The p90 ribosomal S6 kinase (RSK) inhibitor BI-D1870 prevents gamma irradiation-induced apoptosis and mediates senescence via RSK- and p53-independent accumulation of p21WAF1/CIP1

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The p90 ribosomal S6 kinase (RSK) family is a group of highly conserved Ser/Thr kinases that promote cell proliferation, growth, motility and survival. As they are almost exclusively activated downstream of extracellular signal-regulated kinases 1 and 2 (ERK1/2), therapeutic intervention by RSK inhibition is less likely to produce such severe side effects as those observed following inhibition of the upstream master regulators Raf, MEK and ERK1/2. Here, we report that BI-D1870, a potent small molecule inhibitor of RSKs, induces apoptosis, although preferentially, in a p21-deficient background. On the other hand, BI-D1870 also induces a strong transcription- and p53-independent accumulation of p21 protein and protects cells from gamma irradiation (γIR)-induced apoptosis, driving them into senescence even in the absence of γIR. Although we identified p21 in in vitro kinase assays as a novel RSK substrate that specifically becomes phosphorylated by RSK1-3 at Ser116 and Ser146, RNA-interference, overexpression and co-immunoprecipitation studies as well as the use of SL0101, another specific RSK inhibitor, revealed that BI-D1870 mediates p21 accumulation via a yet unknown pathway that, besides its off-site targets polo-like kinase-1 and AuroraB, also does also not involve RSKs. Thus, this novel off-target effect of BI-D1870 should be taken into serious consideration in future studies investigating the role of RSKs in cellular signaling and tumorigenesis.

Ribosomal S6 kinases (RSKs) constitute a family of serine/threonine kinases that in response to various stimuli are almost exclusively activated by extracellular signal-regulated kinases 1/2 (ERK1/2).[1] Thus, RSKs are downstream effectors of the Ras/mitogen-activated protein kinase (MAPK) pathway that is crucial for normal cell proliferation, differentiation, motility and survival. The importance of this pathway is emphasized by the fact that mutations or overexpression of many of the involved signaling components are hallmarks of several human cancers.[2] The RSK family consists of four isoforms (RSK1-4) that are products of separate genes sharing a high degree of sequence homology (75–80% identity). With the exception of RSK4, they are ubiquitously expressed, but are unique among kinases as they possess two distinct and functional kinase domains: the N-terminal kinase domain (NTKD) that is responsible for substrate phosphorylation and the C-terminal kinase domain (CTKD)

required for RSK autoactivation.[1] Activation of RSKs is achieved via a multistep phosphorylation cascade involving ERK1/2-mediated activation of the CTKD that in turn autophosphorylates a hydrophobic turn motif in RSK molecules. This creates a docking site for 3′-phospho-inositide-dependent kinase-1 (PDK1), a constitutively active Ser/Thr kinase, which phosphorylates and activates the NTKD.

Among a long list of proteins targeted by RSKs are, for instance, the pro-survival transcription factors cyclic AMP-responsive element-binding protein-1 (CREB1) and nuclear factor-κB (NF-κB), as well as proteins stimulating cap-dependent translation, including the eukaryotic translation initiation factor-4B (eIF-4B) and ribosomal protein-S6 (rpS6).[3–7] With the phosphorylation and inactivation of several cell cycle-regulatory and pro-apoptotic proteins such as the cyclin-dependent kinase (CDK) inhibitor p27, Chk1, BAD, BimEL, caspase-8 and p53, activated RSKs are

proposed to strongly promote cell cycle progression and survival.[8–13] In line with these conclusions, it was shown that pharmacological or siRNA-mediated inhibition of RSK activity and/or expression caused a G1 cell cycle block in several tumor lines,[14,15] whereas RSK2 overexpression enhanced colony formation even in the absence of tumor promoters.[16] Accordingly, elevated RSK1/2 levels were found in several tissue samples from breast and prostate cancer patients compared with the corresponding normal tissues.[14,15] Furthermore, dominant-negative RSK1 alleles eliminated survival that was mediated by the ERK-activating kinase MEK,[17] whereas constitutively active RSK2 promoted cell survival at levels equivalent to those obtained with an activated MEK enzyme.[10] Together, these data suggest that RSK1/2 are critically involved in cell proliferation and transformation, making them promising therapeutic targets for the treatment of cancer.

One of the most important regulators of cell cycle progression is the CDK inhibitor p21 that mediates DNA damage-induced cell cycle arrest mainly in a p53-dependent manner. In addition, p21 also participates in other diverse processes including transcription, DNA repair, differentiation, senescence and apoptosis.[18,19] Thus, mechanisms are required that tightly control its expression and functional diversity. This is achieved, for instance, by multiple phosphorylations that either result in its stabilization or in proteasomal degradation.[20] In addition, expression of p21 is also controlled at the transcriptional and post-transcriptional level, giving the cell multiple opportunities to interfere with p21 function.[21–24]

Here we show that treatment of cells with the small molecule RSK inhibitor BI-D1870 results in a transcription- and p53-independent accumulation of p21, as well as in senescence induction and protection from γIR-induced apoptosis. Although we subsequently identified p21 as a novel in vitro substrate for RSKs, numerous experiments revealed that BI-D1870 mediates these stress responses via a yet unknown RSK-independent pathway. Thus, our study uncovers novel off-target effects of BI-D1870 that should be taken into consideration in further studies involving this compound.

Results

Effect of MAPK inhibitors on radiation responses of HCT116 cell lines. Recently, we reported that p21 not only inhibits but also activates several kinases including MAPKs in a substrate-dependent manner.[25] As MAPKs are critical components of cellular stress and survival pathways,[26,27] we analyzed their contribution to gamma-irradiation (γIR)-induced stress responses of HCT116 colon carcinoma cells. We have chosen this particular system as p21 determines therein the fate of irradiated cells.[23,28] Whereas γIR drives wild-type HCT116 cells into senescence due to p53-dependent p21 induction, similarly treated checkpoint-deficient (p21 − / − and p53 − / −) HCT116 cells succumb to apoptosis (Figures 1a and b).[23,28] This is because p21 functions not only as an inducer of cellular senescence but also as a potent apoptosis inhibitor.[19] Interestingly, irradiation of p53- and p21-deficient HCT116 cells in the presence of U0126, an inhibitor of the ERK1/2-activating kinases MEK1/2, protected them from apoptosis, as evidenced microscopically (not shown) and by inhibition of caspase-3-like DEVDase activity (Figure 1a). Incubation of the cells with the c-Jun N-terminal kinase (JNK) inhibitor-I or SB203580 targeting p38 had no effect on their radiation responses (Figure 1a). Moreover, although all three MAPK inhibitors did not greatly influence cell cycle distribution of unstressed and irradiated cells (Figure 1c; not shown), p53- and p21-deficient cells rescued by U0126 from γIR-induced apoptosis showed an enlarged and flattened morphology, exhibiting increased senescence-associated β-galactosidase (SA-β-Gal) activities (Figure 1b). Remarkably, these senescence markers were even evident when checkpoint-deficient and wild-type HCT116 cells were treated solely with U0126 (Figure 1b). As inhibition of JNK and p38 affected neither γIR-induced apoptosis (Figure 1a) nor senescence (not shown), our data suggest an involvement of the MEK/ERK pathway in radiation responses of HCT116 cells that is functionally independent of p53 and p21.

Effect of the RSK inhibitor BI-D1870 on radiation responses of HCT116 cell lines. Among a multitude of effectors targeted by ERK1/2 are RSK family members that are almost exclusively activated by these MAPKs.[1] To examine their role in radiation responses of HCT116 cells, we irradiated wild-type and checkpoint-deficient cells in the absence and presence of BI-D1870, a potent RSK inhibitor.[29] BI-D1870 almost completely prevented γIR-induced apoptosis of p53-deficient HCT116 cells (Figure 2a) and, similar to U0126, produced the senescent phenotype in both wild-type and p53-deficient cells regardless of whether or not they were irradiated (Figure 2b). BI-D1870 also induced senescence in MCF-7/casp3 breast carcinoma cells, indicating a more general response to this compound (Supplementary Figure S1). In contrast to U0126, however, BI-D1870 did not exert these effects in p21-deficient cells (Figures 2a and b), although it efficiently induced a transient G2/M arrest in all three non-irradiated HCT116 lines (Figure 2c). In addition, BI-D1870 by itself induced massive apoptosis in p21-deficient cells, whereas wild-type and p53 − / − cells were killed by this compound to a much lesser extent (Figure 2a), suggesting a close functional relationship between RSKs and p21.

BI-D1870 induces transcription and p53-independent p21 accumulation. Therefore, we analyzed p21 expression in BI-D1870-treated cells and found that this compound induced a substantial p21 accumulation in HCT116 wild-type cells albeit to a lesser extent compared with γIR (Figure 3a). In contrast, expression of p27 and p57, two other CDK inhibitors of the CIP/KIP family, remained unaffected (Figure 3b). Interestingly, BI-D1870-mediated p21 accumulation appears to occur independently of p53 that was only stabilized following γIR, but not in response to BI-D1870 (Figure 3a). Consistently, whereas p21 readily accumulated also in BI-D1870-treated p53-deficient cells γIR failed to induce p21 expression in the absence of p53 (Figure 3c). Moreover, only γIR, but not BI-D1870 or DMSO, induced p21 mRNA expression (Figure 3d), demonstrating that
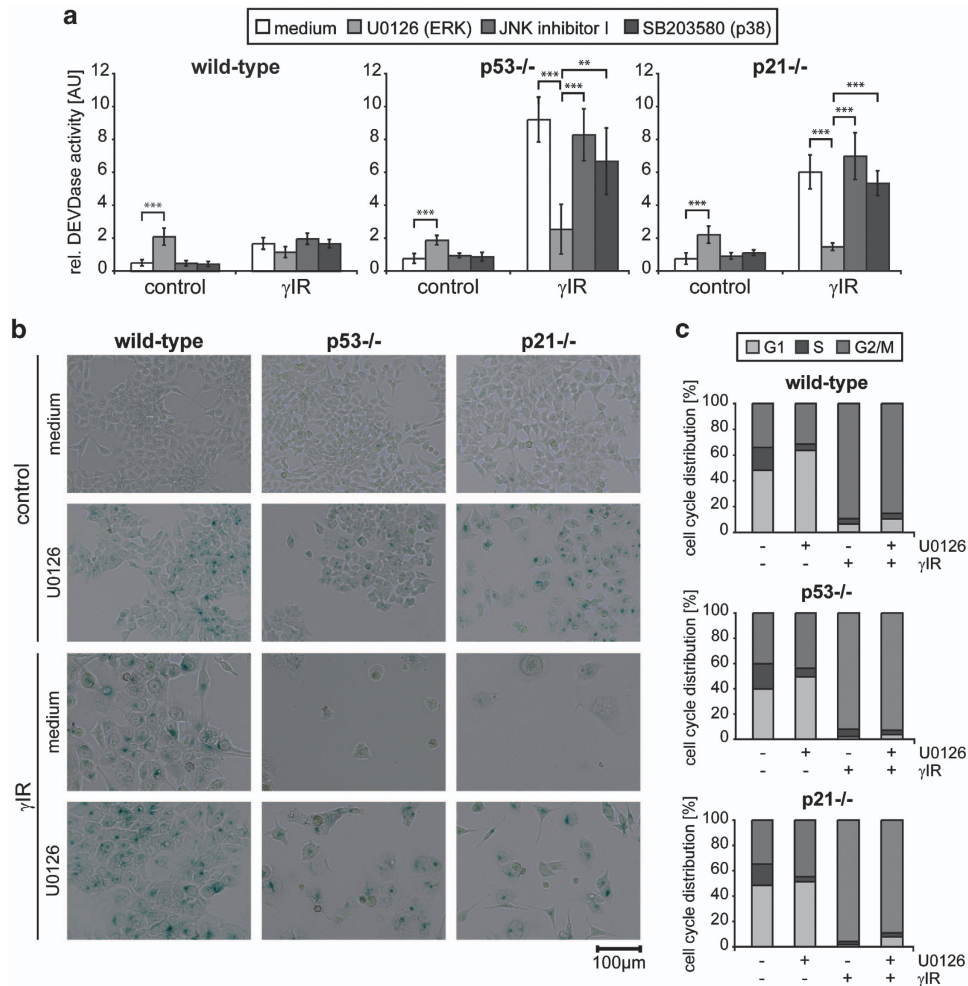
Figure 1 Effect of MAP kinase inhibitors on γIR-induced stress responses. (**a**) U0126 rescues checkpoint-deficient HCT116 cells from γIR-induced apoptosis independently of p21. Determination of DEVDase activities in wild-type and checkpoint-deficient (p53 − / − , p21 − / −) HCT116 cells 3 days after they were either left untreated (control) or γ-irradiated in the absence (medium) or presence of the MEK inhibitor U0126 (20 μM), the JNK inhibitor-I (1 μM) or the p38 inhibitor SB203580 (20 μM). Arbitrary units (AU) shown are the mean of at least four independent experiments + / − S.D. **P<0.01; ***P<0.005, paired Student's *t*-test. (**b**) U0126 induces cellular senescence independently of p21. Determination of SA-β-Gal activity in the indicated HCT116 cell lines 4 days after they were treated as in **a**. One representative experiment out of three is shown. (**c**) Effect of U0126 on cell cycle distribution. The indicated HCT116 cells were treated as in **a** and the number of cells in the different cell cycle phases was determined after 24 h by FACS analyses. Percentages shown are the mean of three independent experiments. For **a** to **c**, cells were preincubated with U0126 for 30 min and with the JNK inhibitor-I and SB203580 for 2 h

BI-D1870-induced p21 upregulation occurs independently of p53 most likely by a post-translational event.

Consistent with our observation that BI-D1870 was able to induce senescence in MCF-7/casp3 cells as well (Supplementary Figure S1), this event was also accompanied by p21 upregulation (Figure 3e). In contrast, we did not detect an increase in p21 expression when MCF-7/casp3 or HCT116/p53 − / − cells were exposed to U0126 (Figures 3e and f), which is in agreement with our observation that U0126, unlike BI-D1870, mediates apoptosis resistance and senescence induction independently of p21 (Figures 1 and 2).

AuroraB and polo-like kinase-1 are not involved in BI-D1870-induced p21 accumulation. Although BI-D1870 was originally postulated to specifically inhibit RSKs, this and another report also demonstrated BI-D1870-mediated inhibition of polo-like kinase-1 (PLK1) and AuroraB, albeit with

lower potencies.[29,30] As PLK1 and AuroraB are crucial regulators of cell division that are often overexpressed in several cancers and whose inhibition elicits severe stress responses such as senescence and apoptosis induction,[31] we determined their involvement in BI-D1870-mediated cellular responses. Although RNA-interference studies revealed that depletion of AuroraB or PLK1 resulted in p21 accumulation in HCT116 wild-type cells, this event was accompanied in both cases by an increase in p53 expression, indicating p53-dependent stress responses (Figures 4a and b, lanes 1–3). Indeed, in the absence of p53, p21 upregulation was only observed when p53-deficient HCT116 cells were treated with BI-D1870, but not in response to control, PLK1 or AuroraB siRNAs (Figure 4b, lanes 4–6; Figure 4c). In addition, silencing PLK1 resulted in massive apoptosis of HCT116 wild-type cells that was much more pronounced than in BI-D1870-treated cells (compare
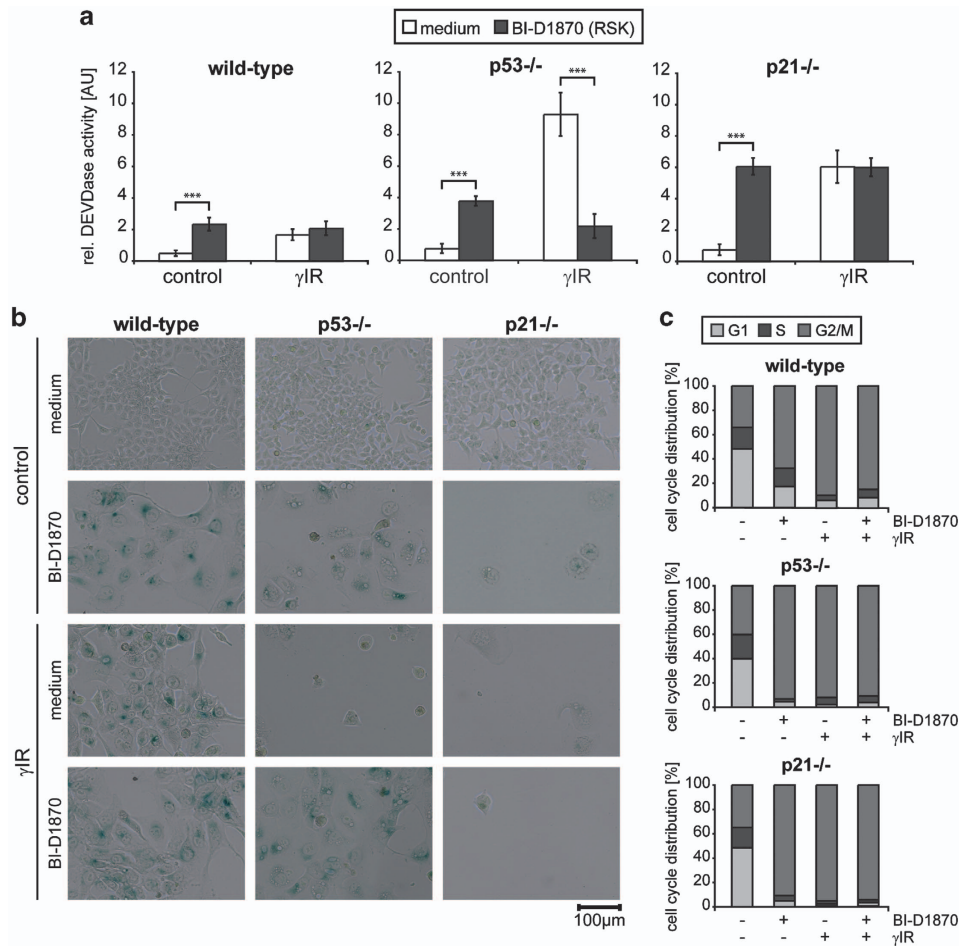
Figure 2 Effect of the RSK inhibitor BI-D1870 on γIR-induced stress responses. (**a**) BI-D1870 rescues HCT116 cells from γIR-induced apoptosis in a p21-dependent manner. Determination of DEVDase activities in wild-type and checkpoint-deficient (p53 $-/-$, p21 $-/-$) HCT116 cells 3 days after they were either left untreated (control) or γ-irradiated in the absence (medium) or presence of BI-D1870 (10 μM). Arbitrary units (AU) shown are the mean of three independent experiments $+/-$ S.D. ***$P < 0.005$, paired Student's *t*-test. (**b**) BI-D1870 induces cellular senescence in a p21-dependent manner. Determination of SA-β-Gal activity in the indicated HCT116 cell lines 4 days after they were treated as in **a**. One representative experiment out of three is shown. (**c**) Effect of BI-D1870 on cell cycle distribution. The indicated HCT116 cells were treated as in **a**, and the number of cells in the different cell cycle phases was determined after 24 h by FACS analyses. Percentages shown are the mean of three independent experiments. For **a** to **c**, cells were preincubated with BI-D1870 for 2 h

Figures 2a and 4d). Together, these data demonstrate that BI-D1870-induced p21 upregulation and the consequent anti-apoptotic and pro-senescent effects do not involve AuroraB or PLK1, but may depend on RSK inhibition.

RSK1-3 isoforms phosphorylate p21 *in vitro* at Ser116 and Ser146. As RSKs phosphorylate and inactivate several anti-apoptotic and cell cycle-regulatory proteins including p27,[8] we investigated whether p21 might be targeted by RSKs. Indeed, *in vitro* kinase assays revealed that recombinant active RSK isoforms (RSK1/2/3) specifically phosphorylate GST-tagged p21, but not GST alone (Figures 5a and b). Furthermore, BI-D1870 inhibited RSK2-mediated phosphorylation of GST-p21 in a dose-dependent manner, verifying the specificity of this reaction (Figure 5c). In order to identify the RSK phosphorylation site(s) in p21, we first reasoned that RSKs might target Thr145 or Ser146, as these residues are known to be phosphorylated by Akt and PKCζ, respectively, which together with RSKs belong to the AGC kinase family

sharing several structural and functional similarities.[32] However, whereas the p21(T145A) mutant was not phosphorylated by Akt, it was even more intensively phosphorylated by RSK2 compared with the p21 wild-type protein (Figure 5d). An inverse picture emerged when we analyzed the p21(S146A) mutant that was phosphorylated by Akt, but hardly by RSK2 (Figure 5d). Thus, we concluded that Akt and RSK2 phosphorylate the adjacent Thr145 and Ser146 sites in p21, respectively.

In addition to Ser146, mass spectrometric analyses of *in vitro* RSK-phosphorylated GST-p21 also identified Ser116 as a phospho-acceptor site for RSK2 (Supplementary Figure S2), a finding that was confirmed by mutagenesis studies (Figures 5c–e). Although the decrease in phosphorylation of the p21(S116A) mutant was not as substantial as that observed with the p21(S146A) mutant, it was still reproducibly less phosphorylated by RSK2 than was the GST-p21 wild-type protein (Figure 5d). Consistently, an additive effect was achieved with the p21 double mutant
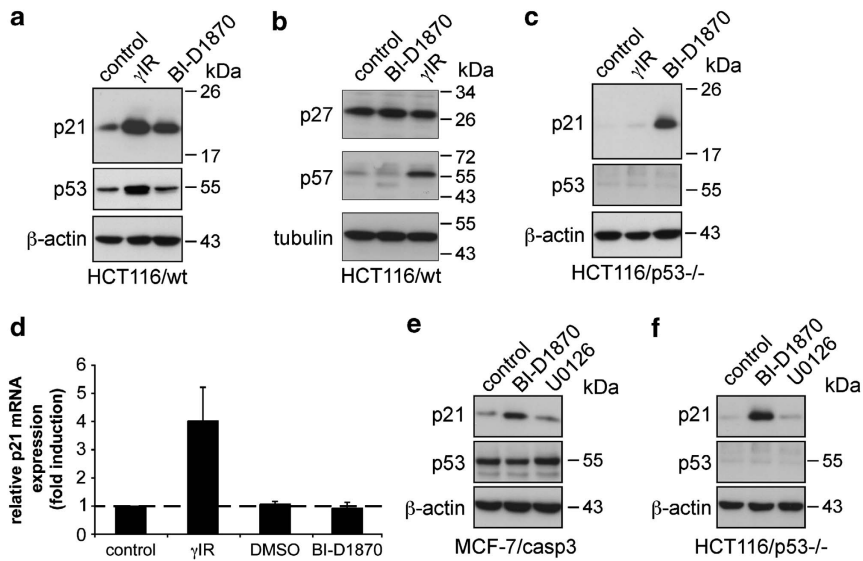
Figure 3 BI-D1870 induces p21 expression in a transcription- and p53-independent manner. (a–c and e and f) Western blot analyses for the status of p21, p27, p57 and p53 24 h after the indicated cell lines were either left untreated (control) or exposed to γIR, BI-D1870 (10 μM) or U0126 (20 μM). Blots shown are representative results of three independent experiments. (d) Real-time PCR for the determination of the expression levels of p21 mRNA in HCT116 wild-type cells that were either left untreated (control) or exposed to γIR, BI-D1870 (10 μM) or DMSO (0.1%). Total RNA was isolated 24 h post treatment and analyzed with transcript-specific probes from Applied Biosystems. Data shown are the mean of three independent experiments $+/-$ S.D.
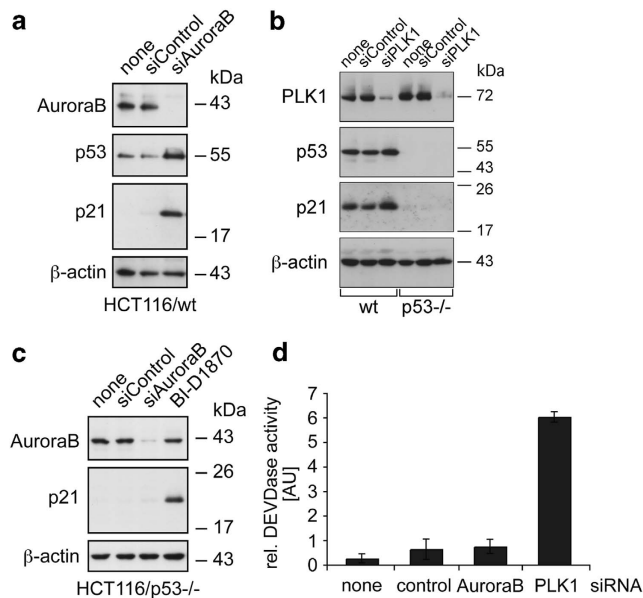

Figure 4 Effect of the knockdown of BI-D1870-inhibitable kinases on p21 expression. (a–c) Western blot analyses for the status of p21 and p53 48 h after the indicated cell lines were transfected with control siRNA, AuroraB siRNA (a, c) or with PLK1 siRNA (b) or following treatment with BI-D1870 (10 μM) (c). Please note that the p21 blot in B is overexposed to show that p21 is indeed not induced by the PLK1 siRNA in p53-deficient cells. Blots shown are representative results of three independent experiments. (d) Determination of DEVDase activity obtained 48 h post transfection of HCT116 wild-type cells with the indicated siRNAs. Data shown are the mean of three independent experiments $+/-$ S.D.

(S116A/S146A) that remained almost completely unphosphorylated in the presence of RSK2, verifying that both residues constitute RSK2 phospho-acceptor sites (Figure 5d). As introduction of these single and double mutations also

compromised p21 phosphorylation by RSK1/3 (Figure 5e), but not by Akt (Figure 5d), our data demonstrate that p21 is phosphorylated *in vitro* at Ser116 and Ser146 by all three RSKs.

In contrast to the amino acid sequence surrounding Ser146 (KRRQTpS), the residue Ser116 (VDLSLpS) does not lie within an RSK consensus sequence (BxBxxpS/pT in which B is a basic aa) that is usually phosphorylated *in vivo* by the NTKD of RSKs. Therefore, we asked whether Ser116 may represent an artificial *in vitro* CTKD target that *in vivo* is not involved in substrate phosphorylation. We incubated the p21(S146A) mutant (in which Ser116 remains RSK phosphorylatable) with recombinant RSK2 in the presence of increasing concentrations of BI-D1870 that exclusively acts as an ATP-competitive inhibitor of the NTKD of RSKs.[29] Similar to phosphorylation of wild-type p21 and the p21(S116A) mutant, the already strongly diminished RSK2-mediated phosphorylation of the p21(S146A) mutant was completely blocked by BI-D1870, whereas DMSO had no effect (Figure 5c). This suggests that both the consensus site at Ser146 and that at Ser116, which is embedded in a non-consensus sequence, are phosphorylated *in vitro* by the NTKD of RSKs.

RSKs are not involved in BI-D1870-induced p21 accumulation. To verify this hypothesis also *in vivo*, we first probed immunoprecipitated Flag-p21wt from MCF-7/casp3 cells treated for 2 hours with PMA, a potent activator of RSKs,[33] with a phospho-Ser146-specific p21 antibody. Indeed, PMA stimulation resulted in RSK activation and in a strong p21(Ser146) phosphorylation that was partially, but significantly, prevented when the cells were co-treated with BI-D1870 (Figures 6a and b). MALDI-MS analysis confirmed PMA-induced Ser146 phosphorylation of Flag-p21wt
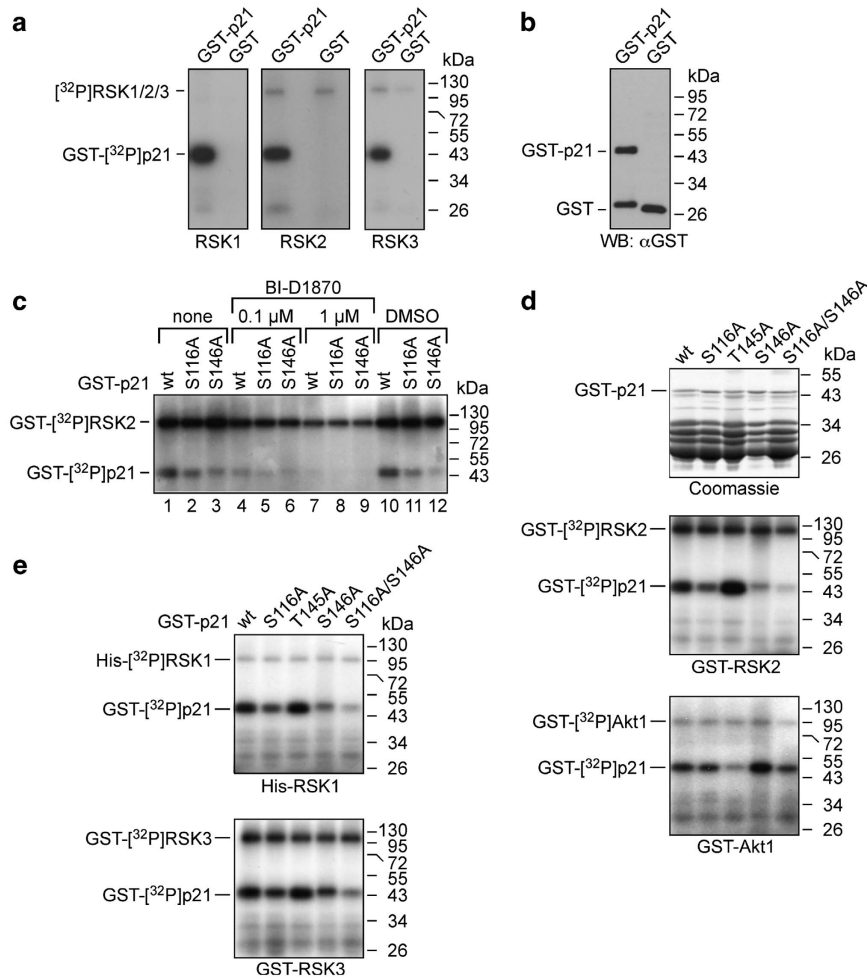
Figure 5 p21 is a novel RSK substrate *in vitro*. (**a** and **b**) p21 is phosphorylated by all three RSK isoforms. Recombinant GST-p21 and GST were either incubated for the *in vitro* kinase assays (**a**) with the indicated active RSK isoforms or were subjected to western blot analysis (**b**) for GST expression. (**c**) BI-D1870 inhibits RSK2-mediated p21 phosphorylation at both phospho-acceptor sites. The indicated GST-p21 wild-type (wt) and mutant proteins were incubated for the *in vitro* kinase assay with active RSK2 in the absence or presence of the indicated concentrations of BI-D1870 or DMSO. (**d**) p21 is phosphorylated by RSK2 at serines 116 and 146. The Coomassie-stained gel (upper panel) shows that approximately equal amounts of the indicated full-length GST-p21 wild-type and mutant proteins were incubated for the *in vitro* kinase assays with active RSK2 (middle panel) or active Akt1 (lower panel). Autophosphorylated RSK2 and Akt1 are indicated. (**e**) p21 is also phosphorylated by RSK1 and RSK3 at serines 116 and 146. Equal amounts of the indicated GST-p21 wild-type and mutant proteins were incubated for the *in vitro* kinase assays with active RSK1 (upper panel) or active RSK3 (lower panel). Autophosphorylated RSK1 and RSK3 are indicated

immunoprecipitated from MCF-7/casp3 and HCT116 cells (not shown), indicating RSK participation in this event. Ser116 phosphorylation, however, was not verified by MALDI-MS (not shown). As also our attempt to generate a phospho-Ser116 p21 antibody was unsuccessful, it is presently unclear whether p21 is phosphorylated *in vivo* exclusively at Ser146 upon PMA stimulation.

Next, we examined p21 expression in HCT116 cells following siRNA-mediated depletion of one or more RSKs. Whereas western blot analyses demonstrated knockdowns of RSK1 and RSK2 in both wild-type and p53-deficient HCT116 cells, because of the lack of reliable RSK3 antibodies real-time PCR analyses were required to assess the successful suppressive effect of RSK3 siRNA (Figures 6c–f). Surprisingly, however, neither individual RSK knockdowns nor their combined depletion resulted in p21 upregulation or in senescence induction (Figures 6c and d; not shown). Moreover, SL0101, another potent RSK inhibitor,[14,15] was

also incapable of upregulating p21 expression and inducing cellular senescence in both wild-type and p53-deficient HCT116 cells (Figure 7a; Supplementary Figure S3). In addition, whereas BI-D1870 rescued only HCT116/p53 − / − cells from γIR-induced apoptosis and by itself induced massive apoptosis in p21-deficient cells, SL0101 displayed no apoptotic potential and protected both checkpoint-deficient cell lines in a p21-independent manner, indicating different target usage (Figures 7b and c).

As these results strongly argued against our initial hypothesis that BI-D1870 induces p21 accumulation and senescence via RSK inhibition, we finally utilized two different approaches to analyze whether the fate of p21 is altered *in vivo* by activated RSKs. First, we compared the half-life of exogenously expressed Flag-p21wt in MCF-7/casp3 cells in the absence and presence of PMA. Although PMA induced phosphorylation and activation of RSK1/2/3, Flag-21wt levels declined with comparable rates regardless of whether or not
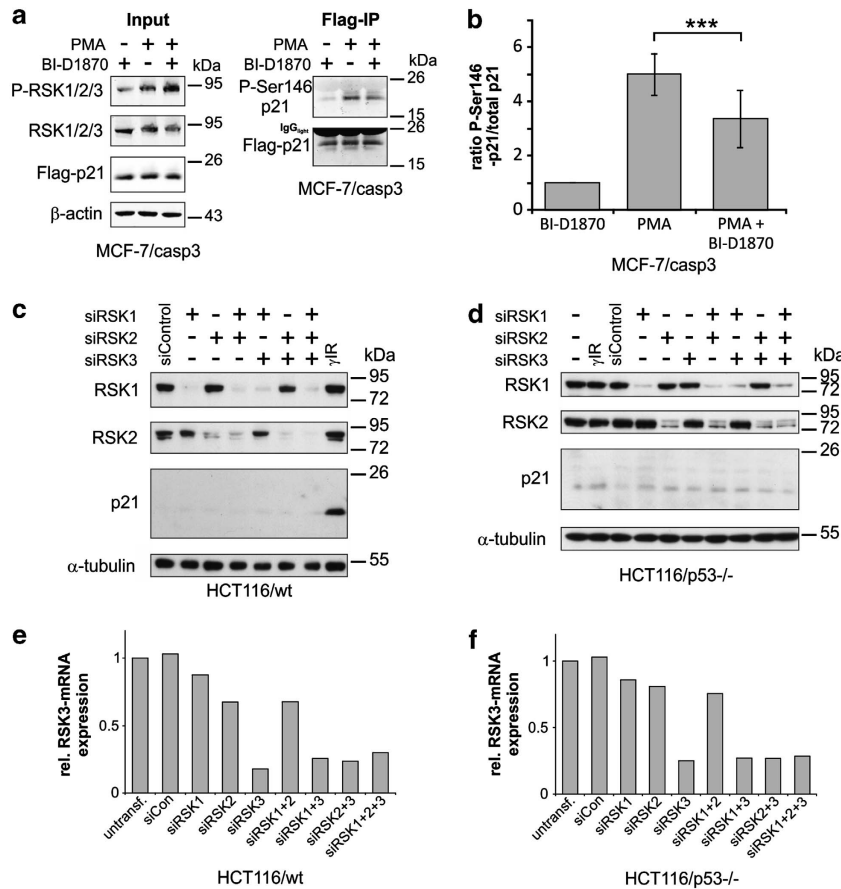
Figure 6 Knockdown of RSKs does not induce p21 expression. (**a** and **b**) PMA induces *in vivo* p21 phosphorylation at Ser146. 24 h after transfection with a Flag-p21 wild-type construct; MCF-7/casp3 cells were treated for 1 h as indicated (PMA, 10 ng/ml; BI-D1870, 10 μM) in the presence of MG-132 (10 μM) before they were harvested and the cellular extracts subjected to immunoprecipitation with Flag-agarose. Both, the cellular extracts (Input) and the precipitates, (Flag-IP) were analyzed by western blotting. One representative experiment out of six is shown. (**b**) Densitometric analysis of the amount of phosphorylated Ser146-p21 relative to the total precipitated p21 protein. Shown is the mean $+/-$ S.D. (***$P < 0.005$, paired Student's *t*-test) of six independent experiments performed as described in (**a**) and normalized to the amount detected in BI-D1870 treated cells. Three experiments were analyzed using scanned ECL-Western Blots and the ImageJ software, whereas three other experiments were analyzed using the LI-COR infrared imaging system and Odyssey software. (**c** and **d**) Western blot analysis for the status of RSK1, RSK2 and p21 48 h (**c**) and 72 h (**d**) after the indicated cell lines were transfected with a control siRNA or with siRNAs targeting RSK1, RSK2 or RSK3. One sample containing gamma-irradiated cells was also included in **c**. One representative experiment out of three is shown. (**e** and **f**) Due to the lack of a reliable RSK3 antibody, we assessed the effect of the RSK3 siRNA by real-time PCR analyses. mRNA was isolated and analyzed with a specific Taqman probe for expression of the RSK3 mRNA. The β-actin probe was used as a normalization control. One representative experiment out of two is shown

the cells were exposed to PMA (Figure 7d). Similar results were obtained in HCT116 cells and when γIR-induced endogenous p21 levels were monitored in both cell lines following their treatment with Chx in the absence or presence of PMA (not shown).

As PMA can also activate other kinases including protein kinase C (PKC) isoforms that are known to phosphorylate p21, thereby altering its stability,[20] we employed h-TERT-immortalized retinal pigment epithelial (RPE1) cells that upon doxycyclin treatment overexpress a constitutively active Flag-RSK2-CA enzyme.[9] Following their exposure to γIR, expression of endogenous p21 was monitored over a period of 10 h in the absence and presence of doxycyclin. However, the massive doxycycline-induced expression of Flag-RSK2-CA had no effect on γIR-induced p21 levels (Figure 7e). Moreover, as also the Chx-mediated decline of exogenously expressed Flag-p21wt proceeded with similar kinetics in the absence and presence of doxycyclin-activated Flag-RSK2-

CA (not shown), these data strongly argue against our hypothesis that p21 becomes degraded following phosphorylation by RSKs. Consistently, whereas several proteins known to interact with p21, including cyclins, CDKs and peripheral cell nuclear antigen (PCNA), were clearly detectable in anti-Flag precipitates of MCF-7/casp3 cells exogenously expressing Flag-p21wt, total or activated (phosphorylated) RSKs were conspicuously absent (Supplementary Figure S4). This suggests that BI-D1870 mediates the observed effects (p21 accumulation, apoptosis modulation, senescence induction) independently of RSK inhibition.

Discussion

Mutational activation of RAS proteins is associated with the occurrence of almost 30% of all human tumors, but due to off-target and severe side effects clinical trials based on small
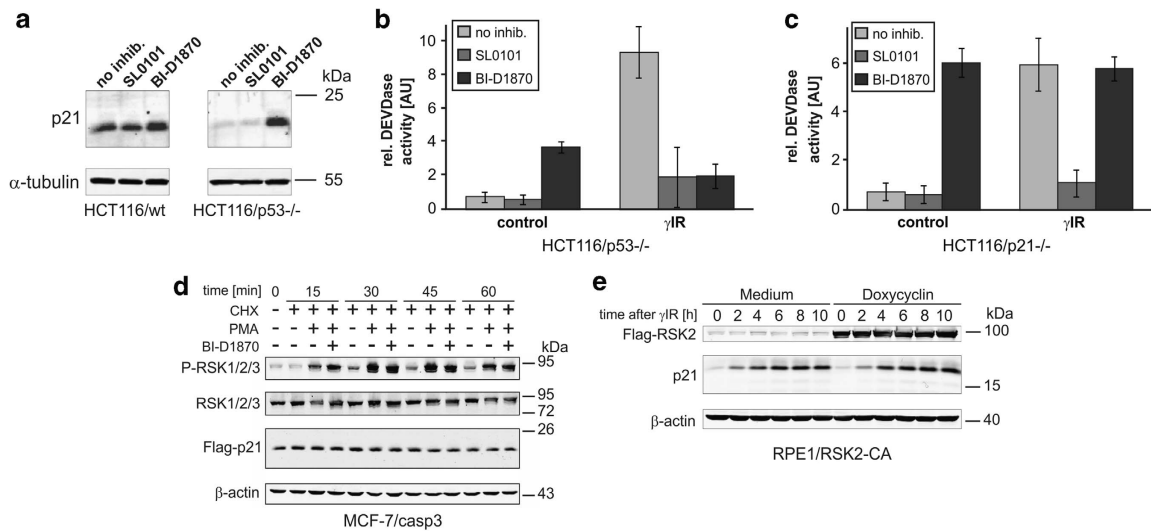
Figure 7 Effects of SL0101 and active RSKs on p21 expression and apoptosis. (**a**) HCT116 wild-type and p53 $-/-$ cells were either left untreated or treated for 24 h with SL0101 (100 μM) or BI-D1870 (10 μM) before their cellular extracts were analyzed by western blotting for p21 expression. One representative experiment out of four is shown. (**b** and **c**) The indicated checkpoint-deficient HCT116 cells were pre-incubated for 2 h with SL0101 (100 μM) or BI-D1870 (10 μM) before they were gamma-irradiated. After 3 days, cells were analyzed for DEVDase-like activities. Shown is the mean $+/-$ S.D. of 4–10 independent experiments. (**d**) MCF-7/casp3 cells were transfected with Flag-p21wt 24 h prior to treatment with PMA (10 ng/ml), CHX (10 μg/ml) and BI-D1870 (10 μM). After the indicated time points cellular extracts were analyzed by western blotting for the status of active P-RSK1/2/3, total RSK1/2/3 and Flag-p21. One representative experiment out of three is shown. (**e**) RPE1 cells that upon doxycyclin treatment express a constitutively active RSK2 mutant (Flag-RSK2-CA) were cultured for 16 h in the absence or presence of doxycyclin (100 ng/ml) before they were gamma-irradiated. At the indicated time points post irradiation, cells were harvested and cellular extracts were analyzed by western blotting for the status of Flag-RSK2-CA and endogenous p21. One representative experiment out of two is shown

molecule inhibitors targeting participating downstream kinases including Raf, MEK and ERK1/2 have so far not been very successful.[34] As a consequence, further efforts are required to identify additional targets that control fewer downstream pathways and that are thus less likely to mediate such severe side effects upon their inhibition. RSKs might represent such targets, as they are almost exclusively activated downstream of ERK1/2, controlling the proliferation and survival of a variety of cancer cell lines.[1,35] Particularly, RSK2 was shown to act as a key regulator in cellular transformation and metastasis.[36–38] Thus, several pre-clinical studies point to RSKs as potential therapeutic targets for various cancer types, including breast, prostate and multiple myeloma.[14,15,34,38–43] However, such an intervention requires detailed knowledge about the *in vivo* effects of RSK inhibitors.[34,44]

Together with SL0101[15] and FMK,[43] the dihydropteridione BI-D1870 was among the first molecules identified inhibiting all four RSK isoforms *in vitro* and *in vivo* with a $>$500-fold greater selectivity over many other kinases tested, including 10 closely related AGC kinases.[29] As BI-D1870 appears to be slightly more potent than SL0101,[30] and because FMK inhibits the C-terminal kinase domain and thus does not affect RSKs activated by a CTKD-independent mechanism,[45] we decided to use BI-D1870 in our study. In various BI-D1870-treated cell lines we observed a massive transcription- and p53-independent accumulation of p21, which, in line with its crucial role as a cell fate modulator,[19,23,28] induced p21-driven senescence and protected p21-proficient cells from γIR-induced apoptosis. On the other hand, BI-D1870 was unable to mediate these effects in p21-deficient cells, which in contrast were efficiently killed by this compound even in the absence of irradiation. As BI-D1870 also induced apoptosis in p21-proficient cells,

albeit to a much lesser extent, our data suggest that this compound bears an important anti-tumorigenic potential that, however, appears to be efficiently counteracted by its own capability to upregulate the expression of anti-apoptotic p21.[19]

As BI-D1870 treatment resulted specifically in p21 upregulation without affecting the expression of other CDK inhibitors such as p27 and p57, we assumed a close functional relationship between RSKs and p21. Indeed, *in vitro* kinase assays identified p21 as a novel RSK substrate, and thus we hypothesized that *in vivo* p21 becomes degraded following phosphorylation by RSKs. To our surprise, however, we accumulated several lines of evidence that clearly argue against this hypothesis. First, co-immunoprecipitation studies failed to reveal an interaction between RSKs and exogenously expressed Flag-p21wt. Second, neither PMA-induced RSKs nor the more specific doxycycline-mediated induction of constitutively active RSK2-CA[9] altered irradiation-induced endogenous p21 levels or those of an exogenously expressed Flag-p21wt protein. Third, comparison of the cellular effects mediated by either BI-D1870 or SL0101, another specific RSK inhibitor,[15] revealed that both compounds most likely target different signaling pathways. Although SL0101 completely failed to increase p21 levels or to drive cells into senescence, both inhibitors prevented γIR-induced apoptosis albeit via p21-independent and p21-dependent pathways, respectively. As also senescence induced by U0126 proceeded in a p21-independent manner, these findings further argue against our initial hypothesis that BI-D1870 mediates the observed cellular stress responses via RSK inhibition. Finally, and most importantly, siRNA-mediated depletion of individual RSKs and even their combined loss did not induce p21 upregulation or senescence. Together, these findings provide strong

evidence that the cellular events observed following BI-D1870 exposure are mediated via an as yet unknown pathway that, besides its known off-targets PLK1 and AuroraB, does also not involve RSKs.

Despite the obvious ineffectiveness of activated RSKs on the expression and stability of p21, it may still be worthwhile knowing whether RSKs target p21 *in vivo*. Unfortunately, we are presently unable to answer this question. MALDI-MS and mutagenesis analyses identified *in vitro* two RSK phospho-acceptor sites in the recombinant GST-p21 protein from which Ser146, but not Ser116, is embedded within an RSK consensus motif. It is not uncommon that kinases including AGC kinases phosphorylate their targets at atypical sites[8,46–48] and that, on the other hand, even perfect matches of recognition sequences do not guarantee *in vivo* phosphorylation by an appropriate kinase.[49] However, MALDI-MS analyses of Flag-p21wt immunoprecipitated from PMA-stimulated MCF-7/casp3 and HCT116 cells only verified *in vivo* phosphorylation of Ser146, but not of Ser116. Although PMA-stimulated S146-phosphorylation of Flag-p21wt was significantly prevented following co-treatment with BI-D1870, due to the above-described off-target effects of this compound it is presently unclear whether RSKs indeed phosphorylate p21 also *in vivo* at Ser146.

Nevertheless, regardless of whether or not RSKs target p21 *in vivo*, according to our data, PMA-mediated p21 phosphorylation at Ser146 alters neither its expression nor its stability. This finding further adds to the controversial effects reported on Ser146-phosphorylated p21.[20] Whereas phosphorylation of this residue by PKCδ increased p21 stability,[50] a decrease in its half-life was observed following Ser146 phosphorylation by the atypical PKCζ.[49] Thus, cell-type specificities may determine the fate of Ser146-phosphorylated p21. Consistent with earlier reports, however, demonstrating that Ser146 does not lie within an Akt consensus motif,[51] was our finding that only Thr145, but not Ser146, is phosphorylated by Akt, whereas Ser146, but not Thr145, is targeted by RSKs, at least *in vitro*. In fact, an alanine substitution of one of these sites appears to enhance phosphorylation of the adjacent residue, suggesting that a conformational alteration at these sites facilitates recognition by the respective kinase. Although this observation also implies that phosphorylation of these adjacent sites might only occur in a mutually exclusive manner, confirmation of this hypothesis under *in vivo* conditions was not within the scope of the present study and requires further investigation.

In summary, we have uncovered for the first time opposite cellular effects induced by the RSK inhibitor BI-D1870: induction of apoptosis *versus* induction of apoptosis resistance and senescence most likely due to the accumulation of p21. Although our data strongly suggest that BI-D1870 mediates these cellular stress responses independently of RSKs, they nevertheless warrant further pre-clinical studies aimed to decipher the signaling pathways involved.

Materials and Methods

Cell lines, reagents and antibodies. HCT116 colon carcinoma cells and their checkpoint-deficient variants (p53 −/− and p21 −/−) were maintained in McCoy's-5A medium (PromoCell; Heidelberg, Germany). MCF-7/casp3 breast carcinoma cells were cultured in RPMI 1640 (PAA Laboratories; Linz, Austria) with 400 μg/ml neomycin.[52] Their identities were confirmed by DNA fingerprinting (DSMZ; Braunschweig, Germany). H-TERT-immortalized retinal pigment epithelial (RPE1) cells that upon doxycyclin treatment overexpress a constitutively active (CA; Y707A) Flag-RSK2 enzyme were cultured in DMEM/F12 (1 : 1) (Gibco, Life Technologies, Darmstadt, Germany).[9] The media were supplemented with 10% heat-inactivated fetal calf serum, 10 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories). The fluorometric caspase-3 substrate Ac-DEVD-AMC and MG-132 were from Biozol (Eching, Germany). The RSK inhibitors BI-D1870 and SL0101 were from Hilary McLauchlan (Dundee, Scotland, UK) and Toronto Research Chemicals Inc. (North York, ON, Canada), respectively. The MAP kinase inhibitors U0126, SB203580 and the JNK inhibitor-I (L)-Form, as well as the p53 monoclonal DO-1 antibody, were purchased from Calbiochem (Bad Soden, Germany). The p21 and p27 monoclonal antibodies were from BD Biosciences (Heidelberg, Germany). From Cell Signaling Technology (Danvers, MA, USA) were the polyclonal rabbit antibodies recognizing AuroraB, p57, RSK1 and RSK2, and the monoclonal rabbit antibodies against all three RSK isoforms (RSK1/2/3) and PLK1. From Neomarkers (Fremont, CA, USA) were the antibodies against cyclin D1 and D3. The polyclonal rabbit antibody toward activated phospho-RSK1/2/3 (Ser380) was from R&D Systems (Wiesbaden, Germany). Monoclonal PCNA and the polyclonal CDK2 and P-Ser146 p21 antibodies were from Santa Cruz Biotechnologies (Heidelberg, Germany). From Sigma-Aldrich (Deisenhofen, Germany) we obtained the β-actin mAb (AC-74), α-tubulin mAb, the polyclonal Flag antibody, X-Gal, cycloheximide (Chx), PMA (phorbol-12-myristate-13-acetate), doxycyclin, the protease inhibitors PMSF, aprotinin, leupeptin and pepstatin and the phosphatase inhibitors sodium orthovanadate and sodium pyrophosphate.

Recombinant proteins, site-directed mutagenesis and kinase assays. The following human recombinant proteins were used: His-RSK1 and GST-Akt1 were from Millipore (Schwalbach, Germany) and Cell Signaling Technology (Danvers, MA, USA), respectively, and the GST-tagged RSK2 and RSK3 kinases were from Biozol. GST-p21 was from Calbiochem. The plasmid encoding the GST-tagged wild-type p21 protein was a kind gift from Mien-Chie Hung (Houston, TX, USA). To generate GST-p21 defective in potential acceptor sites for RSK-/Akt-dependent phosphorylation, serines and threonines were changed to alanines by site-directed mutagenesis using the QuikChange Kit according to the manufacturer's instructions (Agilent Technologies; Santa Clara, CA, USA). Kinase reactions were carried out for 30 min at 30 °C in 5 mmol/l MOPS (pH 7.2), 5 mmol/l MgCl$_2$, 1 mmol/l EGTA, 0.4 mmol/l EDTA, 2.5 mmol/l β-glycerophosphate, 50 μmol/l DTT, 50 μmol/l ATP and 5 μCi [γ-^{32}P]ATP. The reactions were stopped by adding 5x SDS sample buffer and heating (5 min at 95 °C). Following separation on SDS polyacrylamide gels, the gels were fixed in 50% methanol and 10% acetic acid, dried and exposed to X-ray film.

Treatment of cells, assessment of senescence-associated β-galactosidase (SA-β-Gal) activity and flow cytometric analyses. Cells were exposed to γIR (20 Gy at 200 kV) using a Gulmay RS225 X-ray system from IsodoseControl (Bochum, Germany) or to the various kinase inhibitors for the indicated times. Staining for SA-β-Gal activity was performed as described.[28] Pictures were taken on an Axio ObserverA1 microscope using the corresponding AxioVision Software (Carl Zeiss; Göttingen, Germany). For flow cytometric analysis, cells were trypsinized, washed in PBS and resuspended in 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide. After 30 min on ice in the dark, cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson; Heidelberg, Germany). The software CellQuestPro was used to categorize cell cycle distribution based on the relative DNA content.

Immunoprecipitation, cell extracts and western blotting. Extracts of cells transfected with a Flag-p21wt construct (Addgene; Cambridge, MA, USA; #16240) were normalized to equal protein concentrations and volume before they were incubated together with 30 μl Flag-agarose at 4 °C overnight on a rotator. After several washing steps with lysis buffer, the precipitated proteins were analyzed by western blotting. Cell extracts were prepared in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% NP-40 and protease/phosphatase inhibitors, as described.[28] Protein concentrations were determined with the BioRad assay, followed by separation in SDS-polyacrylamide gels and electroblotting onto nitrocellulose membranes (Whatman; Dassel, Germany). Following antibody incubation, proteins were either visualized by enhanced chemiluminescent staining using ECL reagents (Amersham Biosciences; Braunschweig, Germany) or by the

use of the LI-COR infrared imaging system and Odyssey software (LI-COR Biosciences, Bad Homburg, Germany).

Fluorometric determination of caspase-3-like-activity (DEVDase assay). Cell extracts measuring 50 μg were incubated for 3–4 h with 50 μM of the caspase-3 substrate Ac-DEVD-AMC in 200 μl buffer containing 50 mM HEPES (pH 7,4), 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 μM DTT. Release of fluorogenic AMC was measured at an excitation wavelength of 346 nm and an emission wavelength of 442 nm using an Infinite M200 microplate reader (Tecan, Langenfeld, Germany). The detected fluorometric signal that directly correlates to the caspase activity in cell extracts is expressed in arbitrary units (AU). A paired Student *t*-test was employed for statistical analysis.

Transfection of siRNAs and plasmids. ON-TARGET*plus* SMARTpool siRNAs containing four individual siRNAs were purchased from Dharmacon (Lafayette, CO, USA). The knockdown was performed according to the manufacturer's instructions. Forty-eight hours post transfection, cells were analyzed by western blotting and by fluorometric caspase assays for successful knockdowns, for the effect of these knockdowns on p21 expression and DEVDase activity. Cells were transfected with Flag-p21 using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen; Karlsruhe, Germany).

Real-time PCR. Total RNA was isolated using the RNeasy Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol. Reverse transcription was achieved with the High Capacity cDNA Kit (Applied Biosystems; Darmstadt, Germany). Taqman gene expression probes for human p21, RSK3, β-actin and GAPDH mRNA (Applied Biosystems) were employed to analyze their relative expression levels using the 7300 Real-Time PCR system (Applied Biosystems). GAPDH and β-actin mRNA served as endogenous normalization controls. The fold induction of the p21 or RSK3 mRNA was calculated via the $2^{\wedge}(\varDelta(\varDelta Ct))$ method, thereby normalizing all samples to the level of the analyzed RNA in untreated control cells.

Conflict of Interest

The authors declare no conflict of interest.

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