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The NH_2 Terminus of Survivin is a Mitochondrial Targeting Sequence and C-Src Regulator.

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Abstract

Survivin is a cancer-associated protein that exists in several locations in the cell. Its cytoplasmic residence in interphase cells is governed by CRM1-mediated nuclear exportation, and its localisation during mitosis to the centromeres and midzone microtubules is that of a canonical chromosomal passenger protein. In addition to these well-established locations, survivin is also a mitochondrial protein, but how it gets there and its function therein is presently unclear. Here we show that the first 10 amino acids at the NH_2 terminus of survivin are sufficient to target GFP to the mitochondria *in vivo*, and ectopic expression of this decapeptide decreases cell adhesion and accelerates proliferation. The data support a signalling mechanism in which this decapeptide regulates the tyrosine kinase, C-Src, leading to reduced focal adhesion plaques and disruption of F-actin organisation. This strongly suggests that the NH_2 terminus of survivin is a mitochondrial targeting sequence that regulates C-Src, and that survivin acts in concert with C-Src to promote tumorigenesis.

Introduction

Survivin is a cancer-associated protein that inhibits cell death and is essential for mitosis (Altieri, 2008). Although its expression is usually confined to G2-phase and mitosis, survivin is often expressed throughout the cell cycle in cancer. Its abundance in tumours correlates with increased resistance to chemotherapy and radiation, treatments lethal to cells through DNA damage and apoptosis induction. When present in interphase, survivin is predominantly cytoplasmic and is actively shuttled out of the nucleus by CRM1/exportin (Colnaghi et al., 2006; Knauer et al., 2007; Rodriguez et al., 2002; Stauber et al., 2007). Nuclear expression of survivin has been correlated with relapse-free prognosis for some cancer patients (Knauer et al., 2007) and longer survival in others (Okada et al., 2001; Tonini et al., 2005), although several papers argue the opposite, (see Wheatley, 2011). Supporting the cytoprotective role of cytoplasmic survivin, we and others have shown that mutating its nuclear export signal, or forcing nuclear location, abrogates cytoprotection from irradiation and apoptosis (Colnaghi et al., 2006; Connell et al., 2008; Knauer et al., 2007), and may have therapeutic potential (Rexhepaj et al., 2010).

In addition to the cytoplasmic and nuclear pools, in cancer cells some survivin resides in the mitochondria (Dohi et al., 2004). As for other mitochondrial and cytoplasmic proteins (Itoh et al., 2005), when overexpressed the mitochondrial pool of survivin is eclipsed by the abundant cytoplasmic population. However, subcellular fractionation has clearly shown its presence in this organelle, and its abundance increases in response to hypoxia and treatment with adriamycin/etoposide (Ceballos-Cancino et al., 2007; Dohi et al., 2004). Despite its early detection in mitochondria, how survivin enters them and functions therein remain unclear. Kang et al., (2011) showed that a cofactor called aryl hydrocarbon receptor-interacting protein facilitates entry of survivin into mitochondria by interacting with its C-terminal

residue, D142 (Kang et al., 2011). Alternatively survivin may be chaperoned into mitochondria by Hsp90, which interacts with its baculovirus-inhibitor-of-apoptosis repeat domain (Fortugno et al., 2003).

As mitochondria are instrumental in apoptosis, one might expect the primary function of mitochondrial survivin to relate to its status as an inhibitor of apoptosis protein. In fact, Dohi et al. (2004) found survivin had to be released from mitochondria to effectively counter cell death. The functional relevance of mitochondrial survivin may also be linked to its interaction with Hsp90 (Fortugno et al., 2003), as treatment with the survivin-Hsp90 antagonist shepherdin compromises mitochondrial integrity (Hoel et al., 2012; Vishal et al., 2011). Survivin may also influence mitochondrial dynamics by modulating the sculpting proteins, Drp1/Fis1 (Hagenbuchner et al., 2013). Either way, one would expect that compromising mitochondrial integrity would affect apoptosis and metabolism (Hagenbuchner et al., 2013; Rivadeneira et al., 2015).

C-Src is a non-receptor tyrosine kinase that is targeted to the plasma membrane by myristoylation and is frequently overexpressed or aberrantly activated in cancer, particularly epithelial cancers (Frame, 2002; Giaccone and Zucali, 2008). C-Src, the first proto-oncogene identified, was discovered as the endogenous homologue of the oncogene, V-Src. C-Src is involved in many cellular events and, like survivin, interfaces life and death at several levels. At the plasma membrane, C-Src regulates cell-matrix attachment via focal adhesions (FA) and the F-actin cytoskeleton. However, somewhat paradoxically, prolonged C-Src activity prevents FA turnover causing increased adhesion. C-Src can be directed to mitochondria by proline-rich cofactors that interact with its SH3 domain including Dok4 (Itoh et al., 2005), and T-cell leukemia virus type-1 protein (Tibaldi et al., 2011).

This study aimed to determine how survivin enters the mitochondria and its function therein. We report that expression of an NH_2-terminal survivin truncation lacking the first 10 residues causes increased abundance of FA and F-actin in cells, which we attribute to its ability to activate C-Src. Conversely, adhesion is decreased following the expression of the NH_2 terminal decapeptide alone. Finally, we show that the NH_2-terminus is a mitochondrial targeting sequence (MTS) that binds C-Src. Collectively, these data suggest that survivin liaises with C-Src to promote tumorigenesis.

Results and Discussion.

Cells expressing survivin$_{11-142}$-GFP are highly adherent.

We recently showed that HeLa cells expressing an NH_2-terminal truncation of survivin, survivin$_{11-142}$-GFP were resistant to apoptosis and sensitized to irradiation (Wheatley, 2015). During handling we also noticed that they were more adherent than controls, suggesting that FAs were affected. Therefore, we grew cells on glass coverslips, fixed and probed them with anti-vinculin antibodies and counterstained with rhodamine-phalloidin to visualise F-actin. Compared with GFP controls, survivin$_{11-142}$-GFP cells had more prominent FAs and much stronger F-actin fibres (Figure 1A).

The NH_2 terminus of survivin regulates C-Src activity

It is well established that the formation/ dynamics of FAs and the F-actin integrity are dependent on C-Src activity (Frame, 2002). Thus, we examined whether C-Src activity was altered in these cells. Lysates from cells expressing GFP, survivin-GFP or survivin$_{11-142}$-GFP were interrogated for changes in C-Src expression and activity by immunoblotting with pan-C-Src and phospho-C-SrcY416 antibodies, respectively. Strikingly, although C-Src was present at similar levels in all samples, its activity was highly elevated in cells expressing survivin$_{11-142}$-GFP (Figure 1B).

As truncating the first 10 residues of survivin had such a profound effect on adhesion and C-Src activity we turned our attention to the NH_2 terminus itself. Interestingly, 3 of these 10 residues, 4, 6, and 7 are prolines: MGAPPTLPAW. While enrichment of prolines within this decapeptide might explain why structural data was not forthcoming (Verdecia et al., 2000; Sun et al., 2005), from a functional perspective it suggests the potential to interact with SH3 domain-containing proteins, eg. C-Src. To test this, we checked whether survivin$_{1-10}$-GFP

(and survivin-GFP), could immunoprecipitate endogenous C-Src, and gained a positive result in each case (Figure 1C). Mediation of C-Src-survivin interaction by the NH_2 terminus was further corroborated by the inability of survivin$_{11\text{-}142}$-GFP, to co-IP C-Src in this experiment. Thus we conclude that these NH_2 terminal 10 residues are necessary and sufficient to bind to C-Src.

Survivin$_{1\text{-}10}$ is a mitochondrial targeting sequence.

By fusing the first 30 nucleotides of the human survivin gene to GFP –cDNA we engineered MGAPTLPPAW-GFP. When expressed in HeLa cells we discovered that it localised to mitochondria (Figure 2A). To verify this biochemically cells expressing GFP or survivin$_{1\text{-}10}$-GFP were fractionated by differential centrifugation; whole cell extracts and mitochondrial-enriched fractions were probed with anti-GFP antibodies (Figure 2B). Anti-VDAC and anti-tubulin antibodies were used to identify mitochondrial fractions and cytoplasmic contamination respectively. Survivin$_{1\text{-}10}$-GFP was clearly present in the mitochondrial fraction, whereas GFP was excluded (Figure 2B). Next, super-resolution microscopy was used to image survivin$_{1\text{-}10}$-GFP in living cells. Structured illumination showed that survivin$_{1\text{-}10}$-GFP was coincident with MitoTracker throughout the mitochondria rather than simply binding to the exterior (Figure 2C). To ascertain whether survivin$_{1\text{-}10}$-GFP could be targeted to the mitochondria independently of any cofactors, we *in vitro* translated (IVT) ^{35}S-methionine labelled GFP or survivin$_{1\text{-}10}$-GFP to compare their import into isolated mitochondria, using the MTS of cytochrome-c oxidase subunit VIIIA as a positive control (MTS-GFP). Figure 2D shows IVT and radiolabelling, followed by assessment of association of each protein with the mitochondria after washing in buffer (control), after incubation in trypsin (to remove exteriorly bound proteins), or after trypsin and Triton X-100, which eliminates all proteins, GFP acted as negative control; its signal was low after the control

wash, and eliminated by trypsin treatment, highlighting its failure to be imported. In contrast, MTS-GFP, survivin$_{1-10}$-GFP and survivin-GFP were successfully imported into the mitochondria, as evidenced by protein remaining after trypsin treatment. These data suggest that survivin$_{1-10}$-GFP and survivin-GFP are mitochondrial residents and can enter this organelle independently of cofactors.

Mitochondrial targeting sequences are normally NH_2-terminally placed amphiphilic stretches of 17-40 amino acids that tend to form amphipathic α-helices that engage with translocase complexes of the outer and inner mitochondrial membrane. The mitochondrial localisation of survivin$_{1-10}$ and its ability to access isolated mitochondria *in vitro* suggest that it is a *bona fide* MTS despite its short length. Consistent with this, survivin$_{1-10}$ conforms to the amphiphilic requirements of a canonical MTS when mapped on a hydropathy plot (Figure 2E), with hydrophobic residues predominantly on one side, and hydrophilic residues on the other.

The NH_2 terminus of survivin regulates substrate adhesion

Whilst handling we noted that cells expressing survivin$_{1-10}$-GFP grew more rapidly than controls (Figure 2F) and were less adherent (Figure 3A). To determine whether the prolines were critical to adhesion, we substituted them for alanines and transiently expressed this mutated survivin$_{1-10\Delta P}$–GFP into HeLa cells. Proline-alanine substitution restored FAs and F-actin assembly (Figure 3A). Moreover, when observed live the percentage of unspread/floating cells was reduced from 96.2% (N=104) cells expressing survivin$_{1-10}$-GFP, to 10.8% (N=277) in cells expressing survivin$_{1-10\Delta P}$-GFP. Live imaging also revealed that mitochondrial targeting was abolished by PΔA mutation. (Figure 3B). The presence of both a proline-rich sequence and a MTS in the NH_2 terminus fits with a precedent described for Dok4 (Itoh et al., 2005) and HTLV1 (Tibaldi et al., 2011), suggesting that it is a C-Src-

regulator and a mitochondrial chaperone.

Conclusion

We report the novel findings that the NH_2 terminus of survivin is both a C-Src regulator and an MTS. The data suggest that much of what survivin achieves in cancer may be accomplished in collaboration with C-Src.

Materials and Methods.

Unless otherwise indicated, tissue culture reagents were obtained from Invitrogen, cloning enzymes from NEB, and all other reagents from Sigma-Aldrich.

Molecular cloning

Wild type survivin$_{1-10}$-GFP was generated by annealing 2 primers corresponding to the first 30 nucleotides of human survivin cDNA, with 5' EcoRI and 3' HindIII sites. The annealed DNA fragment was ligated into pBS-GFP then shuttled into pcDNA3.1 (Invitrogen) using EcoRI/ Xho1. The triple mutation that translates to MGAaTLaaAW was made by site-directed mutagenesis with the 5′ primer: 5'atgggtgccgcgacgttggccgctgcctgg3' and 3′ primer: 5'ccaggcagcggccaacgtcgcggcacccat3' (Eurofins, MWG Operon), Vent polymerase, dNTPs and survivin$_{1-10}$-GFP cDNA as template, using Stratagene Quickchange II kit (Agilent Technologies). Template was digested with DpnI and nascent cDNA transformed into competent DH5α E.coli cells. All sequences were verified prior to use.

Cell culture and proliferation

HeLa cells (derived from ATCC stock) were cultured at 37°C in 5% CO_2 humidified incubator in Dulbecco's Modified Eagle's Medium (DMEM) with 10% HyClone bovine serum (FBS), L-glutamine (2 mM), 1% penicillin-streptomycin and 1% fungizone. To create lines stably expressing GFP-tagged proteins, cells in antibiotic-free DMEM were transfected with pcDNA3.1 constructs using FuGENE 6 (Promega) in Opti-MEM. To select for positive transformants, G418 (50 μg/ml) was added 24h post-transfection and FACS sorted. Cell number was assessed using a resazurin-based assay in which cells were incubated for 1 h at 37˚C in 10 μg/ml resazurin in DMEM and measured spectrophotometrically (FLUOstar Galaxy, BMG Labtechnologies) with excitation 530 nm and emission 590 nm.

Mitochondrial assays

Fractionation: 10^6 cells were resuspended in mitochondrial isolation buffer (10 mM HEPES, pH 7.5, 200 mM Mannitol, 1 mM EGTA, 70 nM sucrose with protease inhibitors), and lysed with 25 strokes in a $2cm^3$ glass homogenizer. Nuclei were removed by 5 min centrifugation at 1000xg. The supernatant was re-spun (2000xg) to remove contaminating nuclei, then at 10,000xg, (15 min, 4°C) to pellet mitochondria, which were re-washed and pelleted 2 more times to ensure purity.

Import: GFP, MTS-GFP, survivin$_{1-10}$-GFP and survivin-GFP were translated in vitro (IVT) from pcDNA templates using T7 RNA polymerase, incorporating ^{35}S-methionine using a rabbit reticulocyte lysate system (Promega). Radiolabelled proteins were incubated for 1h at 37°C with mitochondria isolated from HeLa cells in import buffer (20 mM HEPES pH7.5, 3% (w/v) fatty acid-free BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$ supplemented with 2 mM ATP and 10 mM sodium succinate), before washing in buffer or incubation in 150 µg/ml trypsin or trypsin plus 1% triton (15 min, on ice).

Immunoblotting

Cell lysates were prepared in M-PER (ThermoFisher, 45 mins, RT), with standard protease (1 µg/ml) and phosphatase inhibitors including 2 mM sodium orthovanadate. Standard procedures were used for SDS-PAGE (12%) and transfer to nitrocellulose (PALL). To detect GFP-tagged proteins, membranes were probed with anti-GFP antibodies (1/1000, Roche). Additional primary antibodies used: tubulin (B512, 1/2000, Sigma); C-Src (SC-18, 1/1000, Santa Cruz); phosphor-C-Src^{Y416}(1/1000, Cell Signalling); VDAC (D73D12, 1/1000, Cell Signalling). Incubations were carried out in PBS with 5% milk and 0.1% Tween 20, except

for phosphor-Src^{Y416}, for which TBST/ 5% BSA was used. Horse-radish peroxidise-conjugated secondary antibodies (DAKO, 1/2000), enhanced chemiluminescence (GeneFlow) and X-ray film (GE Healthcare) were used to detect bands.

Immunoprecipitation

Three million cells were harvested by scraping and lysed in 200µl lysis buffer (10mM Tris-HCl pH 7.5; 150mM NaCl; 0.5mM EDTA; 0.5% NP-40) supplemented with standard protease inhibitors, 2U benzonase and 2 mM $MgCl_2$. Lysates were clarified by centrifugation (20,000 rfg, 2 min, 4ºC) then diluted in dilution buffer (10mM Tris-HCl pH 7.5; 150mM NaCl; 0.5mM EDTA). For every 500µl of extract, 25µl of prewashed GFP-trap_A beads (50% slurry, Chromotek) were added (note; the exact quantity was optimised to the expression of GFP-tagged protein in each sample). Lysates and beads were incubated for 1h at 4ºC with rotation then pelleted by centrifuged 2,500 g for 2 min at 4ºC and washed in ice-cold dilution buffer. Proteins were boiled off the beads (95ºC for 10 min) in lysis buffer with SDS-sample buffer.

Microscopy

Fixed: Cells were cultured on glass coverslips +/- poly-l-lysine, then fixed with 4% formaldehyde, permeabilized using 0.15% Triton-x-100 in PBS and blocked with 1% BSA before immunoprobing with anti-vinculin antibodies (1/1000, Santa Cruz, 1h RT), and Cy5-secondary anti-rabbit antibodies (1/1000, AbCam; 1h RT). Samples were counterstained with 20 nM rhodamine-phalloidin and DAPI, then mounted with Mowiol. Images were acquired using an inverted (Olympus IX71) microscope with 40 x (NA1.2, oil) and 60 x (NA1.4, oil) objectives, DeltaVision software (GE.Healthcare) and a Coolsnap HQ^2 camera

(Photometrics). Maximum projections of deconvolved 0.3 μm Z-stacks prepared in Photoshop are presented.

Live: Cells were grown in glass-bottomed dishes (Willco) +/- poly-l-lysine. Prior to imaging, medium was replaced with MitoTracker® CMXRos (25 nM) in phenol-red free CO_2-independent medium and imaged as above. For super-resolution a Zeiss Elyra PS.1 microscope was used in structured illumination mode, with the following settings: objective Plan-Apochromat 63x/1.4 Oil DIC M27, filter set LBF -488/561, cmos camera exposure time 20 ms. Two imaging tracks were set up in fast frame mode which alternates the excitation lasers (solid state 488nm and 561 nm at 20% and 10% laser power settings, respectively). Channel alignment was confirmed using 100 nm beads scanned with the same settings. Image processing and alignment was carried out using Zeiss Zen Black 2012 software.

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Competing Interests

No competing interests declared.

Author Contributions

LD: Figure 2B and D, RM: Figure 2C, SR: Figure 1B, AT: Figure 1C, EC: Figures 3A and B, SPW all remaining figures, project direction and manuscript preparation.

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Figures

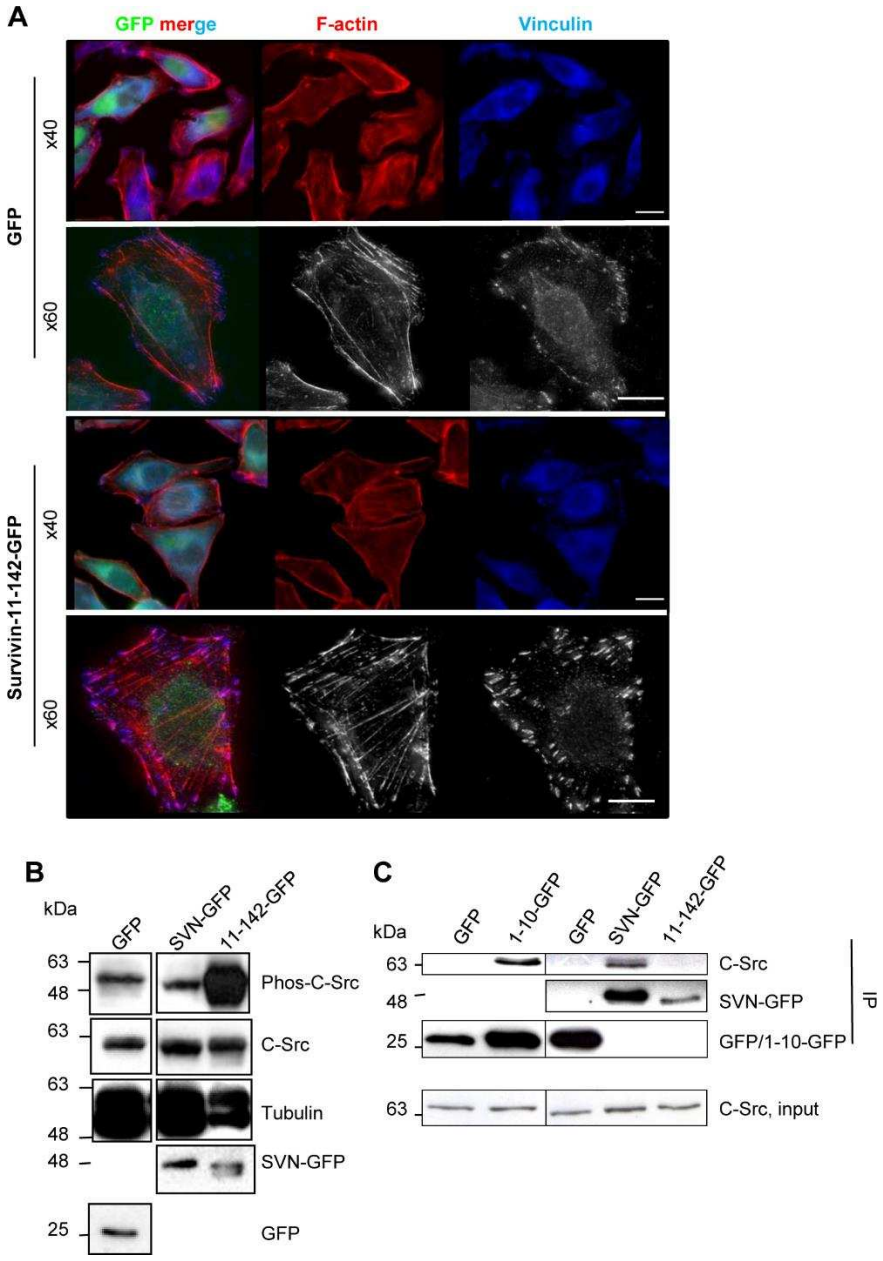

Figure 1: The NH_2 terminus of survivin regulates C-Src. (A) HeLa cells expressing GFP, or survivin$_{11\text{-}142}$-GFP (green) were grown on glass coverslips, fixed and probed to visualise FAs with anti-vinculin antibodies (blue) and F-actin with rhodamine-phalloidin (red), scale bars 5 µm. (B) Asynchronous cell lysates immunoblotted with anti-phospho-C-Src, C-Src and anti-tubulin antibodies. Anti-GFP verified expression of each construct. (C) Using GFP-Trap C-Src co-immunoprecipitated with survivin$_{1\text{-}10}$-GFP and survivin-GFP but not GFP or survivin$_{11\text{-}142}$-GFP.

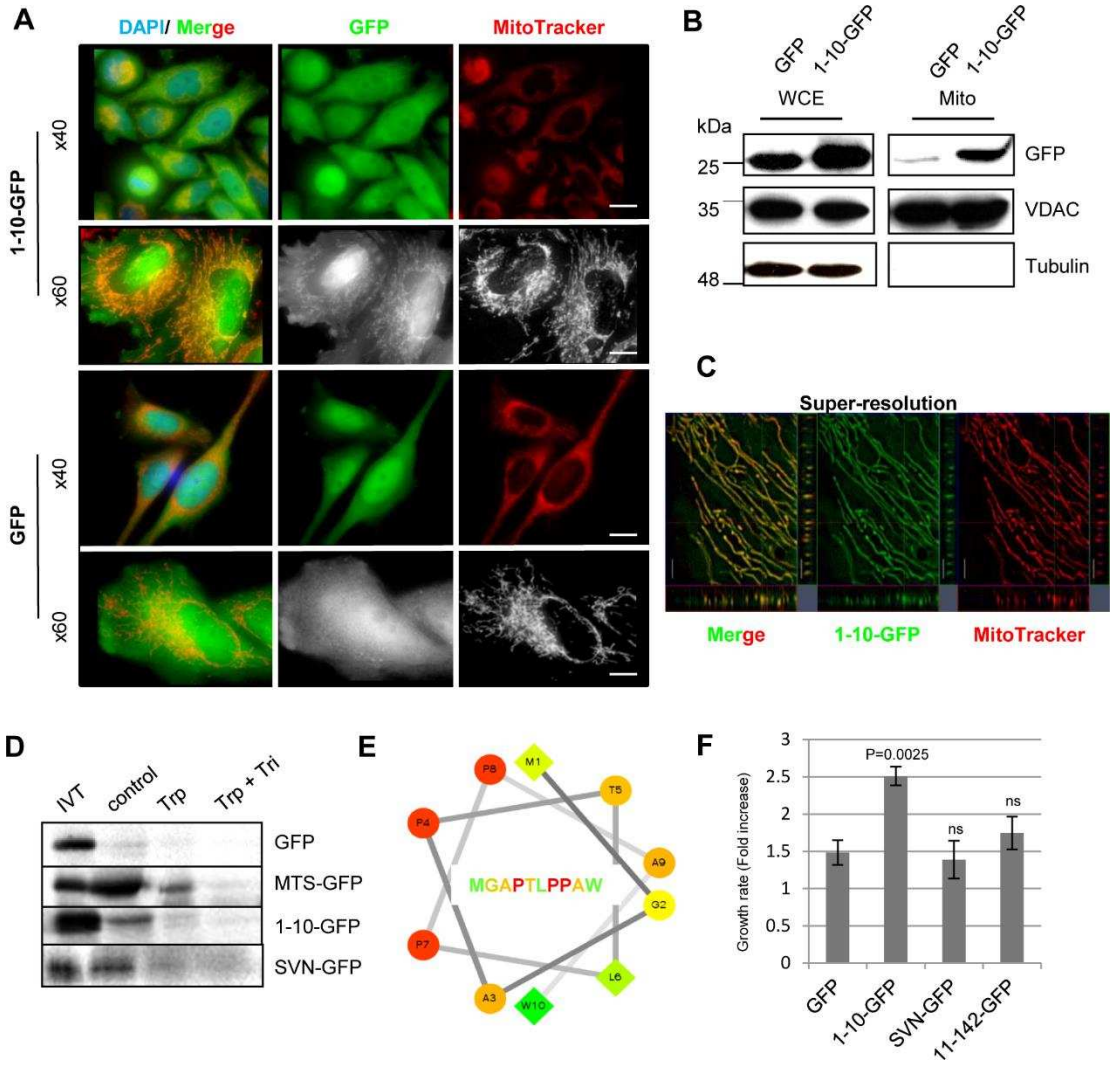

Figure 2: The first 10 amino acids of survivin are an MTS. (A) HeLa cells expressing survivin$_{1\text{-}10}$-GFP or GFP (green) were grown on poly-lysine coated slides, stained with MitoTracker (red) and imaged live. Scale bars 20 µm (upper) and 5 µm (lower). (B) Immunoblot of fractionated cells: 25 µg each whole cell extracts, (WCE) and cytoplasmic fractions (cyto); 8 µg mitochondrial fraction (mito). Anti-GFP detects GFP and survivin$_{1\text{-}10}$-GFP, anti-VDAC and anti-tubulin highlight mitochondrial and cytoplasmic fractions respectively. (C) Structured illumination of mitochondria in live cells expressing survivin$_{1\text{-}10}$-GFP stained with MitoTracker. Scale bar 1 µm. (D) Mitochondrial import assay: GFP, MTS-

GFP, survivin$_{1-10}$-GFP and survivin-GFP translated *in vitro* (IVT), labelled with ^{35}S-methionine then incubated with mitochondria isolated from HeLa cells for 1 h at 37°C. Mitochondria were washed in isolation buffer (control) or treated with 150 µg/ml trypsin (trp) or trypsin and 1% Triton X-100 (tri). Mitochondrial retention of proteins was assessed by SDS-PAGE and phosphor-imaging. (E) Hydropathy wheel plot of the first 10 residues of survivin. Residue type: circles (hydrophilic); diamonds (hydrophobic) Hydrophobicity scale is green (high) to yellow (zero). Hydrophilicity scale is red (high) to orange (low). (F) Rate of cell growth of each line was compared in exponential phase. Mean and standard deviation of three independent experiments is shown. A paired T-test demonstrated that the increased growth rate of 1-10-GFP cells is significantly different from the GFP control, variance in other lines was not significant (ns).

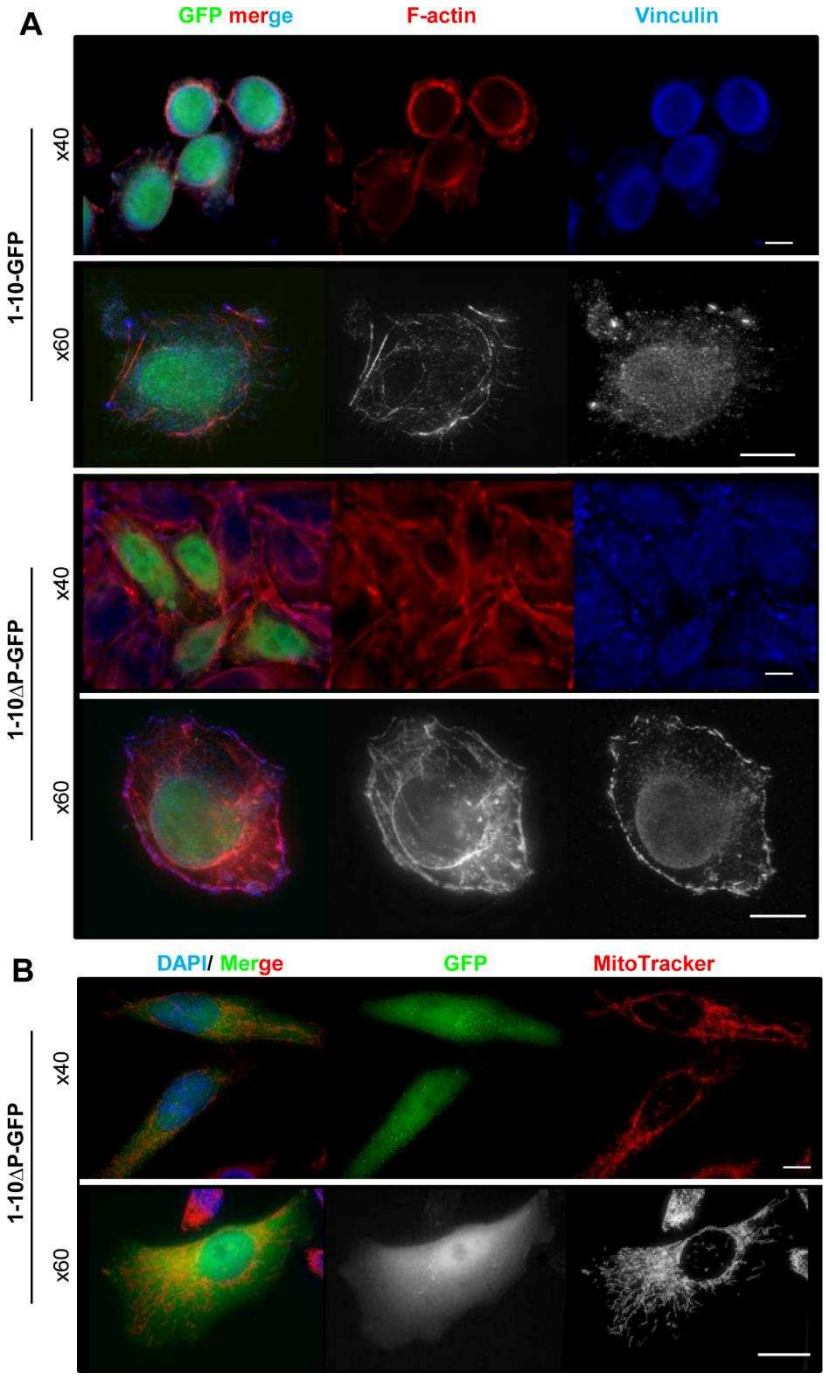

Figure 3: Survivin$_{1-10}$-GFP is a proline-rich sequence that reduces cell adhesion. (A) Cells expressing survivin$_{1-10}$ –GFP or survivin$_{1-10\Delta P}$-GFP (green) were grown on uncoated glass coverslips and stained as in Figure 1A. (B) Live cells expressing survivin$_{1-10\Delta P}$-GFP stained with MitoTracker. Scale bars 10 μm.