Wild type, dEX3 and 2B survivin isoforms localize to the tumor cell plasma membrane, are secreted in exosomes, and interact with extracellular tubulin

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A R T I C L E   I N F O

A B S T R A C T

The Inhibitor of Apoptosis Protein survivin (svn) is upregulated in nearly all types of cancer and represents a promising therapeutic target. Localization to specific subcellular compartments and interactions with various binding partners allow survivin to play diverse roles in apoptosis resistance and mitosis. Survivin has recently been found in two extracellular compartments: the outer plasma membrane and secreted exosomes. In addition to svn-wt, splice variants svn-dEX3 and svn-2B are also overexpressed in human tumors. Here we show that, similarly to svn-wt, svn-dEX3 and svn-2B can be displayed on the outer plasma membrane, and secreted in exosomes. Additionally, we have identified a novel interaction of all three forms of survivin with secreted tubulin.

1. Introduction

Survivin/BIRC5 is an Inhibitor of Apoptosis Protein (IAP) which is absent in terminally differentiated tissues but highly expressed in developing tissues, transformed cells, and in most human tumors [1,2]. Due to its specificity as a tumor associated antigen, survivin represents a highly promising therapeutic target.

Survivin/BIRC5 is encoded by four exons on chromosome 17q25 [1]. The 16.5 kDa survivin protein is comprised of an N-terminal Baculoviral IAP Repeat (BIR) domain (aa 15–89) and a C-terminal alpha helix (aa 100–140) (Fig. 1A) [3]. The BIR domain functions in apoptosis resistance through binding and stabilization of the caspase inhibitor XIAP [4]. The C-terminal alpha helix interacts with INCENP, borealin/Dasra and Aurora B kinase (forming the chromosomal passenger complex, or CPC) to facilitate proper chromosomal segregation during mitosis [5,6]. The C-terminal alpha helix also interacts with the cytoskeletal protein tubulin [7], directing survivin localization to centrosomes, the mitotic spindle, and midbodies [7,8]. Survivin plays a critical role in regulating mitotic spindle formation through its stabilization of growing microtubules [9,10]. In addition to its binding and scaffolding of other proteins,

survivin is known to homodimerize [3]. Regulation of microtubule dynamics by survivin appears to rely on its homodimerization, as a mutant monomeric version of survivin, svn-F101A/L102A, was unable to induce tubulin stabilization in cells [11].

At least five alternative survivin splice variants have been described in humans [12]. Two isoforms, svn-dEX3 and svn-2B, encode non-truncated transcripts which are upregulated in malignancies including renal cell carcinoma, breast cancer, cervical carcinoma, and glioblastoma [13,14]. Svn-dEX3 and svn-2B proteins both show conservation of aa 1–74, a region which contains a mitochondrial targeting sequence (MTS) and Src binding site (aa 1–10), as well as an XIAP binding region (K15-M38) [13–15]. Svn-dEX3 shows deletion of exon 3, generating a unique 64 aa C-terminus. A nuclear localization sequence (NLS) is contained within svn-dEX3's unique C-terminus, which causes it to preferentially reside in the nucleus [16,17]. Svn-2B, which is found mainly in the cytoplasm, includes a cryptic exon generating a 23 aa insertion between aa 74 and 75 (Fig. 1A) [13,17]. Svn-dEX3 and svn-2B both heterodimerize with svn-wt [16], however functional studies have shown opposing roles for these isoforms, with svn-dEX3 preventing apoptosis, and svn-2B promoting it [13,17–19].

Survivin has been identified in small membrane bound extracellular vesicles known as exosomes, which are shed from cancer cells. Survivin-containing exosomes have been isolated from the serum of patients with prostate cancer [20], breast cancer [21] and glioblastoma [22]. Intriguingly, one study identified svn-dEX3 and svn-2B in exosomes isolated from the serum of breast cancer patients [21]. Survivin has also recently been identified on the cell plasma membrane (PM) [23,24]. Our group detected survivin on the outer surface of multiple types of cultured cancer cells in flow cytometry assays using the novel anti-survivin clone 2C2, and found that a distinct fraction of survivin protein was present in PM lipid rafts [24]. Here we present data showing that, in addition to svn-wt, svn-dEX3 and svn-2B can also localize to the outer PM and be secreted in exosomes, and further that all three forms of survivin interact with secreted tubulin.

2. Materials and methods

2.1. Cell lines

HEK293T, HeLa, U87, and A1207 were acquired from ATCC. All cell lines were maintained in DMEM supplemented with 10% FBS plus penicillin/streptomycin, at 37°C in a humidified incubator with 5% CO_2. Cells were confirmed to be mycoplasma negative by PCR.

2.2. DNA constructs

The C-terminally tagged svn-wt-Myc/FLAG construct was previously described [24]. Briefly, svn-wt-Myc/FLAG was generated by PCR cloning the C-terminally Myc/FLAG-tagged survivin open reading frame (Origene) into pcDNA3.1. Svn-dEX3-Myc/FLAG and svn-2B-Myc/FLAG constructs were generated in the same manner using their ORF clones from Origene. N-terminally tagged constructs FLAG-HA-svn-wt, FLAG-HA-svn-dEX3, and FLAG-HA-svn-2B were generated by PCR amplification of ORFs with primers which added a stop codon after the terminal residue of each, then ligating into the plasmid pcDNA-FLAG-HA (Addgene #10792). Sanger sequencing was used to confirm all constructs.

Mutants in FLAG-HA constructs were generated using the NEB Q5 Site Directed Mutagenesis kit according to manufacturer instructions. Mutagenic primers used were: svn-11-142: 5'-CAGCCCTTTCTCAAGGAC CACCGC-3' & 5'-GGCGATCGCGGCGGCAGA-3'; svn-F101A/L102A: 5'-CCTTGGTGAAgctgcGAAACTGGACAGAGAAAG-3' & 5'-GTTAATTCTT-CAAACTGCTTC-3'; svn-1-120: 5'-TGATCTAGAGGGCCCTATTC-3' & 5'-CTTATTGTTGGTTTCCTTTGC-3'. Svn-dEX3dCT was generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) according to manufacturer instructions, with primers 5'-CTGGCCGGAG CTGCCTGGTCCCAtagtctagaCACCACTTCCAGGG-3' & 5'-CCCTGGAAG TGGTGtctagactaTGGGACCAGGCAGCTCCGGCCAG-3'.

2.3. Antibodies and other reagents

The anti-Survivin clone 2C2 was generated through inoculation of mice with the SurVaxM vaccine peptide as described in Ref. [24]. Anti-GAPDH, anti-HA tag, anti-Survivin (clone D8), anti-α-tubulin (mouse), anti-γ-tubulin (mouse) and anti-HSP70 antibodies were purchased from Santa Cruz Biotechnology. Anti-DYKDDDDK (FLAG) (clone L5), APC-conjugated anti-DYKDDDDK (APC-FLAG) (clone L5), anti-DYKDDDDK (clone L5) Affinity Gel, FITC goat anti-rat IgG, FITC goat anti-mouse IgG, Dylight 488 donkey anti-rabbit IgG and Alexa Fluor 647 donkey anti-rabbit IgG were purchased from Biolegend. Anti-survivin clone 60.11 was purchased from Novus. Anti-α-tubulin (rabbit) and anti-β-tubulin (rabbit) antibodies were purchased from Abclonal; anti-Flotillin-1 was purchased from BD Transduction Laboratories; Alexa Fluor 647 goat anti-rat IgG was purchased from Invitrogen. The protease inhibitor MG132 was purchased from Sigma.

2.4. Flow cytometry staining

For staining of cells expressing FLAG-HA-tagged survivin constructs, transfections were performed in 6 well plates using LipoD293 (Signagen), and media was changed after 4 h. 48 h post-transfection, cells were briefly trypsinized, collected and counted before diluting in Cell Staining Buffer (Biolegend). 1 μl of APC-FLAG was incubated with 100,000 cells for 1 h at RT, cells were washed and resuspended in a combination of fixative and Cell Staining Buffer. Stained cells were analyzed on a BD Fortessa flow cytometer running FACSDiva software. Analysis of flow data was performed using FCS Express v7.0 (De Novo). Quantification of APC+ events was performed by gating on SSC v. APC and normalizing to background staining of untransfected cells. For each transient expression experiment, transfected cells were also pelleted and
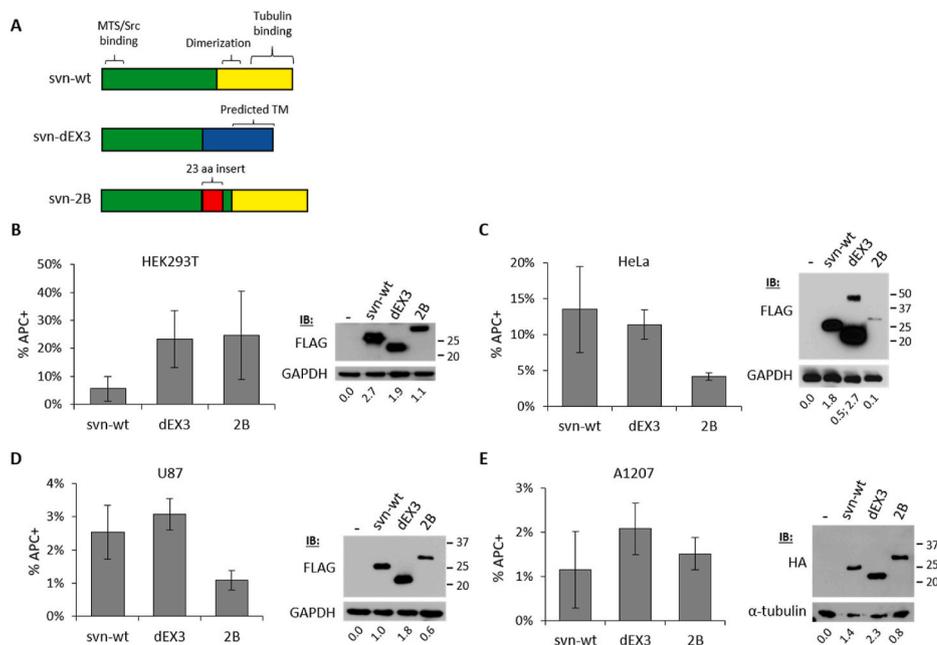

Fig. 1. Survivin isoforms show plasma membrane localization in embryonic and cancer cells. (A) Schematic showing structural features of 3 survivin isoforms. Green = BIR domain; yellow = C-terminal alpha helix; blue = unique C-terminus; red = 2B cryptic sequence. (B–E) Flow cytometry detection of transiently expressed survivin isoforms (N-terminal FLAG-HA constructs) in non-permeabilized cells. Representative WBs (including densitometric quantitation) show total expression of tagged proteins at right. Averages of three or more individual experiments are shown. Bars represent SE. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lysed for direct WB.

2.5. Western blotting

Cells were lysed using RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% triton-X100, 0.1% SDS, 0.5% deoxycholic acid, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, plus protease inhibitors). Protein concentrations were determined using Pierce BCA Assay Reagent, and 10–20 µg of protein was mixed with Laemmli buffer containing beta mercaptoethanol, boiled 5 min and separated using SDS-PAGE. Protein was transferred to PVDF membranes, which were then blocked in 5% milk in TBST for 1 h, and incubated with antibodies diluted 1:1000 in blocker overnight at 4°. Membranes were washed, incubated for 1 h with HRP-conjugated secondary antibody, washed again, incubated with ECL reagent, then exposed to X-ray film. Scanned films were subjected to densitometry analysis using Image J. Densitometry values represent band of interest intensity divided by corresponding GAPDH band intensity.

2.6. Lipid raft fractionation

Triplicate 15 cm dishes of HEK293T were transfected with svn-wt-Myc/FLAG, svn-dEX3-Myc/FLAG or svn-2B-Myc/FLAG plasmids. 24 h later, cells were rinsed with PBS, scraped in PBS, and pelleted. Lipid raft extraction was performed according to the detergent-free lipid raft fractionation method of [25]: pellets were resuspended in 1 mL of a base buffer (20 mM Tris-Cl pH 7.8, 250 mM sucrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM NaF, plus EDTA-free protease inhibitor from Roche), incubated on ice 15 min then homogenized through a 22-gauge needle 20 times, and centrifuged at $1,000 \times g$ for 10 min. Two such extractions were done per sample and the post-nuclear supernatants were pooled on ice. Post-nuclear supernatants were then mixed with equal volumes of 50% Optiprep (Stemcell Technologies) prepared in 1X base buffer, dispensed into 12 mL tubes, and layered with 2 mL each 20%, 15%, and 10% Optiprep in 1X base buffer. Tubes were centrifuged at $52,000 \times g$ for 3 h in a Beckman Coulter L9-55 M Ultracentrifuge equipped with SW28 rotor. Fractions were collected from top to bottom as 0.67 mL, and 25 µl of fractions 1–9 (containing lipid rafts) were subjected to WB.

2.7. Immunofluorescence

48 h after transfection, cells were trypsinized, collected, and resuspended in Cell Staining Buffer before incubating with primary antibodies for 1 h at room temperature. Cells were washed and then incubated with fluorescent secondary antibodies, washed again and resuspended in a combination of Cell Staining Buffer and fixative. 3000 events were collected on the ImageStreamX Mark II Imaging Flow Cytometer (Amnis). For coimmunofluorescent staining of lipid rafts and FLAG-HA-survivin, transfected HEK293T were stained with anti-FLAG for 1 h at room temperature, washed and then incubated with Alexa Fluor 647 goat anti-rat IgG for 1 h. Cells were washed again then subjected to staining, as per product instructions, using the Vybrant Alexa Fluor 488 Lipid Raft Labeling Kit (Molecular Probes). For colocalization, Imagestream data were exported to Ideas software (Amnis), and events positive for exogenous survivin were selected for image export. Overlays were assembled using Image J.

2.8. Exosome isolation

HEK293T cells were transfected with FLAG-HA plasmids. 4 h later, media was removed and replaced with DMEM supplemented with 10% exosome depleted FBS (Gibco). 48 h post-transfection, media was removed from cells, spun at $2,000 \times g$ for 30 min to remove debris, and subjected to exosome extraction using Total Exosome Isolation reagent (Invitrogen). Briefly, 500 µl of reagent was added per mL of media,

samples were incubated at 4° overnight, then centrifuged at $10,000 \times g$ for 1 h at 4°. Exosome pellets were resuspended in 150 µl filtered PBS. For WB, 20 µl of exosomes were boiled with Laemmli buffer plus beta mercaptoethanol prior to loading onto SDS-PAGE gels. Cells were also collected 48 h post-transfection and used for immunofluorescence staining by flow cytometry and assessment of total protein expression by WB.

2.9. Co-immunoprecipitation

Cells were lysed 48 h post-transfection in RIPA buffer. 50–100 µg of lysate was precleared at 4° for 1 h with 50 µl of Protein A/G agarose (Invitrogen), then subjected to immunoprecipitation overnight at 4° with anti-DYKDDDDK (FLAG, clone L5) Affinity Gel. Beads were washed 3 times with RIPA buffer, then 20 µl of 2X Laemmli containing beta mercaptoethanol was applied. Beads were boiled 5 min and protein extracts were loaded onto SDS-PAGE for analysis by WB.

3. Results and discussion

3.1. Svn-wt, svn-dEX3 and svn-2B translocate to the outer plasma membrane

We sought to test whether, in addition to svn-wt, the alternative isoforms svn-dEX3 and svn-2B could localize to the outer PM. Currently, commercial antibodies against survivin variants are either unavailable or are low affinity; we therefore chose to investigate PM localization using transient expression of survivin isoforms driven by a CMV promoter (Fig. 1A). Constructs consisting of the svn-wt, svn-dEX3 and svn-2B open reading frames fused to FLAG tags were generated and used to transfect cells, which were then subjected to flow cytometry staining (without permeabilization) with an anti-FLAG antibody. Upon transient expression, all three isoforms could be detected on the cell surface (Fig. 1). Svn-wt, svn-dEX3 and svn-2B localized to the PM in diverse cell types, including human embryonic kidney (HEK293T), cervical adenocarcinoma (HeLa) and glioblastoma (U87 and A1207). Western blotting (WB) was performed to verify expression levels. No significant differences in PM localization were detected amongst isoforms in any cell type tested. In HeLa and U87 cells, lower levels of svn-2B on the PM correlated with less overall protein expression when compared to svn-wt and svn-dEX3 (Fig. 1C and D). When expressed in A1207, survivin isoforms showed similar overall expression and PM translocation (Fig. 1E). In the embryonic cell line HEK293T, svn-dEX3 and svn-2B showed a trend toward preferential PM translocation compared to svn-wt, despite equivalent expression levels (Fig. 1B). This is interesting given that in the various cancer cells tested, svn-wt translocated as well, if not better than, the alternative isoforms.

In order to identify which structural components contributed to survivin translocation to the outer PM, specific shared or unique sequences were mutated in the expression constructs (Fig. 2A). Svn-F101A/L102A lacks the ability to homodimerize [11]; svn-11-142 lacks a mitochondrial targeting sequence (MTS) and Src-binding site shared by all 3 isoforms [15]; and svn-1-120 lacks the C-terminus shared by svn-wt and svn-2B [26]. When transiently expressed in HeLa cells, all three mutant constructs retained the ability to localize to the PM (Fig. 2B). Svn-F101A/L102A showed a significant decrease in PM localization, however this corresponded with 2-fold lower overall expression by WB. These results suggest that the N- and C-termini of svn-wt (shared amongst all three isoforms or only with svn-2B, respectively), as well as its homodimerization domain, are dispensable for PM localization.

The predictive programs TMPRED and CCTOP were utilized to identify potential transmembrane (TM) sequences within svn-wt, svn-dEX3 and svn-2B [27,28]. Svn-wt and svn-2B were not predicted to contain any TM sequences, however both programs predicted TM helices within svn-dEX3's unique C-terminus, within aa 119–136 (TMPRED)
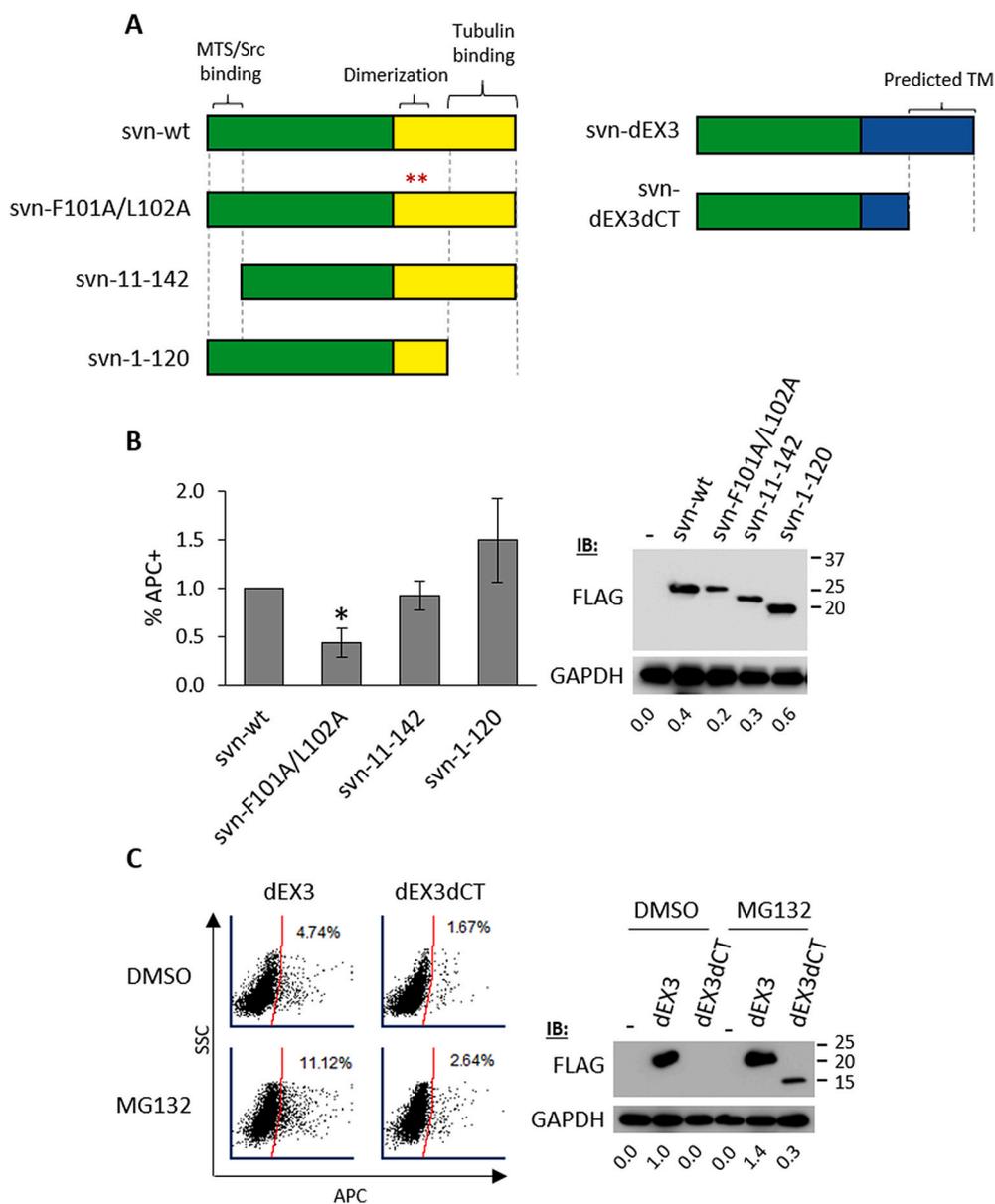
Fig. 2. Structural requirements for survivin PM translocation. (A) Schematic of survivin mutants generated for localization studies. (B) Detection of transiently expressed svn-wt mutants on the surface of non-permeabilized HeLa cells by flow cytometry. *Right*: representative WB showing total expression levels. (C) Flow cytometry detection of transiently expressed svn-dEX3 or svn-dEX3dCT on the surface of non-permeabilized HeLa treated with MG132. *Right*: WB showing total expression levels. Error bars indicate standard error from average of 3 experiments. *, $p \leq 0.05$.

or aa 113–134 (CCTOP) (Fig. 2A). To examine whether this region could direct svn-dEX3 to the PM, we generated a mutant in which aa 114–135 were deleted (svn-dEX3dCT). This mutant did not express protein in most cell backgrounds, however treatment of HeLa cells with the proteasome inhibitor MG132 stabilized its expression to detectable levels (Fig. 2C). Upon treatment with MG132, svn-dEX3dCT was identified on the PM of transfected, non-permeabilized cells. Compared to svn-dEX3, svn-dEX3dCT showed 4-fold lower PM expression in MG132-treated cells, correlating with a 4-fold lower expression level seen by WB. Overall, the C-terminus of svn-dEX3 does not appear to be required for PM localization. Altogether these data suggest that the sequence shared by all three isoforms, contained within aa 1–74 and comprising 3/4 of the BIR domain, directs localization to the PM. Given the conservation of the BIR domain, it is reasonable to speculate that additional survivin isoforms, or other IAP proteins, may also localize to the PM.

3.2. Svn-wt, svn-dEX3 and svn-2B localize to lipid rafts and are secreted in exosomes

Previously, we detected survivin in lipid raft microdomains on the

plasma membrane [24]. To test whether all three survivin isoforms could localize to lipid rafts, we subjected transiently transfected HEK293T cells to lipid raft isolation using discontinuous Optiprep gradient fractionation. Svn-wt, svn-dEX3 and svn-2B could all be found in flotillin-containing low density lipid raft fractions (Fig. 3A). Additionally, we performed immunofluorescent staining of the exogenously expressed isoforms in combination with cholera toxin subunit B (CT-B) lipid raft staining (Fig. 3B). Visualization using Imagestream flow cytometry showed that svn-wt, svn-dEX3 and svn-2B all colocalized in PM areas with lipid rafts.

We hypothesized that the presence of survivin isoforms on the PM may be associated with their secretion in lipid raft enriched exosomes. To test this, we isolated exosomes from the conditioned media of transfected HEK293T cells. Following transfection, svn-wt and svn-dEX3 showed greater overall expression than svn-2B by WB, and greater localization of svn-wt and svn-dEX3 to the PM, as assayed by flow cytometry (Fig. 3C). All three isoforms could be found in exosomal extracts by WB, however the relative amounts of the isoforms in exosomal lysates did not correlate well with their overall expression or PM localization. While svn-dEX3 expressed robustly overall in HEK293T
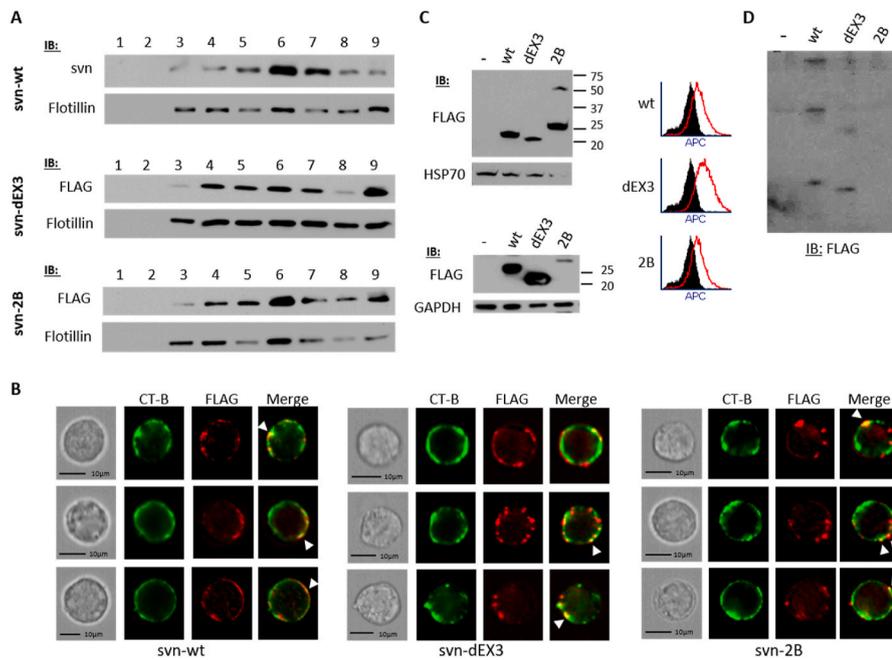
Fig. 3. Survivin isoforms localize to lipid rafts and are secreted in exosomes. (A) WBs showing partition of transiently expressed survivin isoforms (C-terminal Myc/FLAG constructs) to low density lipid raft fractions in HEK293T cells. (B) Localization of transiently expressed survivin isoforms (N-terminal FLAG-HA constructs) to cholera toxin B (CT-B) labeled lipid rafts in 293T cells. Arrows indicate regions of coincident staining. 3 individual cells per sample were captured using Imagestream imaging flow cytometry. (C) Expression of survivin isoforms in exosomes secreted from transfected HEK293T cells. *Upper WB*: exosomal lysates; *Lower WB*: Total cell lysates; *Right*: Isoform PM localization in non-permeabilized cells assayed by flow cytometry. (D) WB of exosomes collected from transiently transfected U87 cells.

and localized strongly to the PM, much less svn-dEX3 protein could be detected in secreted exosomes compared to svn-wt and svn-2B. Conversely, svn-2B was expressed at much lower levels overall and showed less PM localization, but was present in substantial amounts in secreted exosomes. In WB of exosomal lysates, svn-2B also showed a band at 2X its molecular weight, indicating the potential presence of a dimer (Fig. 3C).

Transfected U87 glioma cells secreted exosomes containing svn-wt and svn-dEX3, but not svn-2B (Fig. 3D). Interestingly, variable svn-2B secretion has also been shown among breast cancer exosome isolates [21]. The finding that svn-dEX3 and svn-2B secretion varies across cell types is particularly interesting in light of their differing anti- and pro-apoptotic functions, respectively [13]. The secretion of survivin variants may provide a level of plasticity in tumor cell communication with the microenvironment. Further development of dEX3 and 2B as potential biomarkers will require the development of reliable specific antibodies or other detection reagents.

3.3. Survivin isoforms interact with secreted tubulin on the cell surface

Tubulin has previously been found on the outer PM of primary rat neuronal and glial cells [29], lymphoid and monocyte-derived cell lines [30–32]. Using flow cytometry, we found that HEK293T cells showed both alpha and beta tubulin on their surface, whereas gamma tubulin was detected only in permeabilized cells (Fig. 4A). To test whether PM-survivin interacts with PM-tubulin, HEK293T cells were transfected with tagged survivin isoforms then assessed for both survivin and tubulin by immunofluorescence using Imagestream flow cytometry. Svn-wt, svn-dEX3 and svn-2B all showed multiple areas of colocalization with tubulin on the PM (Fig. 4B–D). Staining of endogenous PM-svn in U87 glioblastoma cells using anti-survivin clone 2C2 likewise showed strong colocalization with PM tubulin (Fig. 4E). Pulldown of survivin isoforms from transfected cell lysates co-immunoprecipitated tubulin, indicating that all three survivin isoforms complexed with tubulin (Fig. 4F). Altogether these findings suggest that novel survivin-tubulin complexes exist on the cell surface. The role of these complexes is
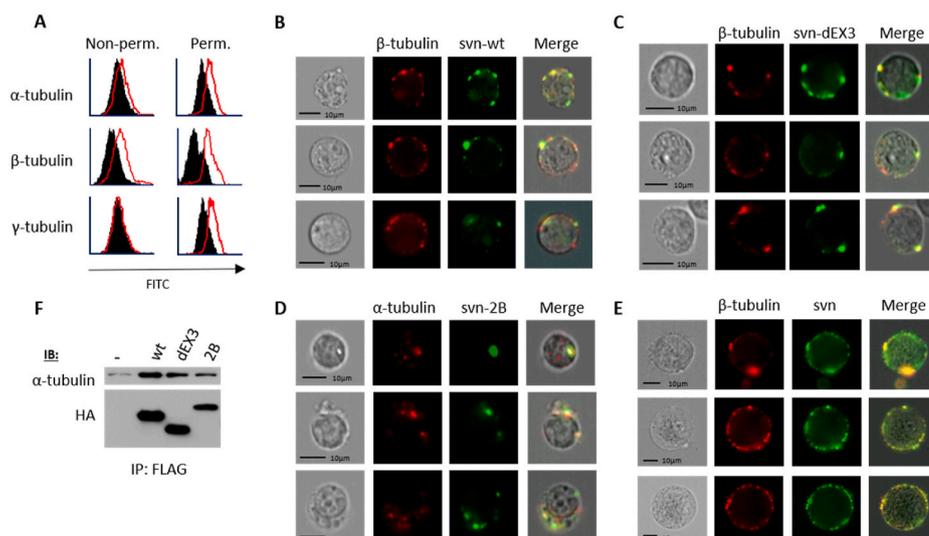

Fig. 4. Survivin isoforms interact with tubulin on the outer PM. (A) Flow cytometry analysis of intact or permeabilized HEK293T cells stained for α, β, and γ tubulin. Solid histogram = IgG control; red = tubulin. (B–D) Co-immunofluorescent staining of tubulin and FLAG-tagged survivin isoforms (using anti-survivin/60.11 for svn-wt, anti-FLAG for svn-dEX3 and svn-2B) in HEK293T cells. (E) Co-immunofluorescent staining of tubulin and endogenous survivin (using anti-survivin/2C2) in U87 cells. (F) Co-immunoprecipitation of tubulin with exogenously expressed survivin from HEK293T lysates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unclear at present; however we speculate that survivin may serve to scaffold additional proteins with PM-tubulin, and/or to regulate extra-cellular microtubule dynamics.

4. Conclusion

In this work we have found that, in addition to svn-wt, the alternative isoforms svn-dEX3 and svn-2B could be localized on the outer PM. This phenomenon was not cell type specific, as it was seen in multiple different types of human cancer cells and immortalized human embry-onic cells. The PM localization of svn-wt, svn-dEX3 and svn-2B appeared to be driven by their shared N-terminus rather than by unique features such as the svn-wt homodimerization domain or with the non-canonical C-terminus of svn-dEX3. All three isoforms localized to lipid rafts within the PM, and were secreted in exosomes. Finally, we observed that all three isoforms strongly colocalized with secreted tubulin on the outer PM of cells. Further studies are needed to examine the role of survivin on the cell surface and the function of the PM survivin-tubulin complex.

Declaration of competing interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101174.

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