent experiments $\pm$ SD. Statistical analysis was performed using one-way ANOVA. * $p < 0.05$. **b)** The Androgen receptor is not differentially localized. LNCaP and C4-2 cells were separated into nuclear and cytosolic fraction by ultracentrifugation. The amount of Androgen receptor in each fraction was analysed by Western blotting. **c) & d)** The influence of Rac and PAK on the AR activity in C4-2 (black) and LNCaP (white) cells was analysed following incubation for 18 h in the presence of 4 nM CNFY and the inhibitors EHT1864 (Rac inhibitor) and IPA3 (PAK inhibitor). Data shown represent three independent experiments $\pm$ SD. Statistical analysis was performed using one-way ANOVA (**** $p < 0.0001$).

V Androgen Withdrawal Leads to Upregulation of Rac and Rac1b

Generation of Rac seems to represent an important ADT escape mechanism. To analyse whether hormone depletion leads to an enhancement of Rac mRNA in LNCaP cells, we cultivated LNCaP cells in medium containing charcoal-treated (hormone depleted) calf serum (Life Technologies) for 8 weeks (C/S), purified mRNA from the culture and analysed the respective cDNA for the presence of total Rac (including Rac1b) and Rac1b by qPCR. As shown in (Figure 5a), depletion of hormones increased the level of Rac and Rac1b mRNA to 150% and 300%, respectively. This further indicates a role of Rac in androgen-independent survival of PCa cells. Moreover, cultivation of LNCaP cells in the presence of the Enzalutamide (20 μ M, an androgen receptor inhibitor) and Abiraterone (10 μ M, an inhibitor of androgen synthesis), respectively, increased the protein level of Rac and Rac1b (quantification shown in Figure 5b, western blot in Figure S2) with only a moderate effect of the receptor inhibitor Enzalutamide.

To verify the clinical relevance of our findings, we also analysed PCa tissue: One primary tumor and matched lymph node metastases of a patient who has been treated with goserelin acetate to repress androgen production. First, we isolated mRNA and proved the presence of Rac1b (Figure 5c). The presence of Rac1b in the tissues was further verified by western blot analysis. In line with the quantitative qRT-PCR results, Rac1b expression is particularly high in the matched lymph node metastasis (Figure 5d). Considering the fact that Rac1b is known to promote and execute MMP-3 induced EMT in breast and lung cancer, this finding is not surprising.

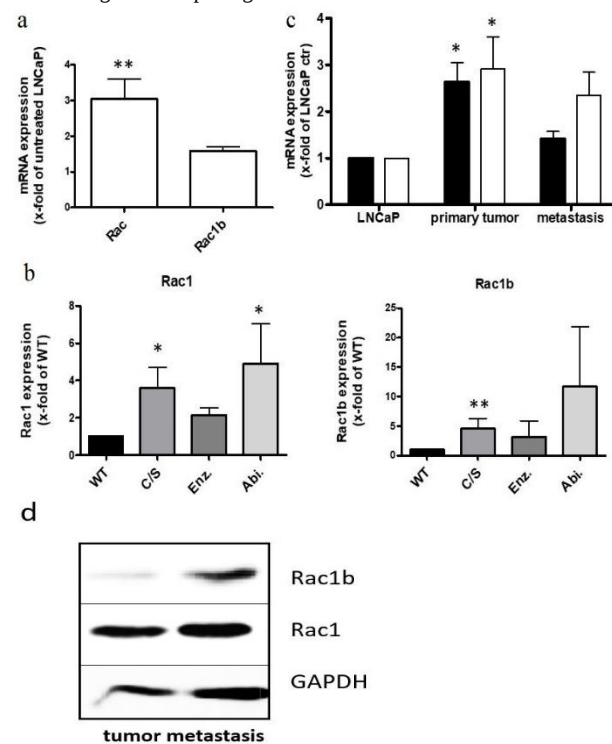


Figure 5: Androgen withdrawal induces expression of activated Rac. LNCaP cells were cultivated in medium containing charcoal-treated (hormone depleted) calf serum (C/S) or in the presence of Enzalutamide or Abiraterone for 8 weeks. **a)** cDNA was analysed for the presence of total Rac (including Rac1b) and Rac1b by qPCR. **b)** Rac1 (left) and Rac1b (right) protein was detected by western blotting. Shown is the quantification of 3 independent experiments depicted in (Figure S2). Clinical relevance: analysis of PCa tissue: One primary tumor and matched lymph node metastases of a patient who has been treated with Goserelin was analysed for **c)** Rac (black) and Rac1b (white) mRNA by qPCR and for **d)** Rac and Rac1b protein by western blotting with specific antibodies.

Discussion

Targeted therapies have successfully evolved for selected advanced solid tumors, including e.g. non-small cell lung carcinomas (NSCLC) or colorectal carcinomas (CRC). Analysis of mutations in a few therapy-relevant genes, e.g., EGFR exons 18-21 (NSCLC) or KRAS/NRAS exons 2-4 (CRC), is currently the prerequisite for therapy decision along with diagnostic guidelines. Besides mutations, also alternative splicing allows the formation of cancer-driving proteins. Splicing based non-genetic variability of gene expression is known to promote isoform

switching in the vast majority of oncogenes such as Rac potentially enabling new mechanisms of therapy resistance. For example, the anti-apoptotic effects of active Rac may support the survival of single tumor cells cycling within the blood vessels. The self-activating splice form Rac1b has been identified to promote tumor progression in several cancer entities. It induces the formation of ROS and ROS-dependent genetic instability in mammary epithelia [20]. Moreover, it stimulates epithelial to mesenchymal transition (EMT) in lung cancer [21]. However, the role of Rac1b in PCa is still very poorly investigated. Like many other solid tumors, PCa is a heterogeneous tumor, with different susceptibility to androgen withdrawal. Following a first response with diminished cancer tissue, frequently androgen-independent cancer cells develop (castration-resistant cancer), resulting in increased morbidity and reduced prognosis.

We detected Rac1b in the tissue of a goserelin treated PCa patient as well as in androgen independent PCa cells. We were further able to show that Rac1b expression is sufficient to induce AR-dependent gene synthesis. Thereby Rac1b can mediate androgen withdrawal-induced cancer cell death. Different from a mutation, Rac1b is generated by mRNA splicing. Splicing based non-genetic variability of gene expression promotes isoform switching in oncogenes such as Rac potentially enabling new mechanisms of therapy resistance. For example, the anti-apoptotic effects of active Rac may support the survival of single tumor cells cycling within the blood vessels. In this study, Rac1b influenced androgen dependence and survival of PCa cells and blocked pro-apoptotic signalling pathways. Selective knockdown of Rac1b or inhibition of Rac-dependent pathways re-induced apoptosis. Rac may, therefore, be considered a double-edged sword supporting two pro-tumorigenic functions in PCa, apoptosis and androgen-dependence. Moreover, our data suggests inhibition of Rac1b or Rac-dependent signalling (for example, PAK-inhibition) to be a pharmacological strategy for the treatment of castration-resistant PCa. The presence of an additional 19 amino acids, not present in Rac wildtype or other Rho GTPases, may allow the development of specific small molecular Rac1b inhibitors.

Ethics Statement

The use of human tissue for molecular analysis was approved by the ethics statement 311/12 by the Ethics Committee of the University Medical Center Freiburg, Engelbergerstr. 21, 79106 Freiburg (09.08.2012). All authors read the manuscript and agreed with publication (written consent obtained).

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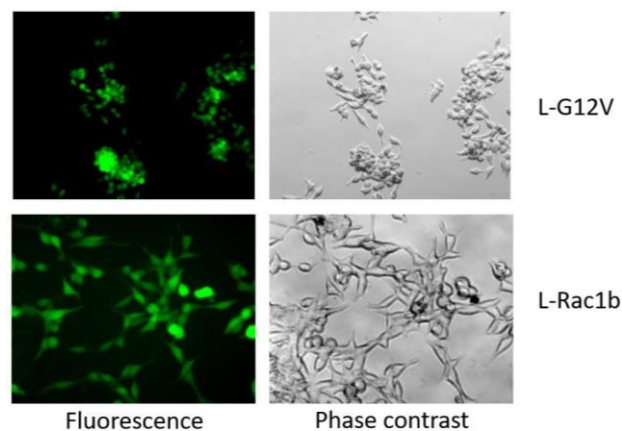


Figure S1: Rac and GFP expressing cell lines: GFP positive cells sorted using FACS.

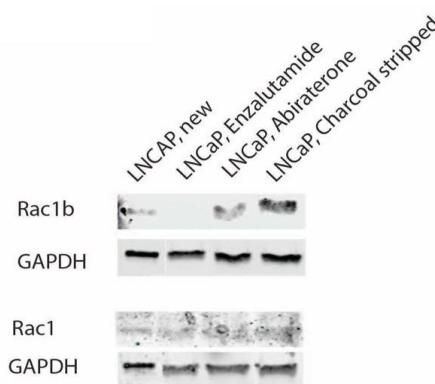


Figure S2: LNCaP cells were cultivated in medium containing charcoal-treated (hormone depleted) calf serum (C/S) or in the presence of Enzalutamide or Abiraterone for 8 weeks. **A)** cDNA was analysed for the presence of total Rac (including Rac1b) and Rac1b by qPCR. Rac1 and Rac1b protein was detected by western blotting (shown is a typical blot out of three).

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E et al. (2011) Global cancer statistics. *CA Cancer J Clin* 61: 69-90. [[Crossref](#)]
2. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H et al. (1983) LNCaP model of human prostatic carcinoma. *Cancer Res* 43: 1809-1818. [[Crossref](#)]
3. Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S et al. (1994) Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. *Int J Cancer* 57: 406-412. [[Crossref](#)]
4. Thalmann GN, Anezinis PE, Chang SM, Zhou HE, Kim EE et al. (1994) Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res* 54: 2577-2581. [[Crossref](#)]
5. Thalmann GN, Sikes RA, Wu TT, Degeorges A, Chang SM et al. (2000) LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. *Prostate* 44. [[Crossref](#)]
6. Vega FM, Ridley AJ (2008) Rho GTPases in cancer cell biology. *FEBS Lett* 582: 2093-2101. [[Crossref](#)]

7. Karlsson R, Pedersen ED, Wang Z, Brakebusch C (2009) Rho GTPase function in tumorigenesis. *Biochim Biophys Acta* 1796: 91-98. [[Crossref](#)]
8. Jaffe AB, Hall A (2002) Rho GTPases in Transformation and Metastasis. *Adv Cancer Res* 84: 57-80. [[Crossref](#)]
9. Fiegen D, Haeusler LC, Blumenstein L, Herbrand U, Dvorsky R et al. (2004) Alternative splicing of Rac1 generates Rac1b, a self-activating GTPase. *J Biol Chem* 279: 4743-4749. [[Crossref](#)]
10. Melzer C, Hass R, Lehnert H, Ungefroren H (2019) RAC1B: A Rho GTPase with Versatile Functions in Malignant Transformation and Tumor Progression. *Cells* 8: 21. [[Crossref](#)]
11. Aktories K (2006) Toxins as Tools. The Comprehensive Sourcebook of Bacterial Protein Toxins. *Clin Immunol Aspects Applications Bacterial Protein Toxins* 976-990.
12. Schmidt G, Goehring UM, Schirmer J, Lerm M, Aktories K (1999) Identification of the C-terminal part of Bordetella dermonecrotic toxin as a transglutaminase for Rho GTPases. *J Biol Chem* 274: 31875-31881. [[Crossref](#)]
13. Lang AE, Schmidt G, Sheets JJ, Aktories K (2011) Targeting of the actin cytoskeleton by insecticidal toxins from *Photobacterium luminescens*. *Naunyn Schmiedeberg's Arch Pharmacol* 383: 227-235. [[Crossref](#)]
14. Schmidt G, Sehr P, Wilm M, Selzer J, Mann M et al. (1997) Gln63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor 1. *Nature* 387: 725-729. [[Crossref](#)]
15. Flatau G, Lemichez E, Gauthier M, Chardin P, Paris S et al. (1997) Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* 387: 729-733. [[Crossref](#)]
16. Hoffmann C, Aktories K, Schmidt G (2007) Change in substrate specificity of Cytotoxic Necrotizing Factor (CNF) unmasks proteasome-independent down-regulation of constitutively active RhoA. *J Biol Chem* 282: 10826-10832. [[Crossref](#)]
17. Hoffmann C, Pop M, Leemhuis J, Schirmer J, Aktories K et al. (2004) The Yersinia pseudotuberculosis cytotoxic necrotizing factor (CNFY) selectively activates RhoA. *J Biol Chem* 279: 16026-16032. [[Crossref](#)]
18. Augspach A, List JH, Wolf P, Bielek H, Schwan C et al. (2013) Activation of RhoA,B,C by Yersinia Cytotoxic Necrotizing Factor (CNFY) induces apoptosis in LNCaP prostate cancer cells. *Toxins (Basel)* 5: 2241-2257. [[Crossref](#)]
19. Sotiropoulos A, Gineitis D, Copeland J, Treisman R (1999) Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* 98: 159-169. [[Crossref](#)]
20. Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM et al. (2005) Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* 436: 123-127. [[Crossref](#)]
21. Stallings Mann ML, Waldmann J, Zhang Y, Miller E, Gauthier ML et al. (2012) Matrix metalloproteinase induction of Rac1b, a key effector of lung cancer progression. *Sci Transl Med* 4: 142ra95. [[Crossref](#)]