Gene 469 (2010) 9–17

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Cloning and functional characterization of the guinea pig apoptosis inhibitor protein Survivin

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ARTICLE INFO

Article history:
Accepted 9 August 2010
Available online 19 August 2010

Received by Dixie Mager

Keywords:
Chromosomal passenger protein
Cytoprotectivity
Inhibitor of apoptosis
Animal models
Mitosis
CRM1

ABSTRACT

Background: The guinea pig is widely used as a model to study (patho)physiological processes, such as neurodegenerative disorders. Survivin's dual function as an apoptosis inhibitor and a mitotic regulator is crucial not only for ordered development but its modulation seems crucial also under disease conditions. However, data on the expression and function of the guinea pig Survivin protein (Survivin$_{Gp}$) are currently lacking.
Results: Here, we here report the cloning and functional characterization of Survivin$_{Gp}$. The respective cDNA was cloned from spleen mRNA, containing a 426 bp open reading frame encoding for a protein of 142aa. Survivin$_{Gp}$ displays a high homology to the human and murine orthologue, especially in domains critical for function, such as binding sites for chromosomal passenger complex (CPC) proteins and the nuclear export signal (NES). Notably, phylogenetic analyses revealed that Survivin$_{Gp}$ is more related to humans than to rodents. Ectopic expression studies of a Survivin$_{Gp}$-GFP fusion confirmed its dynamic intracellular localization, analogous to the human and murine counterparts. In interphase cells, Survivin$_{Gp}$-GFP was predominantly cytoplasmic and accumulated in the nucleus following export inhibition with leptomycin B (LMB). A typical CPC protein localization during mitosis was observed for Survivin$_{Gp}$-GFP. Microinjection experiments together with genetic knockout demonstrated that the NES is essential for the anti-apoptotic and regulatory role of Survivin$_{Gp}$ during cell division. *In vivo* protein interaction assays further demonstrated its dimerization with human Survivin and its interaction with human CPC proteins. Importantly, RNAi-depletion studies show that Survivin$_{Gp}$ can fully substitute for human Survivin as an apoptosis inhibitor and a mitotic effector. Immunohistochemistry, immunofluorescence, and western blotting were employed to detect Survivin expression in guinea pig tissues. Besides its expression in proliferating tissues, such as spleen and liver, we also found Survivin in terminally differentiated cell types. Importantly, Survivin was detectable also in the cochlea, suggesting a potential role for Survivin in the auditory system.
Conclusions: We provide the first experimental evidence for the expression of Survivin in the guinea pig. As Survivin$_{Gp}$ can substitute for known functions of human Survivin, the guinea pig model will now also allow investigating Survivin's (patho)physiological role and to test Survivin-directed potential therapeutic strategies.

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1. Introduction

Animal models have aided in the identification of factors and molecular circuitries involved in development, aging, and disease. Also, the guinea pig model is used as a clinically relevant facsimile of human diseases, particularly in the area of hearing research (Canlon et al., 2007; Bahekar et al., 2008). However, the current lack of molecular tools represents a bottleneck to fully exploit the potential of this animal model. In particular, disease patterns and therapeutic intervention strategies often involve the rational modulation of mitotic or apoptotic processes (Fadeel and Orrenius, 2005; Canlon

Abbreviations: α, anti; aa, amino acids; Ab, antibody; BCL-2, B-cell lymphoma 2; BIR, baculovirus IAP repeat; bp, base pairs; CPC, chromosomal passenger complex; CRM1, chromosomal region maintanance; GFP, green fluorescent protein; Gp, guinea pig; GST, glutathione S-transferase; Hu, human; IAP, inhibitor of apoptosis protein; INCENP, inner centromere protein; IHC, immunohistochemistry; LMB, leptomycin B; mut, mutant; NES, nuclear export signal; NLS, nuclear import signal; PCD, programmed cell death; RING, Really Interesting New Gene; RNAi, RNA interference; scr, scrambled control; siRNA, small interfering RNA; wt, wild type.

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doi:10.1016/j.gene.2010.08.007

et al., 2007; Youle and Strasser, 2008). Deregulation of these processes culminating in cell loss, include stroke, neurodegeneration and hearing impairment research (Canlon et al., 2007; Bahekar et al., 2008), or disease characterized by a failure to eliminate harmful cells like cancer and autoimmunity (Fadeel and Orrenius, 2005).

In general, modulation of programmed cell death (PCD) can be achieved *inter alia* by the dynamic expression of pro- and anti-apoptotic BCL-2 protein family members as well as of apoptosis inhibitor proteins (IAP) (Stauber et al., 2007; Altieri, 2008; Youle and Strasser, 2008).

In humans, the *Survivin* gene on chromosome 17q25 potentially gives also rise to four alternatively spliced transcripts (Li, 2005). However, not all variants have been unambiguously shown to be transcribed or even expressed *in vivo*, and there are conflicting reports concerning their potential biological functions (Li, 2005; Noton et al., 2006; Knauer et al., 2007a). Human wild type Survivin (16.5 kDa), the smallest member of the IAP family, comprising of 142 amino acids, is characterized by a single baculovirus IAP repeat (BIR), a carboxy-terminal coiled-coil domain, the absence of a carboxy-terminal RING finger domain, and appears to exist as a homodimer (Sun et al., 2005). Survivin is expression is low in the majority of non-malignant interphase cells, whereas there is a pronounced upregulation of Survivin during the G2/M phase of the cell cycle (Lens et al., 2006; Altieri, 2008). Survivin is one of the chromosomal passenger complex (CPC) proteins and interacts with Aurora-B kinase, Borealin and the inner centromere protein (INCENP) in order to execute essential roles during cell division (Lens et al., 2006; Ruchaud et al., 2007). In interphase cells, Survivin seems to inhibit apoptotic executors, e.g., caspases, due to its cytoplasmic localization (Knauer et al., 2007c). It is actively exported into the cytoplasm as Survivin contains a canonical nuclear export signal (NES) interacting with the transport receptor CRM1 and the RanGTP/GDP axis (Knauer et al., 2007c; Stauber et al., 2007; Connell et al., 2008).

Survivin expression is critical for normal embryonic development (Uren et al., 2000). Furthermore, Survivin is highly expressed in most human tumors, and expression appears to correlate with increased resistance to cancer therapy (Stauber et al., 2007; Altieri, 2008; Mehrotra et al., 2010). Notably, recent evidence suggests that Survivin is also expressed in non-malignant tissues, potentially executing cytoprotective functions against various stress conditions (Johnson et al., 2005; Fukuda and Pelus, 2006; Kindt et al., 2008).

Although Survivin is under intense investigation in human medicine, comparatively little is known regarding its expression and molecular function in mammalian animal models except mouse. Consequently, we here present the cloning and functional characterization of the guinea pig Survivin and performed a functional comparison with the human orthologue. Our results indicate that also the guinea pig model is applicable to study the (patho) physiological functions of Survivin.

2. Results

2.1. Cloning of the guinea pig Survivin cDNA

For cloning, we generated cDNA from guinea pig spleen tissue and subjected it to PCR amplification steps using primers, which were predicted to bind to highly conserved sequences in *Survivin* genes from mammals (Additional files 1 and 3). In total, we analyzed six partially overlapping regions by means of "cDNA walking." Sequence analysis finally revealed an open reading frame showing 86% nucleotide identity to the human orthologue, encoding for a protein of 142aa (Fig. 1A, and Additional file 1; GenBank accession number: GQ496319). The Survivin$_{Gp}$ protein displays a high homology to the human and murine orthologue, especially in domains critical for function, such as the nuclear export signal (NES), protein interaction domains, and posttranslational modification sites (Ruchaud et al., 2007; Stauber et al., 2007; Altieri, 2008) (Fig. 1A). Sequence comparison with Survivin from other species in terms of amino acid conservation (Additional file 1) as well as in form of a phylogenetic tree (Fig. 1B), revealed that despite its evolutionary affiliation to the rodents, Survivin$_{Gp}$ shows a higher similarity to the human than to the murine counterpart (Fig. 1A). As the expression of human and mouse Survivin splice variants in cancer cells has been shown on the mRNA level, we performed RT-PCR to examine the presence of Survivin$_{Gp}$ splice forms in adult guinea pig tissues. We could only detect a PCR product corresponding to wt *Survivin*$_{Gp}$ and no additional bands indicative of the expression of *Survivin*$_{Gp}$ isoforms were detectable in the spleen, heart or cochlea (Additional file 1D, and data not shown). Hence, it can be assumed that if expressed at all, the guinea pig Survivin variants appear to be expressed at very low levels.

2.2. The Survivin$_{Gp}$ localizes as a typical CPC protein capable of interacting with human CPC members

To compare the functional properties of the guinea pig Survivin protein with those of its human homologue, we first examined its localization during mitosis. In HeLa cells transiently expressing Survivin$_{Gp}$-GFP, immunofluorescence analysis revealed that Survivin$_{Gp}$-GFP correctly localized during mitosis, i.e., at the centromeres from pro- to metaphase, at the spindle midzone during anaphase and at the midbody during telophase and cytokinesis (Fig. 2; Additional file 2A). Survivin's mitotic functions critically depend on its interaction with the other CPC members, which is at least partially reflected by their correct colocalization (Vagnarelli and Earnshaw, 2004; Knauer et al., 2006). Indeed, the human CPC proteins AuroraB kinase, Borealin and INCENP colocalized with Survivin$_{Gp}$-GFP as know for human Survivin (Fig. 2B–D; Additional file 2A). Immunoprecipitation experiments further verified complex formation between Survivin$_{Gp}$-GFP and the human CPC members (data not shown). Hence, we concluded that Survivin$_{Gp}$-GFP is capable of interacting with human CPC members and can assemble in a functional CPC requested to guide cells through mitosis.

As Survivin dimerization appears to be another criterion required for biological function, we applied our translocation-based protein interaction assay to probe heterodimer formation of Survivin$_{Gp}$ with Survivin$_{Hu}$ in living cells (Knauer et al., 2005b). Fluorescence microscopy shows that Survivin$_{Gp}$-GFP is a predominantly cytoplasmic in interphase cells, and its localization nicely concurs with that of human Survivin (Survivin$_{Hu}$; Fig. 3A). In contrast, Fig. 3B illustrates that the cytoplasmic Survivin$_{Gp}$-GFP prey is tethered to the nucleolus upon coexpression of the nucleolar anchored Survivin$_{Hu}$-RevBFP bait (Fig. 3B, upper panel). Similar results were obtained upon coexpression of the cytoplasmic Survivin$_{Gp}$-GFP prey with the Survivin$_{Hu}$-RevBFP bait (data not shown). As a control, no colocalization was observed upon co-expression of Rev-BFP only (Fig. 3B, lower panel), confirming the assay's specificity. Also, Survivin$_{Gp}$-GFP is capable of interacting with the human isoform Survivin3B$_{Hu}$, as ectopic expression of Survivin3B$_{Hu}$-RevBFP results in their colocalization at the nucleolus (middle panel).

2.3. The biological function and localization of Survivin$_{Gp}$ depend on its active nuclear export signal (NES)

Previously, we showed that the functionality of a CRM1-dependent NES in human and murine Survivin is essential for its localization and function as an apoptosis inhibitor and mitotic effector (Knauer et al., 2006; Stauber et al., 2006). However, whether such a requirement is also true for Survivin orthologs from other species has not been examined. First, to demonstrate that also the localization of Survivin$_{Gp}$ depends on the NES/CRM1 interaction, interphase cells showing cytoplasmic Survivin$_{Gp}$-GFP were treated with the export inhibitor leptomycin B (LMB), resulting in the nuclear accumulation of

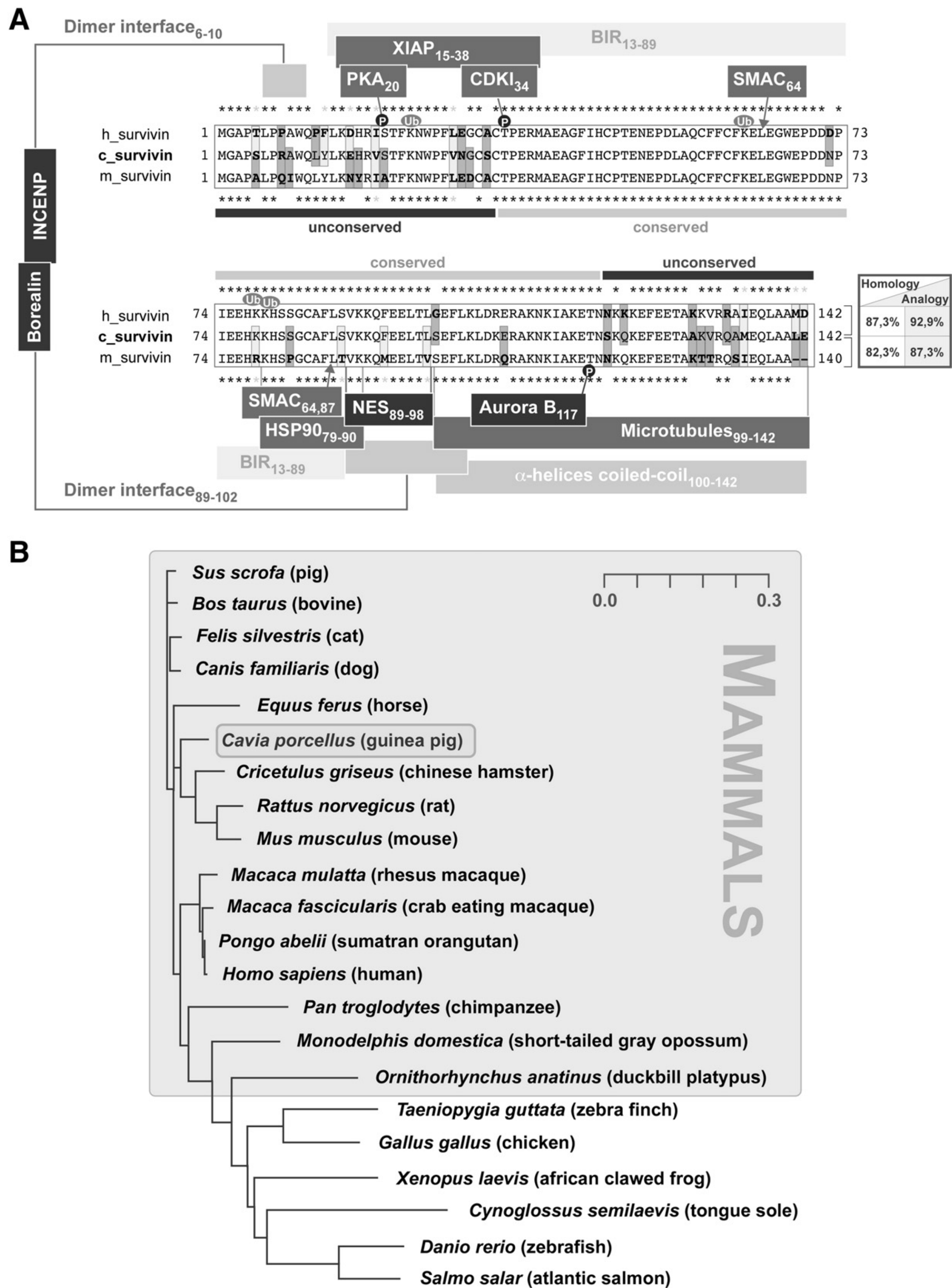

Fig. 1. Molecular cloning and phylogenetic sequence analysis of the guinea pig Survivin. (A) Comparison of the deducted amino acid sequence of the guinea pig Survivin protein with its human and murine homologue. Organisms, amino acid (aa) positions, protein identity calculations as well as interaction and modification sites known for the human Survivin protein are indicated. Asterisks mark identical amino acids, dark gray boxes mark non-conserved residues, light gray boxes indicate equivalent aa substitutions. BIR, baculovirus IAP repeat; CDKI, cyclin-dependent kinase inhibitor; HSP90, heat shock protein 90; NES, nuclear export signal; PKA, protein kinase A. (B) Phylogram constructed on the basis of amino acid sequences depicting the evolutionary relationships among Survivin proteins of different species (*TreeDomViewer*, http://www.bioinformatics.nl/tools/treedom/), including various mammalian members (highlighted in grey). Distances on the *X*-axis correspond to the grade of sequence homology, distances on the *Y*-axis are arbitrary.

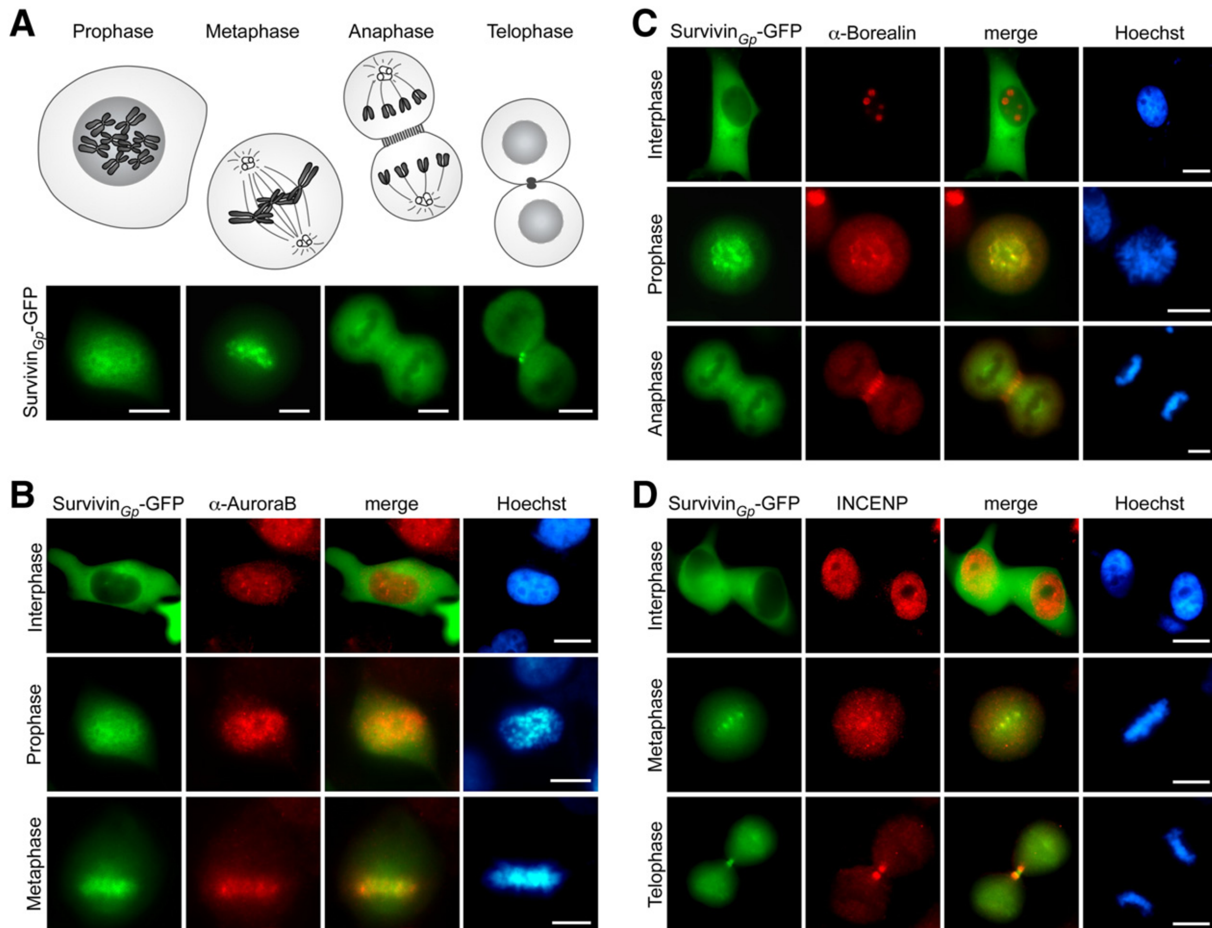

Fig. 2. Survivin$_{Gp}$ behaves as a typical chromosomal passenger complex protein during mitosis. (A) Survivin$_{Gp}$-GFP (green) localizes to the centromeres from pro- to metaphase, at the spindle midzone at anaphase, and at the midbody during telophase and cytokinesis. (B–D) Survivin$_{Gp}$-GFP correctly colocalizes with human CPC proteins during mitosis. HeLa cells were transfected with the Survivin$_{Gp}$-GFP expression construct. Aurora-B, Borealin, and INCENP were detected by immunostaining (red). Survivin$_{Gp}$-GFP was visualized by fluorescence microscopy (green). DNA was stained with Hoechst (blue). Colocalization is indicated in the overlay (yellow). CPC proteins were detected by immunostaining (red) using α-AuroraB (B), α-Borealin (C) and α-INCENP (D) Abs. Whereas the CPC proteins localize to different cellular compartments during interphase, they correctly colocalize at the different phases of mitosis. Scale bars, 10 µm.

Survivin$_{Gp}$-GFP (Fig. 3C, upper panel) as also shown for Survivin$_{Hu}$-GFP (Fig. 3C, lower panel). Second, we examined the export activity of the Survivin$_{Gp}$ NES using microinjection, a highly stringent system that allows the quantification of active transport in living cells (Knauer et al., 2005a). Due to the size of the GST-GFP fusion (54 kDa), the localization of the recombinant autofluorescent transport substrate is not flawed by passive diffusion, and the protein remains at the site of injection (Knauer et al., 2005a). In contrast, GST-Survivin$_{Gp}$NES-GFP (^{89}**V**KKQ**F**EE**L**T**L**^{98}) was actively exported following nuclear injection in Vero cells (Fig. 3D, upper panel). As a stringent control, a signal, in which essential residues in the NES were mutated (GST-Survivin$_{Gp}$NES$_{mut}$-GFP; ^{89}VKKQPEEATA^{98}, mutated residues underlined), was inactive under identical experimental conditions (Fig. 3D, lower panel). Likewise, ectopically expressed NES-deficient full-length Survivin$_{Gp}$ (Survivin$_{Gp_NESmut}$-GFP) was equally distributed between the nucleus and the cytoplasm, comparable to the localization of Survivin$_{Gp}$-GFP following chemical export inhibition, and did not further respond to LMB treatment (Additional file 2B, and data not shown). Collectively, these results identify the NES comprising aa89–98 (Fig. 1A) and exclude the presence of additional NESs as well as of an active nuclear import signal (NLS) in Survivin$_{Gp}$.

To finally analyze whether the NES is also required for the cytoprotective activity of Survivin$_{Gp}$, HeLa cells ectopically expressing human or guinea pig Survivin-GFP fusions, were exposed to apoptosis-inducing stimuli. Fig. 4A shows that overexpression of both proteins counteracted induction of apoptosis by treatment with UV-B or cisplatin. In contrast, cells expressing Survivin$_{Gp_NESmut}$-GFP were not protected against cell death. Similar expression levels of Survivin-GFP fusion proteins were confirmed by immunoblot analysis using α-GFP Ab (data not shown).

Next, we also demonstrated in guinea pig fibroblasts that dominant negative export deficient human Survivin (Survivin$_{Hu_NESmut}$-GFP) (Knauer et al., 2007a, 2007b) inhibits the function of endogenous guinea pig Survivin *in trans*. Guinea pig fibroblasts overexpressing Survivin$_{Hu\text{-}NESmut}$-IRES-GFP or IRES-GFP were generated by retroviral transduction. Fig. 4B shows that the number of multi-nucleated cells, indicative of mitotic disturbance, increased upon expression of dominant negative export deficient human Survivin.

2.4. Survivin$_{Gp}$ can functionally substitute for the human orthologue

Albeit the above experiments indicate that Survivin$_{Gp}$ is active also in human cells, cytoprotection may be mediated by heterodimers between the different orthologues. To provide evidence that Survivin$_{Gp}$ can indeed functionally replace human Survivin, we used RNAi to deplete endogenous human Survivin. Various reports demonstrated defects in cell cycle progression following downregulation of Survivin resulting in mitotic arrest and polyploidy (Ruchaud et al., 2007; Stauber et al., 2007; Altieri, 2008). Whereas transfection of GFP-expressing HeLa cells with Survivin siRNA resulted in an increased number of

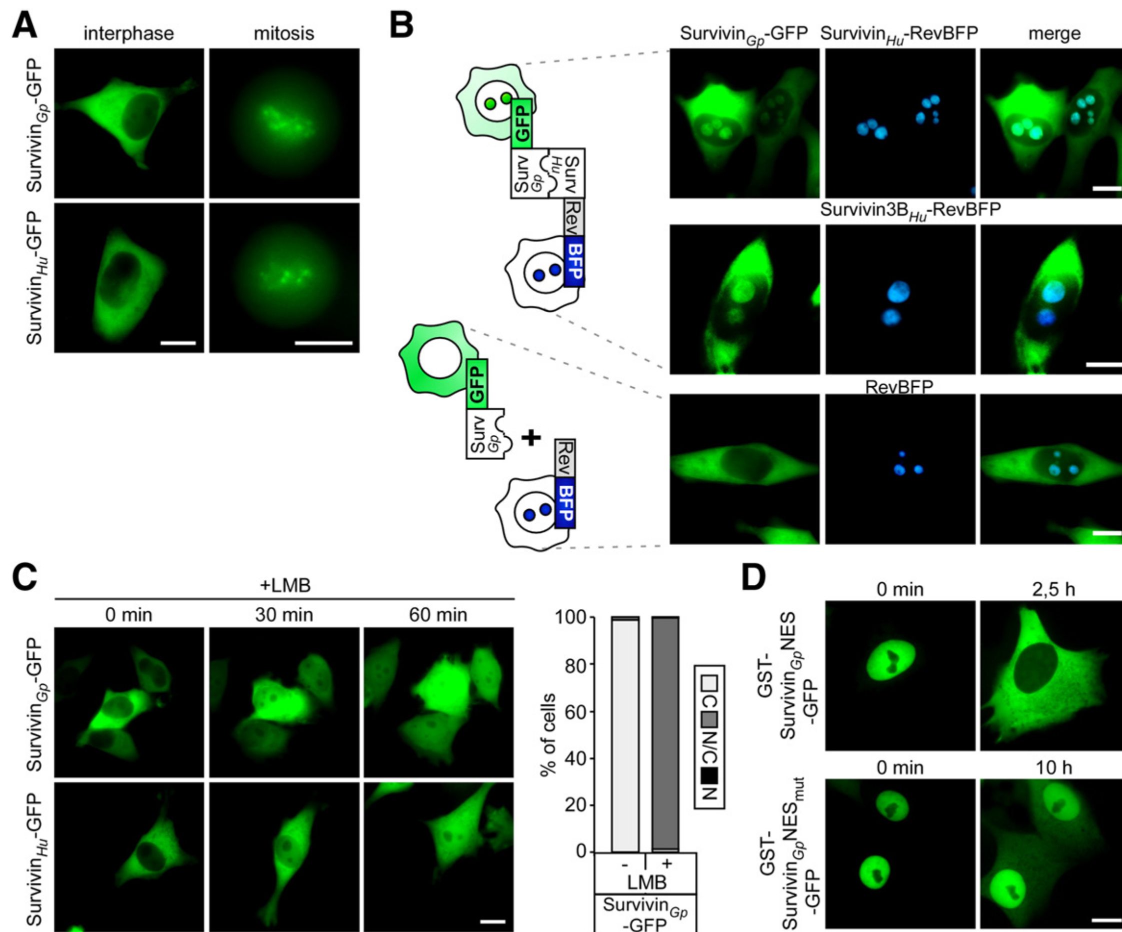

Fig. 3. Functional characterization of Survivin$_{Gp}$. Survivin-GFP fusions were detected by fluorescence microscopy. DNA was stained with Hoechst (blue). Bars, 10 µm. (A) Localization of Survivin$_{Gp}$- and Survivin$_{Hu}$-GFP in transiently expressing interphase and mitotic HeLa cells. (B) Survivin$_{Gp}$ can efficiently interact with Survivin$_{Hu}$ and the human isoform Survivin3B$_{Hu}$ *in vivo.* HeLa cells co-transfected with the indicated expression constructs (2 µg each) were analyzed 24 h later. Upon co-expression, nucleolar anchored Survivin$_{Hu}$-RevBFP recruited cytoplasmic Survivin$_{Gp}$-GFP and Survivin3B$_{Hu}$-GFP to the nucleoli (upper and middle panel, respectively). As a control, co-expression of nucleolar Rev-BFP did not affect the localization of Survivin$_{Gp}$-GFP (lower panel). The corresponding expression constructs used in this *in cell*-interaction assay are indicated schematically on the left. (C) The localization of Survivin$_{Gp}$-GFP is regulated by Crm1. In living HeLa transfectants, the indicated Survivin-GFP proteins are predominantly cytoplasmic and accumulate in the nucleus upon treatment with the CRM1 inhibitor LMB (10 nM) within the indicated time scale (left panel). 1 h after LMB addition, the cells were fixed, and the number of cells showing cytoplasmic (C), cytoplasmic and nuclear (N/C) or nuclear (N) fluorescence were counted in at least 200 Survivin-GFP-expressing cells (right panel). Results and images from a representative experiment are shown. (D) Survivin$_{Gp}$ contains an active NES. Recombinant GST-Survivin$_{Gp}$NES-GFP protein was actively exported into the cytoplasm upon microinjected into the nucleus. Conversely, a export-deficient Survivin$_{Gp}$NES$_{mut}$ containing substrate remained nuclear. Approximately 100 cells were injected, and representative examples are shown.

multinuclear cells, no mitotic disturbance was observed for Survivin$_{Gp}$-GFP expressing cells (Fig. 3C). In contrast, dominant negative export deficient guinea pig Survivin (Survivin$_{Gp_NESmut}$-GFP) was unable to compensate for the depletion of endogenous human Survivin. Additionally, depletion of endogenous Survivin$_{Hu}$ by RNAi was rescued by Survivin$_{Gp}$-GFP but not by GFP complementation, protecting the cells against UV-B- or cisplatin-induced cell death (Fig. 4D). RNAi-mediated depletion was confirmed by immunoblot analysis, and no effect was evident upon transfection of a scrambled siRNA control (Additional file 2C).

2.5. Survivin expression in guinea pig tissues

The guinea pig model is used as a clinically relevant facsimile of human diseases, including the area of hearing research (Canlon et al., 2007; Bahekar et al., 2008). First, we examined Survivin's expression in various guinea pig tissues. The evolutionary conservation of Survivin proteins throughout mammals (Fig. 1, and Additional file 1) encouraged us to employ an α-Survivin Ab previously used to investigate expression and function of human and murine Survivin (Fig. 2, and (Knauer et al., 2006, 2007a; Engels et al., 2007). A typical CPC protein localization could be visualized for endogenous Survivin$_{Gp}$ by indirect immunofluorescence in isolated proliferating guinea pig fibroblasts in different phases of mitosis (Fig. 5A). Also, a single band with the molecular weight predicted for Survivin was also detectable by immunoblot analysis in whole cell lysates from liver, lung, spleen, brain, heart, kidney and intestine (Fig. 5B). Cell lysates from proliferating mouse and guinea pig fibroblasts as well as from a human tumor were used as a control (data not shown). Although the expression of human and mouse Survivin splice variants has been shown in tumor cells on the mRNA level, we did not detect additional bands besides wt Survivin by immunoblots analysis. Hence, it can be assumed that if expressed at all, the guinea pig Survivin variants appear to be expressed at very low levels (Additional file 1D). Employing our established IHC protocol (Engels et al., 2007), Survivin was specifically detectable as a cytoplasmic and nuclear protein in various guinea pig tissues, albeit at low levels (Fig. 5C, and data not shown).

2.6. Survivin expression in terminally differentiated cells of the guinea pig's auditory system

As hearing impairment is often the consequence of cell death in the cochlea, and the guinea pig is widely used as an animal model in

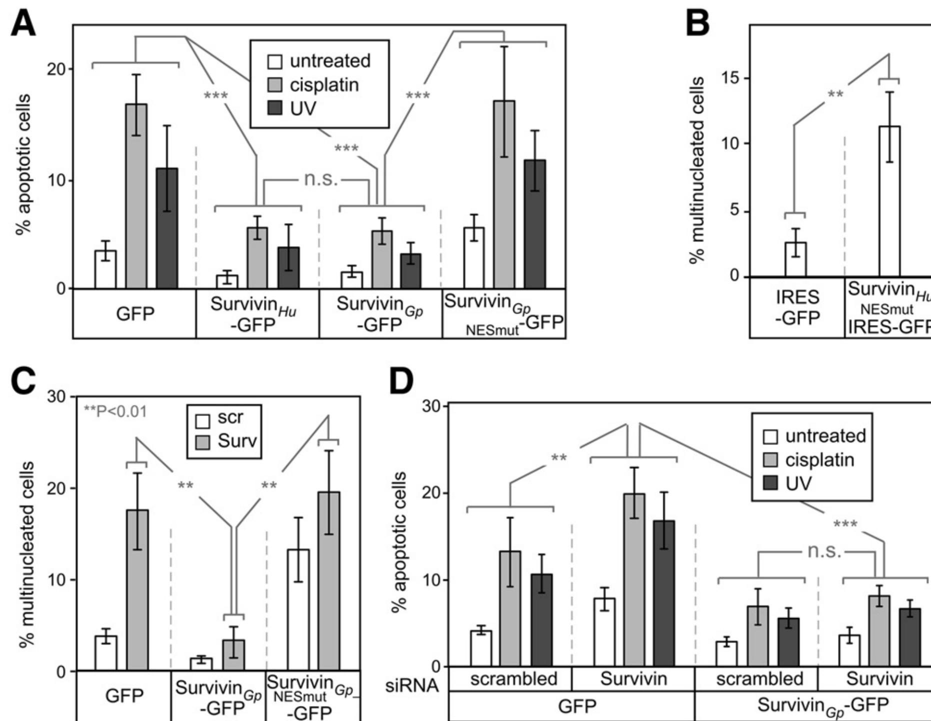

Fig. 4. (A) Survivin$_{Gp}$ is cytoprotective. HeLa cells expressing the indicated proteins were either untreated, treated with cisplatin or UV-B irradiated. 24 h later fragmented nuclei were visualized by staining with Hoechst, and the percentage of apoptotic cells counted in at least 500 cells. (B) Dominant negative export deficient human Survivin (Survivin$_{Hu_NESmut}$-GFP) inhibits the mitotic effector function of endogenous guinea pig Survivin *in trans*. Guinea pig fibroblasts overexpressing Survivin$_{Hu-NESmut}$-IRES-GFP or IRES-GFP were generated by retroviral transduction. 48 h later, the number of multi-nucleated cells was counted in at least 300 cells. (C/D) Survivin$_{Gp}$ can functionally substitute for human Survivin. HeLa cells expressing the indicated proteins were transfected with human Survivin- (surv) or a scrambled control-siRNA (scr). (C) RNAi-mediated ablation of endogenous human Survivin leads to the formation of multi-nucleated cells, which could be rescued by ectopic expression of Survivin$_{Gp}$-GFP, but not by export-deficient Survivin$_{Gp_NESmut}$-GFP or GFP. (D) Survivin$_{Gp}$-GFP but not GFP protects RNAi-depleted human cells against cisplatin- or UV-B-induced cell death. Cells were either untreated, treated with cisplatin or UV-B 24 h later, and analyzed after 48 h. Fragmented nuclei were visualized by staining with Hoechst, and the percentage of apoptotic cells counted in at least 500 GFP-expressing cells. Columns, mean; bars, ±SD from three independent experiments, $^{**}P<0.01$, $^{***}P<0.001$, n.s., not significant.

hearing research (Canlon et al., 2007), Survivin expression was examined in the cochlea. Interestingly, IHC analysis of mid-modiolar cross-sections revealed that Survivin was detectable in the organ of Corti, the lateral wall, the interdental cells of the Limbus as well as in cells of the cochlear nerve and the spiral ganglions (Fig. 5D). No immunoreactivity was observed in cells of the inner and outer sulcus and the Reissner's membrane. As a control for staining specificity, no IHC signal was detectable upon omission of the primary α-Survivin Ab or preabsorption of the α-Survivin Ab with recombinant human Survivin-GFP protein (data not shown).

3. Discussion

The guinea pig model is used as a clinically relevant facsimile of human diseases, particularly in the area of hearing research (Canlon et al., 2007; Bahekar et al., 2008). The anatomy and physiology of the human and the guinea pig is quite similar in several aspects and thus, easier accessible to surgical manipulations compared to mouse models. An important prerequisite for intensifying the use of this model in translational research is certainly the just completed sequencing of the guinea pig genome. However, data concerning the developmental and (patho)physiological function of factors relevant in the human system are largely missing for this organism. Here, we present the cloning as well as the molecular and functional characterization of the guinea pig Survivin, and demonstrate that this IAP member can mimic biological functions known for the human orthologue.

The guinea pig *Survivin$_{Gp}$* gene encodes a protein with high homology to the human and murine ortholog, especially in domains critical for functions (Altieri, 2006; Lens et al., 2006; Stauber et al., 2007; Altieri, 2008) (Fig. 1A). These include interaction domains for CPC proteins, sites for posttranslational modifications, such as for phosphorylation and ubiquitination, as well as the nuclear export signal regulating Survivin's localization to distinct subcellular compartments. These *in silico* predictions were confirmed experimentally by analyzing the dynamic localization of endogenous Survivin$_{Gp}$ and Survivin$_{Gp}$-GFP fusions in interphase and mitotic cells. Notably, this report is the third example showing that the NES-mediated interaction with CRM1 is critical for Survivin's dual activity as an apoptosis inhibitor and mitotic effector, underlining the evolutionary conservation of this regulatory mechanism in mammals (Stauber et al., 2007).

As indicated by the correct localization of Survivin$_{Gp}$ in human cells together with its capability to interact with human CPC proteins and with human Survivin, Survivin$_{Gp}$ can functionally substitute for the human orthologue. Ectopic expression studies combined with RNAi-mediated ablation of endogenous human Survivin indeed demonstrated that Survivin$_{Gp}$ is cytoprotective and can fully function as a mitotic regulator.

To date, several human and mouse Survivin splice variants have been identified (Li, 2005; Noton et al., 2006). Although not all variants have been unambiguously shown to be transcribed or even expressed *in vivo*, the human transcripts potentially encode proteins of 74 (Survivin$_{-2\alpha}$), 120 (Survivin$_{-3B}$), 137 (Survivin$_{-\Delta Ex3}$), 142 (Survivin), or 165 (Survivin$_{-2B}$) amino acids (aa) (Li, 2005; Knauer et al., 2007a). However, there are conflicting reports concerning the biological functions of Survivin isoforms in adult tissue, as most conclusions are based on massive ectopic overexpression studies in tumor cell lines (Li, 2005; Noton et al., 2006; Altieri, 2008). Summarizing our current knowledge on human Survivin splice variants it is suffice to say that

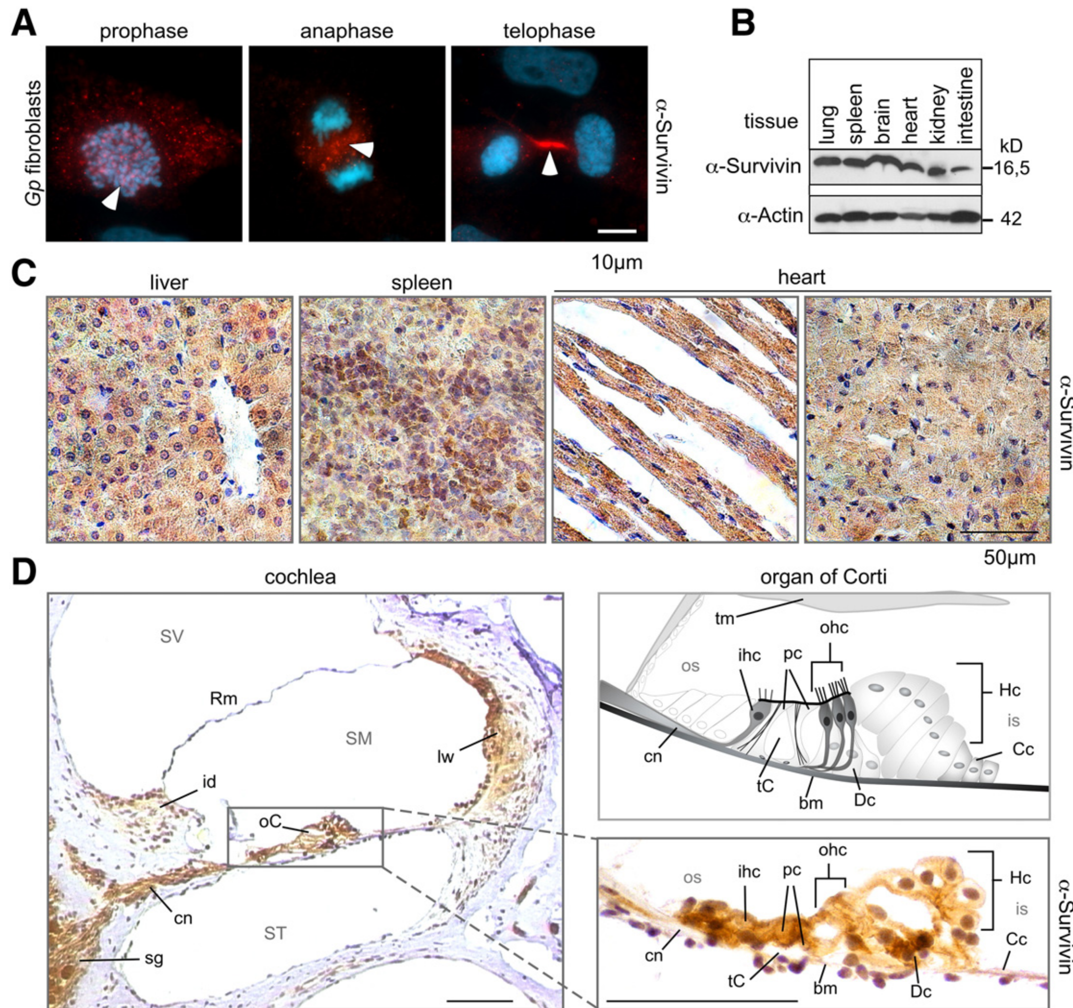

Fig. 5. Survivin expression and tissue distribution in the guinea pig. (A) A typical Survivin localization (red) could be visualized by indirect immunofluorescence in mitotic guinea pig (*Gp*) fibroblasts. DNA was stained with Hoechst (blue). Scale bar, 10 µm. (B) Survivin expression in the indicated guinea pig tissues was visualized by immunoblot. Actin served to control loading of cell lysates. (C) IHC detection of endogenous Survivin$_{Gp}$ in various guinea pig tissues. Scale bars, 50 µm. (D) Survivin expression in a mid-modiolar section of the guinea pig cochlea visualized by IHC (left panel). Immunoreactivity was observed in the lateral wall (lw), organ of Corti (oC), interdental cells (id) of the Limbus as well as in cells of the cochlear nerve (cn) and the spiral ganglions (sg). Survivin was detectable in all cell types in the organ of Corti (right panel). Abbreviations: bm, basilar membrane; Cc, Claudius cells; cn, cochlear nerve; Dc, Deiters' cells; Hc, Hensen cells; id, interdental cells; ihc, inner hair cell; is, inner sulcus; lw, lateral wall; oC, organ of Corti; ohc, outer hair cells; os, outer sulcus; pc, pillar cells; Rm, Reissner's membrane; sg, spiral ganglion; sl, spiral ligament; SM, scala media; ST, scala tympani; sv, stria vascularis; SV, scala vestibuli; tC, tunnel of Corti; tm, tectorial membrane.

wt Survivin and Survivin isoforms are low or not expressed in normal tissue and expression appears to be induced in tumors. To date, studies showing convincingly the overexpression of Survivin isoforms in non-malignant cells on the protein level are still missing. The antibodies used to detect Survivin isoforms in cell lines (Noton et al., 2006; Caldas et al., 2007; Knauer et al., 2007a) show cross-reactivity with wt Survivin and will unlikely be able to discriminate among Survivin variants. Hence, the question whether Survivin isoforms are expressed and what biological function they execute in mammals, e.g., during development, remains to be resolved.

In contrast, numerous studies have clearly shown that wt Survivin has a bifunctional role for cellular division and survival decisions. As a consequence, the paradigm of an oncofetal expression pattern and function of Survivin has emerged. As such, Survivin is broadly believed to play restricted roles in embryonic development and tumor biology. However, recent reports including ours demonstrate Survivin expression in a number of adult cells and tissues (Fukuda and Pelus, 2006; Lechler et al., 2007). As Survivin is critical for correct mitosis and highly up-regulated during the G_2/M phase of the cell cycle, expression in cells and tissues with relatively high proliferation rates, like cells of the immune system or the gastric mucosa may simply reflect a requirement for high proliferation rates (Fukuda and Pelus, 2006; Lechler et al., 2007). But, as others and we detected Survivin also in organs composed of cells believed to be quiescent and postmitotic, expression can therefore not be explained solely by mitotic activity (Fukuda and Pelus, 2006; Lechler et al., 2007). Next to regulation of cellular division, Survivin's second role is the inhibition of apoptosis. However, in organs composed of differentiated cells, very little apoptosis can be found unless the organ is in diseased state. In such a setting, regulation of apoptosis can be both important for pathogenesis and critical for repair mechanisms. Notably, several pathological models in different organs indicated expression, and implicated functions of Survivin in adult tissues. Survivin was shown to be up-regulated in brain trauma or ischemia, potentially executing cytoprotective functions against various stress conditions (Conway et al., 2003; Johnson et al., 2005; Kindt et al., 2008). Intriguingly, Survivin has been reported to interact with HSP90, a molecular chaperone, with central role in cellular stress responses (Fortugno et al., 2003). We found Survivin expressed in transducers of acoustic signals from and to the central nervous system, such as hair cells, as

well as the spiral ganglion neurons. Also, Survivin was detectable in non-neuronal cell populations, including the stria vascularis, critical for the maintenance of cochlear homeostasis and thus, hearing perception (Brown et al., 2008). As the balance of pro- and anti-apoptotic mechanisms are known to contribute to hearing impairment (Cheng et al., 2005; Kalinec et al., 2005; Jiang et al., 2006; Canlon et al., 2007), it is tempting to speculate that Survivin may represent an otoprotective factor in the auditory system (Atar and Avraham, 2005). Yet, this hypothesis awaits experimental validation. Continuing investigations of mechanisms modulating Survivin expression and function during homeostasis and disease in multiple animal models will aid to pinpoint differences in Survivin function, exploitable to selectively targeting Survivin as therapeutic strategies.

4. Methods

4.1. Animals and tissue sources

Male, two-week-old BFA pigmented guinea pigs (Charles River Laboratories) were used. Animals were kept at 22 °C ± 1 °C under a 12:12 h light–dark cycle, and fed chow and water ad lib. Experimental protocols were approved by the local Animal Care and Use Committee. Animals were sacrificed by pentobarbital-sodium (Narcoren) injection before tissue removal. Small tissue pieces from lung, spleen, brain, heart, kidney and intestine were promptly removed. Probes were either processed directly for primary cell culture, snap frozen in liquid nitrogen and stored at −80 °C for further biochemical analyses or alternatively, specimens were fixed for subsequent immunohistochemical staining.

4.2. Primary cell culture

For primary cell culture, lung tissue was disaggregated in fibroblast medium (199/Ham's F-12; 20% fetal calf serum, antibiotics, 10 μg/mL insulin; Gibco BRL, Invitrogen) containing 0.1% collagenase III (Sigma Aldrich). Cells were seeded into tissue culture plates and further cultivated in fibroblast medium.

4.3. Antibodies (Ab), reagents and treatment

Ab were: Polyclonal rabbit and monoclonal mouse α (anti)-Survivin (NB-500-201/NB-500-205, Novus Biologicals; AF886, R&D Systems); α-actin and appropriate Cy3/FITC-conjugated secondary Ab (Santa Cruz Biotechnology). Cells were treated with cisplatin (10 μM), the export inhibitor leptomycin B (LMB) (10 nM) or were UV-B irradiated (150 J/m^2) as described (Stauber et al., 2006; Knauer et al., 2007b).

4.4. Protein extraction, immunoblot analysis and immunofluorescence

Preparation of cell lysates and tissue extraction were carried out as described (Kramer et al., 2009, Fetz et al., 2009a). Protein concentrations were determined by the Bradford protein assay and 5 μg were used for each analysis. Immunoblotting and immunofluorescence were performed as described (Knauer et al., 2006).

4.5. Tissue preparation and immunohistochemistry (IHC)

Tissues were processed for IHC as described (Engels et al., 2008; Fetz et al., 2009a). Cochleae were removed and fixed with 0.2% picric acid, 4% para-formaldehyde and 0.1% glutardialdehyde for 24 h at 4 °C. For mid-modiolar cross-section preparation, cochlea were decalcified (EDTA for three weeks, 4 °C), dehydrated by ethanol and xylene and embedded in paraffin according to Heinrich et al. (2008). Antigen retrieval was performed in a pressure cooker (Survivin: EDTA buffer, 10 mM, pH 9.0). For visualization of Survivin (polyclonal α-Survivin Ab-diluted 1:1250), the EnVision® detection system (Dako GmbH) was used as described (Engels et al., 2008; Fetz et al., 2009a). Sections were counterstained with hematoxylin. Negative control slides without primary Ab were included for each staining. Specificity of the α-Survivin Ab was tested by preincubation of 5 μg α-Survivin Ab with 100 μg immobilized recombinant GST-Survivin-GFP protein (4 h, 4 °C). The supernatant was subsequently tested by IHC. Recombinant GST-Survivin-GFP protein was prepared as described (Knauer et al., 2006).

4.6. RNA extraction, cDNA cloning and Survivin expression plasmids

Total RNA extraction, purification and cDNA synthesis were performed as described (Schlingemann et al., 2005). Based on sequence homologies between the published Survivin cDNA sequences of human [GenBank:NM_001168], mouse [GenBank:NM_009689], rat [GenBank:NM_022274], cat [GenBank:NM_001009280], dog [GenBank:NM_001003019], wild boar [GenBank:NM_214141], and cattle [GenBank:NM_001001855], the cDNA of the guinea pig *Survivin* gene was initially amplified using degenerative PCR primers designed from highly conserved sequence domains. Resulting PCR products were subcloned into pGEM-T-easy (Promega) to obtain sequence information from both strands, and cDNA walking was performed in both directions (Additional file 1A). Multiple rounds of sequencing, amplification and cloning finally led to the verified full length cDNA sequence of the Survivin_{Gp} open reading frame (ORF) which was submitted to GenBank at the NCBI [GenBank:GQ496319]. For ectopic expression, the coding region of $\textit{Survivin}_{Gp}$ was amplified by PCR using specific primers bearing *Bam*HI and *Nhe*I restriction sites (Additional file 3), respectively and cloned into the pcDNA 3.1 plasmid (Invitrogen) as a C-terminal GFP fusion as described (Knauer et al., 2006). The resulting expression plasmid pc3Survivin_{Gp}-GFP was verified by sequence analysis according to Knauer et al. (2006). Potential nuclear export/import signals were cloned into the bacterial expression vector pGEX-GFP according to Knauer et al. (2005a), resulting in the plasmids GST-Survivin_{Gp}NES-GFP (^{89}VKKQMEELTV^{98}) and GST-Survivin_{Gp}NESmut-GFP (^{89}VKKQMEEATA^{98}). To generate a NES deficient full-length Survivin mutant, the respective critical aa were changed by mutagenesis as described (Knauer et al., 2005a). Eukaryotic expression constructs for GFP, GFP-tagged and untagged versions of human and murine Survivin as well as retroviral vectors were reported before (Stauber et al., 2006; Engels et al., 2007).

4.7. Phylogenetic studies

Alignments and clustalW analyses were performed using BLAST (http://www.blast.ncbi.nlm.nih.gov/) and the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit) with either Survivin cDNA sequences of guinea pig and human [GenBank:NM_001168] or the amino acid sequences of guinea pig Survivin and its species homologues available on NCBI databases (human [GenBank: NP_001159]; mouse [GenBank:NP_033819]; rat [GenBank:NP_071610]; hamster [GenBank:ABJ98055]; pig [GenBank:NP_999306]; bovine [GenBank:NP_001001855]; cat [GenBank:NP_001009280]; dog [GenBank:NP_001003348]; horse [GenBank:XP_001504809]; rhesus macaque [GenBank:XP_001083183]; crab eating macaque [GenBank:BAE00908]; sumatran orangutan [GenBank:NP_001125727]; chimpanzee [GenBank:XP_001156642]; opossum [GenBank:XP_001380429]; platypus [GenBank:XP_001517041]; zebra finch [GenBank:XP_002198931]; chicken [GenBank:NP_001012318]; clawed frog [GenBank:NP_001089984]; sole [GenBank:ACA60823]; zebrafish [GenBank:NP_919378]; salmon [GenBank:ACI66178]). A phylogenetic tree was conducted by the neighbor-joining method (Saitou and Nei, 1987), using TreeDomViewer (University of Wageningen, Netherlands) (Alako et al., 2006).

4.8. Cells, transfection and RNAi

HeLa and Vero cells were maintained under conditions and transfected as described (Knauer et al., 2006; Fetz et al., 2009b). Double-stranded siRNAs (Eurogenetec) directed against human wt Survivin (Surv) or a scrambled control (scr) were used to downregulate endogenous Survivin as described (Fetz et al., 2009a). The targeted regions in human Survivin are: 5'-CTGGACAGAGAAAGAGCCA-3'. Cells were treated in parallel with an unspecific scrambled (scr) control siRNA duplex: 5´-GGTGTGCTGTTTGGAGGTC-3'. Generation of recombinant retroviruses and retroviral transduction was performed as outlined (Engels et al., 2007).

4.9. Microscopy, image analysis and microinjection

Observation, image analysis and quantification of protein localization were performed as described (Knauer et al., 2006; Fetz et al., 2009b). DNA/cell nuclei were visualized by staining with Hoechst 33258 (Sigma Aldrich) according to Knauer et al. (2006). At least 100 fluorescent cells in three independent experiments were examined. Recombinant GST-GFP hybrid proteins were expressed and purified from bacteria and microinjected into Vero cell nuclei as described (Knauer et al., 2005a).

4.10. Measurement of apoptosis and cell vitality

Assessment of apoptosis was performed by analysis of mitochondrial integrity using the *PromoKine Mitochondrial Apoptosis Staining Kit* (PromoCell) and quantitation of caspase-3 activity as described (Fetz et al., 2009a; Kramer et al., 2009). Apoptotic, fragmented nuclei were visualized by staining with Hoechst dye, and their percentage determined in at least 500 cells as described (Stauber et al., 2006). Cell viability was analyzed using the electric sensing zone method according to Fetz et al. (2009a).

Supplementary materials related to this article can be found online at doi:10.1016/j.gene.2010.08.007.

Acknowledgments

Grant support: The Peter and Traudl Engelhorn-, Wilhelm-Sander Foundation, Head&Neck Cancer Foundation, Medical University Mainz Priority Program (BIOMATICS), ZMB Innovation Funds. We thank Sandra Friedl and Britta Blees for excellent technical assistance.

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