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Oncogenic c-H-ras deregulates survivin expression: An improvement for survival

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Abstract Survivin protein accomplishes two basic functions: cell cycle regulation and control of apoptosis. It is only expressed in G2/M phase and it influences rescue pathways in apoptosis-induced cells. Overexpression of constitutive active c-H-ras in HeLa, or induction of c-H-ras in a stable HeLaDiR cell line, led to sustained survivin expression in all cell cycle phases and even protected cells from drug induced apoptosis. siRNA-mediated silencing of survivin reversed this protection. Here we link the anti-apoptotic property of survivin to its cell cycle (in)dependent regulation via the activity of oncogenic c-H-ras.

Keywords: Survivin; c-H-ras; HeLa; Cell cycle; Drug-induced apoptosis; Chemotherapy

1. Introduction

In the past few years research on the inhibitor of apoptosis protein (IAP) survivin has elucidated a variety of aspects concerning regulation of microtubule dynamics [1,2], control of mitosis and cell cycle [3–6], interaction with and control of apoptotic machinery [6,7], pathways in cancer development [8] and its potential as a new target in anticancer interventions [9,10].

Highly expressed during embryonic development, survivin is not detectable in most differentiated tissues [11,12], with the exception of primitive hematopoetic cells, T lymphocytes and vascular endothelial cells [13]. Noticeable is its reexpression in the majority of human tumors [12]. Moreover, detection of high level expression provides prognostically relevant information, as it is predictive of tumor progression as well as significantly shorter patient survival [14,15]. High expression levels of survivin also correlate with an increased rate of tumor recurrence and resistance to chemo- and radiotherapy [16,17]. Particularly this fact corroborates the theory, that regulation of survivin signaling interactions may differ in normal versus cancer cells [18], supporting its aforementioned potential in anticancer treatment. In first experimental settings the inhibition of survivin expression by anti-sense or dominant-negative mutants triggers apoptosis as well as defects in cell division [9,10].

Oncogenic mutations in the ras gene are present in approximately 30% of all human cancers [19]. Mutated, constitutive activated RAS drives signaling pathways that block apoptosis and enhance cell cycle [20]. The ras/survivin axis was described as an oncogenic pathway that leads to upregulation of survivin gene-expression [21].

In survivin biology two basic modes of operation – in mitosis as well as survival – are described [22]. Its role in mitosis and the cell cycle periodicity is controlled on transcriptional [23] and post-translational levels [24], which result in peak expression at mitosis. In this phase survivin is found localised at various components of the mitotic apparatus like centrosomes, microtubules and midbodies [21,25].

An additional question in this context is how survivin (de)regulation converges with its role in preventing apoptosis. Survivin is supposed to interfere with the apoptotic machinery by directly inhibiting caspases, cell death specific proteases, or their activation [26,27]. A recently described mitochondrial localisation of survivin in tumor cell lines has been linked to apoptosis resistance and therefore enhanced tumorigenicity [7]. Growing evidence indicates that survivin also acts in a caspase-independent manner [28,29]. A central question to understand the contribution of survivin to cancer development is its cell cycle independent gene-expression, identified in various cancers [30] and cell systems [21,31].

In the present work we characterised in a human carcinoma cell line the ras/survivin axis [20,21] as a possible connection between the anti-apoptosis property of survivin and its (de)regulated expression during the cell cycle, that entails an improvement for survival and consequently might influence therapeutic strategies.

2. Materials and methods

2.1. Chemicals and antibodies

Anti-survivin (A-19) and anti-caspase 3 (H-277) were purchased from Santa Cruz, anticleaved caspase 3 (9661) from Cell Signaling, anti-p21^{ras} from DAKO Diagnostics, anti-actin from Sigma.

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Abbreviations: IAP, inhibitor of apoptosis protein; DiR, dexamethasone inducible ras; Dex, dexamethasone

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Peroxidase-secondary antibodies and Doxorubicin were purchased from Calbiochem, Complete® Protease Inhibitor Cocktail from Roche Diagnostics, Lipofectamine™ 2000 from Invitrogen, propidium iodide, RNaseA, L-mimosine, thymidine and nocodazole from Sigma.

2.2. Cell culture and transfection

To obtain stable cell lines HeLa cells were transfected with a MMTV-rasA construct, expressing a dexamethasone (Dex)-inducible constitutive active, oncogenic mutation (V12) of the human c-H-ras gene (dexamethasone inducible Ras; DiR) [32] together with a neoresistance gene by Lipofectamine™ 2000. Transfected HeLa cells were selected with G418 (800 μg/ml) and Dex (1 μM) in RPMI supplemented with penicillin/streptomycin and 10% FCS. Individual clones were isolated (HeLaDiR clones) and cultured in the presence or absence of Dex. For cell cycle arrest, growing cells were treated for 24 h with 1.5 μM nocodazole to arrest in G2/M, 400 μM mimosine to arrest in G1 and 2 mM thymidine to arrest in G1/S.

2.3. Western blotting, Northern blotting

Western blotting was performed as previously described [21]. Briefly, cells were harvested by scraping, washed twice in ice-cold PBS and lysed in RIPAII. Lysates were sonicated, centrifuged at $12.000 \times g$ and supernatants were used as total cell lysates. Twenty micrograms total protein was separated on SDS–PAGE. Northern blot analysis of 10 μg total RNA was performed as described in [33].

2.4. FACS analysis

After arresting cells as described in Section 2.2, cells were harvested by trypsinisation and fixed overnight in 70% ice-cold ethanol, washed in PBS and resuspended in 1 ml PBS containing 5 μg/ml propidium iodide and 100 μg/ml RNaseA. Samples were incubated for 60 min at 37 °C and placed at 4 °C before flow cytometry analysis. Samples were analysed with a Becton Dickinson FACSCalibur system using CELLQuest software.

2.5. Determination of apoptosis

The adequate time point for these experiments was determined in untransfected, G1 arrested HeLa cells. After 18 h of mimosine treatment, Doxorubicin was added in a time course up to 24 h and cells were prepared for Western analysis of cleaved caspase 3. For transient ectopic expression of human c-H-ras^{Val12} a plasmid construct with the cDNA under control of the cytomegalovirus promoter (CMVras) [34] was applied. HeLa cells were seeded into 24-well plates (4×10^5/well) 24 h before transient transfection (1 μg DNA/well, Lipofectamine™ 2000) with CMVras or pUC19 as vector control. 4 h after transfection, cells were arrested in G2/M, G1 and in S for 24 h. For the last 8 h cells were treated with Doxorubicin (4 μM). Cells were fixed with 3% formaldehyde/PBS and DNA was stained with DAPI (1 μg/ml). As apoptotic marker condensed and fragmented nuclei were used. Per condition, about 1000 cells were counted in randomly chosen microscopic fields (Nikon Eclipse TE300 microscope, 60×).

2.6. siRNA experiments

FITC labeled siRNA oligos (VBC-Genomics, Austria) were described in [35]. Briefly, the sequence of SRi-2 was GCGCCUGCACCCCGGAGCGTT and for scraSRi CAGUCGCGUUUGCGACUGGTT and the respective inverse complementary sequences for in vitro annealing. HeLa cells were seeded into 24-well plates (2×10^5/well) 24 h before transient Lipofectamine™ 2000 transfection with either 1 μg/well pUC19 as transfection control alone or together with 2 μM of FITC labeled siRNA construct against survivin mRNA (SRi-2). A FITC labeled, scrambled RNA construct (scraSRi) was used as siRNA control. Transfection rate was determined by fluorescence microscopy. Four hours after transfection cells were arrested in G2/M for 24 h. For the last 8 h cells were treated with 4 μM Doxorubicin. Cells were fixed with 3% formaldehyde/PBS and DNA was stained with DAPI. Per condition, 10 randomly chosen microscopic fields were counted and apoptosis rate was determined. For determination of silenced survivin expression, HeLa cells were transiently transfected with pUC19 alone or together with SRi-2 and scraSRi. 28 h after transfection cell lysates were prepared for immunoblotting.

3. Results

3.1. Ras induced survivin protects against cytotoxic effects throughout the cell cycle

HeLa cells express high levels of survivin in a cell cycle regulated manner [11]. Therefore, we chose this well characterised tumor cell line for our experiments. We determined the adequate concentration of Doxorubicin by MTT-assay. We calculated an ID_{50} of 4 μM after 24 h for untreated HeLa cells (data not shown). The adequate time span of 8 h to induce apoptosis in untransfected, G1 arrested cells was determined by counting condensed and fragmented nuclei in a time course experiment. Apoptosis rate was elevated from initially 5% to approximately 10% within 4 h in the presence of Doxorubicin and increased in a linear range up to 30% until 8 h (data not shown). The earliest time point to detect cleavage of caspase 3 was 6 h after Doxorubicin addition. The amount of cleaved caspase 3 increased up to 24 h, whereas at this time point also HeLa cells treated with mimosine only became apoptotic. Level of procaspase 3 slightly decreased after 4 h of Doxorubicin treatment (Fig. 1a). We transiently overexpressed c-H-ras in HeLa cells and compared the cytotoxic effects of Doxorubicin in different cell cycle stages by determining apoptosis rates (Fig. 1b). Arresting cells in different cell cycle phases, without any additional treatment, resulted in an apoptosis rate between 1% and 7%, compared to a basic level of approximately 6% in untreated cells (Fig. 1a and data not shown). Doxorubicin treatment of unarrested cells resulted in about 20% apoptosis in CMVras as well as in control transfected cells. Compared to untransfected HeLa (Fig. 1a), apoptosis rates were generally higher in the transfection experiments. This may be an effect of this additional treatment. In control transfected cells, Doxorubicin treatment of G2/M arrested onces led to an increase of apoptosis rate of about 10% (Fig. 1b, bars 5 and 6), whereas in G1 and G1/S arrested cells apoptosis rate increased dramatically to about 50% (Fig. 1b, bars 7 and 8). In contrast, in CMVras transfected cells, apoptosis rate remained at the level of unarrested cells with evanescent aberrations, remarkably even in G1 and G1/S arrested cells (Fig. 1b, bars 1–4). Similar results were obtained using 1 μM Staurosporine (data not shown). Ras and survivin protein expression was compared in control and CMVras transfected, arrested cells (Fig. 1c). In control transfected HeLa cells, strongest survivin expression was detected in G2/M (Fig. 1c, lane 6), whereas expression in G1 and G1/S phase was severely reduced (Fig. 1c, lanes 7 and 8). Endogenous ras expression was not observed in control transfected cells (Fig. 1c, lanes 5–8). In contrast, CMVras transfected cells exhibit high survivin expression in all cell cycle phases, with a remarkable increase in G1/S (Fig. 1c, lanes 1–4). Taken together, we found that ectopic expression of c-H-ras^{Val12} leads to survivin expression also in G1 and G1/S phase, independent of its otherwise strict cell cycle regulated expression in G2/M. This c-H-ras induced survivin expression protects HeLa cells against cytotoxic effects in all cell cycle phases.

3.2. Prevention of drug-mediated apoptosis is a specific effect due to survivin expression

To verify the protective effect of survivin expression against chemotherapeutic substances, we silenced survivin expression using siRNA. We transfected HeLa cells with siRNA oligos for survivin mRNA (SRi-2), a scrambled construct (scraSRi)

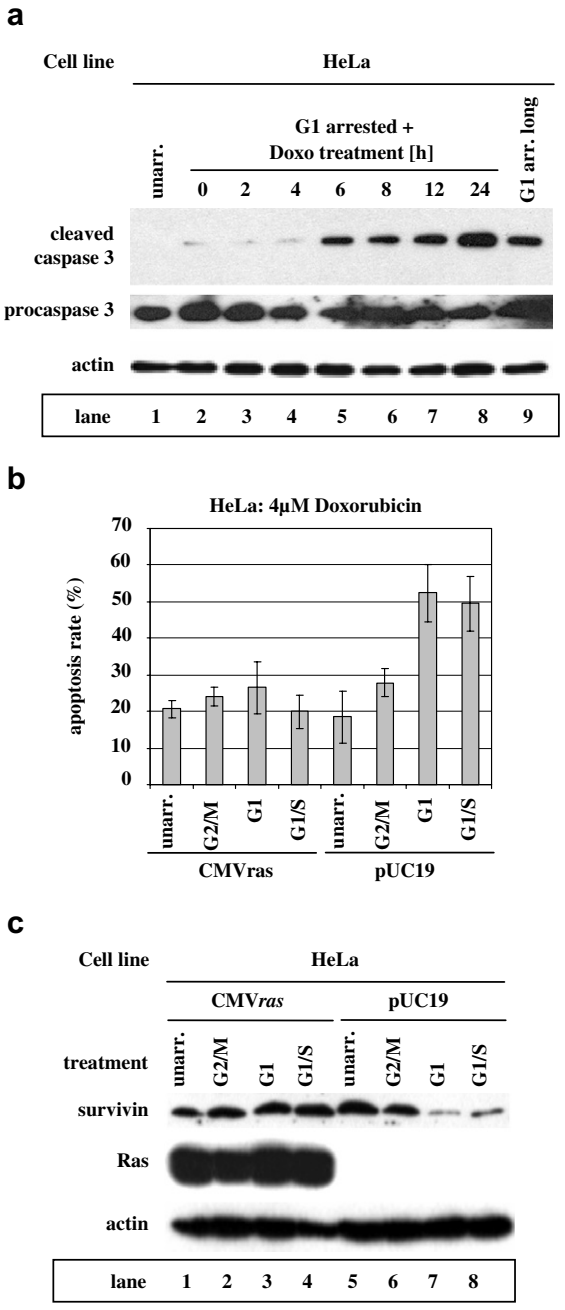

Fig. 1. Ras induced survivin protects against cytotoxic effects throughout the cell cycle. (a) HeLa cells were arrested in G1 for 18 h and 4 μM Doxorubicin was added for 2, 4, 6, 8, 12 and 24 h, respectively. Western analysis of a cleaved caspase 3 and procaspase 3 is shown. Actin was used as loading control. Unarrested (unarr.) cells were used as control. HeLa cells arrested in G1 for 18 + 24 h (G1 arr. long) are shown in lane 9. (b) HeLa cells were seeded into 24-well plates and transfected with CMVras or pUC19 as vector control. Four hours after transfection cells were arrested in G2/M, G1 and G1/S for 24 h. Unarrested cells were used as control. For the last 8 h cells were treated with Doxorubicin. Cells were fixed and DNA was stained with DAPI. Per condition, about 1000 cells were counted in randomly chosen microscopic fields and apoptosis rate (%) was determined. The bars represent S.D. (c) Survivin expression is cell cycle independent in ras expressing, cell cycle arrested HeLa cells. HeLa cells were transiently transfected with CMVras or pUC19 as vector control. Cells were treated as described in (a) except addition of Doxorubicin. Protein lysates were prepared and subjected to immunoblotting with antibodies against survivin, Ras and actin as loading control.

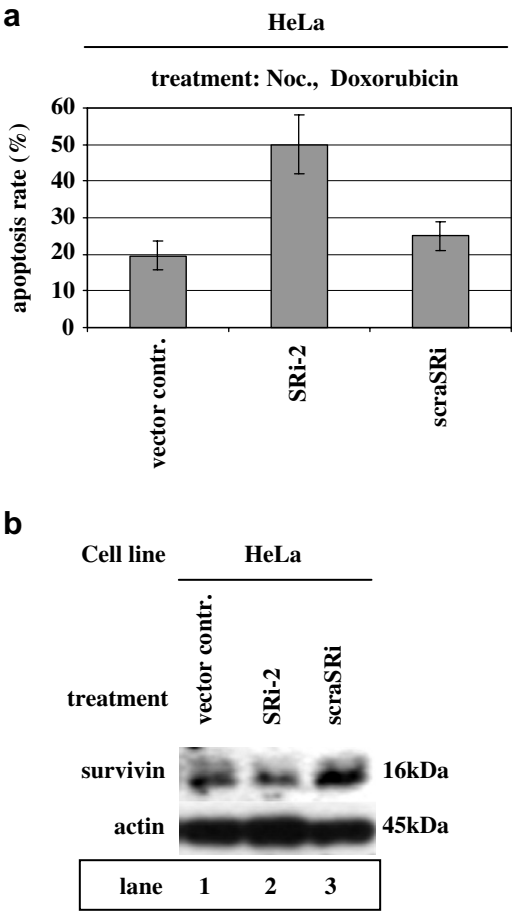

Fig. 2. Prevention of drug-mediated apoptosis is a specific effect due to survivin expression. (a) HeLa cells were seeded into 24-well plates 24 h before transient transfection with a vector (pUC19) alone or together with a siRNA construct against survivin mRNA (SRi-2). A scrambled RNA construct (scraSRi) was used as siRNA control. Four hours after transfection cells were arrested in G2/M for 24 h. For the last 8 h cells were treated with Doxorubicin. Cells were fixed and DNA was stained with DAPI. Per condition, 10 randomly chosen microscopic fields were counted and apoptosis rate was determined. The bars represent S.D. (b) Silencing of survivin expression by SRi-2 was confirmed by immunoblotting. HeLa cells were transiently transfected with a vector (lane 1) alone as transfection control or together with SRi-2 (lane 2) and scraSRi (lane 3), respectively. Twenty-eight hours after transfection cell lysates were prepared and subjected to immunoblotting with antibodies against survivin and actin as loading control.

as siRNA control [35] and a vector alone as transfection control. Cells were arrested in G2/M phase, where they express high levels of survivin (Fig. 1b, lane 6). After treatment with Doxorubicin the number of apoptotic cells was determined (Fig. 2a). Apoptosis rate of vector control transfected, G2/M arrested cells was 20% (Fig. 2a, bar 1). Transfection with SRi-2 increased the number of apoptotic cells to about 50% (Fig. 2a, bar 2), whereas apoptosis rate of scraSRi transfected cells was about 25%. To confirm silencing of survivin mRNA and consequently protein expression, immunoblot analysis was performed (Fig. 2b). Survivin protein level of SRi-2 transfected cells decreased remarkable compared to vector control (Fig. 2b, lanes 1 and 2). Using scraSRi slightly increased survivin protein level (Fig. 2b, lane 3). Thus, protection against cytotoxic effects can be correlated with survivin mRNA expression.

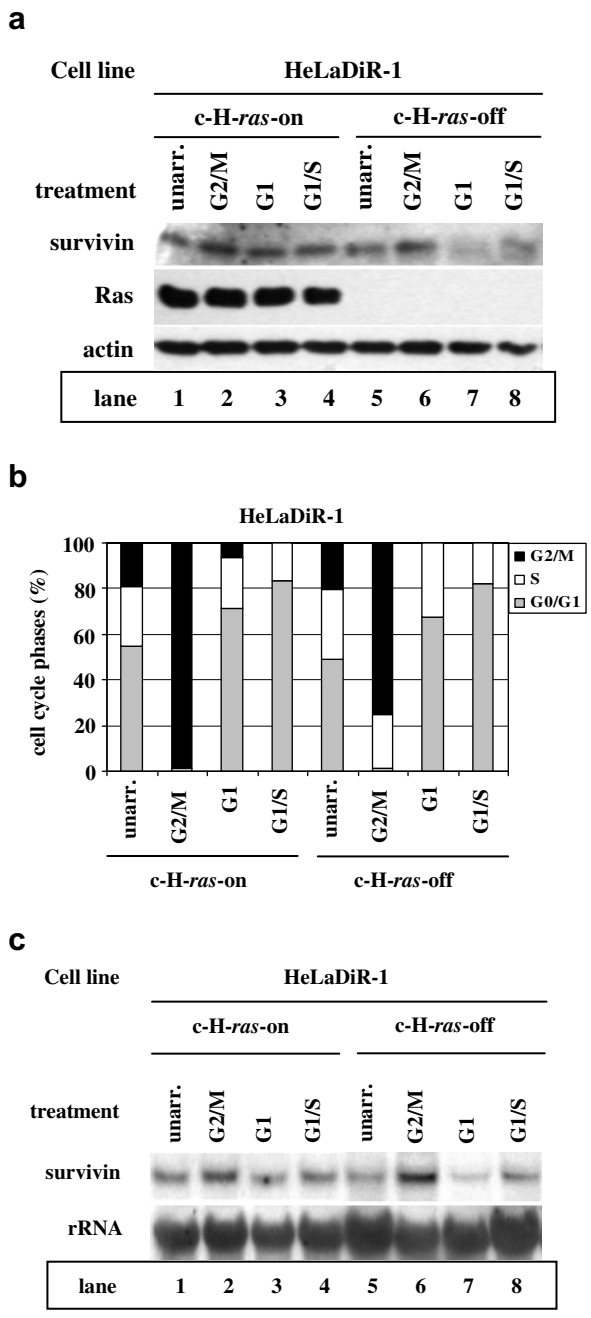

Fig. 3. Sustained survivin expression is connected to oncogenic, activated c-H-ras. (a) Western blot analysis of cell cycle arrested c-H-Ras expressing and non-expressing HeLaDiR-1 subclone. Cells were arrested in G2/M, G1 and G1/S for 24 h. Unarrested cells (unarr.) were used as control. Cell lysates were prepared and subjected to immunoblotting with antibodies against survivin, Ras and actin as loading control. (b) Cell cycle distribution of growing and arrested c-H-Ras expressing and non-expressing HeLaDiR-1 cells. FACS analysis was performed as control for cell cycle arrest. Viable cells are shown. (c) Northern blot analysis of growing and cell cycle arrested c-H-Ras expressing and non-expressing HeLaDiR-1 cells using a probe for survivin and normalised to 28S rRNA. Cells were treated as described in (a). Total mRNA was isolated and used for Northern blot analysis.

3.3. Sustained survivin expression is connected to oncogenic, activated c-H-ras

To specify the effect of c-H-ras being responsible for sustained survivin expression, we analysed this interdependence taking the advantage of a HeLa cell line stably expressing a Dex-inducible, oncogenic c-H-ras. To confirm the effectiveness of ras-induction by Dex we performed immunoblotting (Fig. 3a). In induced cells ras was constantly expressed, whereas it was undetectable in uninduced cells. Survivin expression was compared at different cell cycle phases. Induced (c-H-ras-on) and non-induced (c-H-ras-off) HeLaDiR-1 cells were arrested in G2/M, G1 and in G1/S phase. These treatments only slightly influenced the viability of cells (as described in Fig. 1). The efficiency was determined by FACS analysis (Fig. 3b). In unarrested HelaDiR-1 cells expression of c-H-ras had no effect on cell cycle distribution. Differences between c-H-ras-on and -off cells were found in G2/M and G1 arrested cells. G1 arrest in c-H-ras-on cells led to a remaining fraction in G2/M (Fig. 3b, compare bars 3 and 7) whereas block in G2/M of c-H-ras-off cells was incomplete (Fig. 3b, compare bars 2 and 6). In spite these leakages, again we found in c-H-ras-off cells highest survivin protein expression in G2/M phase (Fig. 3a, lanes 5–8), whereas in c-H-ras-on cells survivin was additionally expressed in G1 and G1/S phase (Fig. 3a, lanes 1–4). Interested in the regulatory level of this interdependence, we investigated the influence of activated ras on survivin expression at transcriptional level using Northern blot analysis. Survivin mRNA was upregulated in G1 and G1/S arrested, c-H-ras-on (Fig. 3c, lanes 1–4), compared to c-H-ras-off HeLaDiR-1 cells (Fig. 3c, lanes 5–8). Thus, decoupling of survivin from the cell cycle to a sustained expression was shown to be regulated at transcriptional level via the c-H-ras/survivin axis in a human cancer cell line.

4. Discussion

Survivin overexpression is frequently detected in tumors [11] and in various tumorderived cell lines, as in HeLa cells (Fig. 1c) [36]. Here we demonstrate that mutated, constitutive active c-H-ras reflects the missing link between the anti-apoptotic property of survivin to its (de)regulated expression during the cell cycle and thus acts at the interface between proliferation and cell death decision. Cell cycle phase independent, sustained survivin expression driven by activated c-H-ras, protected HeLa cells against apoptosis induced by the chemotherapeutic substance Doxorubicin. Although K-Ras is the more important ras family member in human tumorigenesis, we performed our experiments with c-H-Ras to refer to previous studies, where we analysed a series of c-H-Ras transformed rat cell lines [21].

Endogenous survivin reexpression was described in a series of human malignancies compared to normal tissues [11,37], which confirms cancer specific survivin promoter activity [38], but no correlation was drawn to oncogenic mutations. These findings might support observations of survivin expression independent of the mitotic index [39]. Survivin was found to be expressed independently of cell cycle progression after stimulation by cytokines in hematopeotic cells, i.e. $CD34^+$ stem cells [40]. In a more recent study from the same authors, they show that interleukin-stimulated survivin expression decreased upon inhibition of endogenous ras in these $CD34^+$ cells. Furthermore, in murine Ba/F3 cells overexpression of activated H-ras was found to upregulate survivin in G0/G1 cells independent of interleukin [20]. Recently published data

show that even T cell expansion is driven by sustained survivin expression. The authors describe that upon stimulation, survivin was upregulated in late G1 and thus responsible for an increased S phase progression during T cell proliferation [31]. These data reflect also a physiological role of survivin especially in hematopoetic cells. In cells of epithelial and mesenchymal origin, cell cycle independent survivin expression was found in malignancies only. In prostate carcinoma cell lines survivin was increased in all cell cycle phases by IGF-1 [41]. We demonstrated previously in a c-H-ras transformed, mesenchymal cell line that cell cycle dependent survivin expression was deregulated [21]. In this work the overexpression of oncogenic c-H-ras in a human carcinoma cell line led to sustained survivin expression throughout all cell cycle phases. Remarkably, transient ras overexpression led to highest survivin levels in G1/S arrested HeLa cells (Fig. 1c). It was shown that survivin interacts with cyclin-dependent kinases such as Cdk2 and Cdk4, which are known to regulate G1 to S transition [42] or that survivin is induced by E2F, a transcriptional regulator also at G1/S transition [43], respectively. Hence, it should be reasonable to speculate that, in addition to the here described activity of mutated c-H-ras in malignant cells, also a physiologically activated ras pathway might provide the missing answer to the question how sustained survivin expression could be driven [44].

Growing evidence has indicated that survivin expression plays an essential role in drug resistance and that genetic or pharmacological modulation of survivin expression affects drug effectiveness in apoptosis induction [6]. We observed in c-H-ras-transfected HeLa cells a decreased apoptosis rate in all cell cycle phases after Doxorubicin treatment (Fig. 1b, bars 1–4). Whereas in mock-transfected HeLa cells apoptosis rate increased dramatically in G1 and G1/S arrested cells (Fig. 1b, bars 5–8), in correlation with low levels of endogenous survivin (Fig. 1c, lane 5–8). Targeted inhibition of high survivin level in G2/M arrested HeLa cells by siRNA, led to increased apoptosis rate after Doxorubicin treatment (Fig. 2a). Recently it was published that cell cycle independent survivin expression in prostate carcinoma cells conferred resistance to Flutamide treatment [41]. The authors also concluded that targeted inhibition of survivin enhances the therapeutic effects of Flutamide. Their observations also support our data and the approach that susceptibility to chemotherapeutic drugs in tumors with sustained and elevated survivin expression could be improved by pretargeting survivin.

Taken together, we describe an important interdependence between the oncoprotein ras and survivin as a possible answer to the outstanding questions how this protein is able to merge its dual functions, in cell cycle regulation and in survival pathways. We show a ras-dependent deregulation of survivin expression that severely impacts survival of carcinoma cells and their susceptibility to chemotherapeutic substances.

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