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Nuclear survivin has reduced stability and is not cytoprotective

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NUCLEAR SURVIVIN HAS REDUCED STABILITY AND IS NOT
CYTOPROTECTIVE.

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Running head: Nuclear survivin is rapidly degraded.

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Survivin is an essential mitotic protein that is over expressed in many cancers and its presence is correlated with increased resistance to radiation and chemotherapy. Here we demonstrate that sending survivin into the nucleus accelerates its degradation in a *cdh1* dependent manner, abolishes the radio resistance normally conferred to cells by its over expression, and prevents survivin from inhibiting apoptosis without affecting its mitotic localisation. Our data suggest that targeting survivin to the nucleus provides an efficient means of eliminating it from the cell and may prove a novel strategy in cancer treatment, particularly in combination with radiotherapy.

Introduction.

Survivin is an essential mitotic protein that can also inhibit apoptosis. It is up regulated in the vast majority of human cancers and, unlike in normal proliferating cells, in cancer cells it can be present throughout interphase, indicating a loss of cell-cycle regulation. Deregulated survivin expression has been reported at both mRNA and protein levels and correlates with increased resistance to radio- and chemotherapies. In tumour biopsies, survivin has been localised to the nucleus and cytoplasm or both, and a number of studies have implied that differences in patient prognosis correlate with differences in nuclear or cytoplasmic compartmentalisation. However, there is no clear consensus from these studies (1).

We, and others, have recently shown that survivin is a nuclear-cytoplasmic shuttling protein, which is predominantly cytoplasmic due, in part, to an active nuclear exportation signal (NES) in its linker region (2-8). Importantly, unlike wild type survivin, NES mutants are unable to protect cells against X-irradiation or TRAIL induced apoptosis (4), suggesting that relocating survivin to the nucleus during interphase may be key to inhibiting its

cytoprotective activity. These data highlight the importance of regulating, not only the level, but also the localisation of survivin in cancer cells.

To date, any link between sub cellular compartmentalisation and survivin stability has not been addressed; such regulation would have implications in terms of the protein's behaviour, both in the etiology of tumourigenesis and in the design of chemotherapeutic targeting of the protein. To examine the consequences of expressing survivin in the nucleus rather than the cytoplasm, we have fused wild type survivin-GFP to nuclear localisation signals (NLS). We herein report that nuclear survivin is subject to accelerated proteosomal degradation and an abrogation of the cytoprotection otherwise afforded by its overexpression. Together our results suggest a possible mechanism for eliminating survivin from interphase cells with a concomitant sensitisation to apoptotic stimuli.

Experimental Procedures.

Unless otherwise stated tissue culture reagents were from Invitrogen, and all other chemicals from Sigma.

Cell culture and generation of stable lines.

HeLa cells were maintained at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum, penicillin/streptomycin, 500 µg/ml G418 and fungizone. Lines made specifically for the study were survivin_{NLS(LANA)}-GFP, survivin_{NLS(SV40)}-GFP and GFP_{NLS}-GFP. All other lines have been described previously (4,9). Proteins of interest were expressed by FuGene-6 (Roche) mediated transfection of pcDNA3.1 constructs and selected with G418 (500 µg/ml). Cells stably expressing proteins of interest were maintained similarly but were grown in the presence of G418. Prior to experimentation, lines were sorted using an LSRII fluorescence activated cell

sorter (BD Biosciences) to ensure homogeneous populations, and used within five passages of sorting.

Nuclear-cytoplasmic fractionation.

Cells were harvested, washed in PBS then resuspended in ice-cold hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, plus protease inhibitors). Cells were sheared by passage through a 25 gauge needle 15 times. The lysates were centrifuged at 11,000g for 20 minutes at 4°C and the supernatant was collected. The pellet was resuspended in 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM DTT, plus protease inhibitors. Lysates were centrifuged at 21,000g for 5 minutes to obtain nuclear fractions. Protein concentration was determined using a Bradford Reagent protein assay (BioRad) and equal concentration of cytoplasmic and nuclear extracts were used for immunoblotting analyses.

Immunoblotting.

Standard procedures were followed for SDS-PAGE, immunoblotting and enhanced chemiluminescent detection (GE Healthcare). Antibodies used were goat anti-survivin (1/500; R and D Systems), anti-myc (9E10, 1/500), anti-tubulin (1/2000; B512), anti-GFP (1/500; 3E1; CR-UK), anti-XRCC1 (a gift from K.Caldecott), anti-aurora-B kinase (anti-AIM1, 1/250, Transduction Labs), and anti-cdh1 (AbCam). Horse-radish peroxidase conjugated secondary antibodies were from Dako Cytometrics and were used at dilutions of 1/1000-1/5000.

Radiolabelling and Immunoprecipitation.

In vivo labelling was carried out by incubating 10⁶ cells with 50 mCi/ml ³⁵S-methionine. To determine the rate of protein turnover, cells were pulsed as above and chased for up to 16 h in the presence of an excess of unlabeled amino acids. After radiolabelling cells were lysed for 30 minutes on

ice in 500 µl RIPA buffer (20 mM Tris (pH 8), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate with 1 mM β-glycerophosphate, and 1 µg/ml each of the protease inhibitors AEBSF, chymostatin, leupeptin, antipain, pepstatin A), containing 2mM MgCl₂ and 25U/ml benzonase (VWR). Lysates were then cleared and supernatants incubated for 1.5 h at 4°C with 2 µg of a polyclonal anti-survivin (Novus) antibody. Protein G Sepharose beads were then added (40 µl of a 50% slurry in lysis buffer), and samples incubated for a further 2 h at 4°C. Samples were then washed, separated by SDS-PAGE, and band intensities quantified from the dried gel using a Storm 860 PhosphorImager (GE Healthcare). Cold immunoprecipitations were performed similarly and analysed by immunoblotting.

Fluorescence microscopy.

Cells were grown on poly-l-lysine coated coverslips then fixed with 4% formaldehyde (Science Services), and permeabilised with 0.15% triton, both in PBS for 5 and 2 minutes respectively (37°C). Interphase cells were probed with anti-lamin B antibodies (C20: 1/500, Santa Cruz), and mitotic cells were probed with anti-tubulin antibodies (1/2000; B512), followed by anti-goat or anti-mouse Texas red secondary antibodies (1/200; Vector Labs). Cells were mounted in Vectashield with DAPI and viewed using an inverted Olympus microscope fitted with an x 63 oil immersion lens, (NA 1.35). Images were captured using a Hammamatsu CCD camera and Delta Vision Spectris software (Applied Precision). JPEG snap shots were prepared as 3D projections of deconvolved z-stacks. Fields of cells were photographed using a Zeiss Axioplan microscope, fitted with an x 40 objective and operated using Simple PCI software.

RT-PCR.

RNA was extracted from 10^7 asynchronously growing HeLa cells using the RNAqueous kit (Ambion). RNA samples were incubated for 1 h at 37°C with RNAase free DNAase (Promega) to eliminate any contaminating DNA. After inactivation of the DNAase (70°C for 10 minutes), RNA was precipitated with 1 volume of isopropanol, and then resuspended in RNAse free water. 4 µg of each sample was used for cDNA synthesis using First-Strand cDNA synthesis kit (GE Healthcare). cDNA for exogenous survivin-GFP (and variants) was amplified using a forward primer, which annealed to the 5' end of survivin open reading frame and a reverse primer, which annealed to the 5'-end of GFP open reading frame.

Drug Treatments, Cell

Synchronisation and FACS Analysis.

To inhibit protein translation cells were treated with 50 µg/ml cycloheximide. To inhibit protein degradation mediated by the proteasome, cells were treated with 50 µM MG132 for 6 h, or 20 µM MG132 for 16 h. When working with the NLS tagged versions of survivin MG132 treatment prior to cycloheximide treatment was necessary to enable detection of these proteins at the outset of the experiment. To inhibit CRM1 dependent nuclear export, cells were treated with 6-10 ng/ml leptomycin B (LMB; VWR) for 4 h, 6 h or 12 h as indicated. For G1 synchrony, cells were treated overnight with 400 µM mimosine. Cell cycle distribution was determined by measuring the DNA content using flow cytometry. Briefly, 10^5 cells were harvested, washed and fixed with 70% ice cold ethanol. Cells were then washed with PBS, and resuspended in 200 µl of propidium iodide solution containing 50 µg/ml PI and 100 µg/ml RNase A (MP Biomedicals, UK). Propidium iodide stained cells were analysed with a FACScan cytometer using CellQuest software (Becton Dickinson).

Analysis of APC/C modulators.

To over express cdc20 and cdh1, pcDNA-cdc20-myc and pcDNA-cdh1-myc (gifts from Dr. Katya Ravid, University of Massachusetts, Boston, USA) were transiently transfected into HeLa cells using FuGene-6 (Roche) and expression assessed 24 h later by immunoblotting using anti-myc antibodies (9E10).

To deplete cdh1 predesigned cdh1 siRNA oligos (Ambion, ABI Biosystems), were transfected into HeLa cells using Hyperfect (Qiagen). Depletion was assessed by immunoblotting with anti-cdh1 antibodies (AbCam), 24 h post-transfection.

X-irradiation and clonogenic survival.

Cells were seeded at low density (500-1000 cells per dish) in 9 cm² petri dishes and allowed 2 h to attach, before exposure to X-irradiation using an Hs-X-Ray System (A.G.O. Installations Ltd., Reading, UK). Seven days post-irradiation, colonies were stained with methylene blue (1 h room temperature), dried, then rinsed with H₂O and colonies of 50 cells or more were counted.

Apoptosis Assays.

To induce apoptosis by the extrinsic caspase-8/caspase-3 pathway, exponentially growing cells were treated with 250 µg/ml recombinant human TRAIL (Pepro Tech EC Ltd) for 60 or 90 minutes. Cells were lysed (45 minutes, RT) in mammalian protein extraction buffer, MPER (Pierce), supplemented with 1 mM EDTA, 1 µg/ml pepstatin A and 1 mM AEBSF, at a concentration of 10^6 cell equivalents per ml. Lysates were then cleared, snap frozen in liquid nitrogen and stored at -80°C.

To determine apoptotic activity, tetrapeptide cleavage assays were performed in a 96 well plate. Briefly, 5 ng/ml of the caspase-3 specific tetrapeptide substrate (DEVD-AMC; Biomol) was incubated at 37°C for 1 h with 20-50 µl of whole cell lysate prepared in MPER (Pierce), in 20 mM HEPES (pH 7.5) with 10% glycerol and 1 mM DTT. Relative fluorescence

release was measured using a Spectramax Gemini fluorimeter (Molecular Devices) with excitation set at 380 nm and emission at 440 nm.

Cell Viability Assay.

Cells were seeded at a density of 10^4 per well, in a 24 well dish, then irradiated at the doses indicated. Seven days later, cells were incubated with thiazolyl blue tetrazolium bromide (MTT) and cell viability assessed using a Spectramax Gemini fluorimeter (Molecular Devices).

Results.

Endogenous survivin is preferentially degraded in the nucleus.

It has previously been shown that survivin is subject to proteasome-mediated degradation, as levels of endogenous survivin increase after treatment with MG132 (17). This increase is not due to accelerated synthesis as immunoprecipitation of endogenous survivin pulse labelled with ^{35}S methionine for 2 h, actually showed decreased incorporation of ^{35}S (thus decreased synthesis) in the presence of MG132 (data not shown). To investigate any dependence of survivin stability on sub cellular compartmentalisation, we have fractionated asynchronous HeLa cells following MG132 treatment and analysed the level of endogenous survivin in nuclear and cytoplasmic fractions (Figure 1A). Using tubulin and XRCC1 as markers of cytoplasmic and nuclear fractions respectively, we observed a selective increase in nuclear levels of endogenous survivin following MG132 treatment. Consistent with this finding, when endogenous survivin was sequestered in the nucleus by treatment with the exportin inhibitor LMB, its expression was reduced (Figure 1B) by approximately 30%. Together, these data suggest that nuclear survivin may be less stable than survivin localised to the cytoplasm.

Generation of stable lines expressing survivin_{NLS}-GFP.

Survivin is a nuclear-cytoplasmic shuttling protein, which is primarily cytoplasmic when over expressed. To further investigate post-translational regulation of survivin levels we sought to send survivin to the nucleus. To this end we fused full length human survivin to two separate NLS sequences, the bipartite LANA sequence RRHERPTTTRIRHRKLRS (10), and the monopartite SV40 T-antigen NLS sequence PKKKRKV (11), hereinafter referred to as survivin_{NLS(LANA)}-GFP and survivin_{NLS(SV40)}-GFP respectively. As these survivin constructs are expressed from the CMV promoter, they are not subject to transcriptional regulation, thus they enable us to investigate changes in protein level attributed solely to posttranslational regulation. HeLa cell lines were generated that stably over expressed these versions of survivin and were FACS sorted to homogeneity prior to use. As shown in Figure 2A survivin-GFP was predominantly cytoplasmic while both survivin_{NLS(LANA)}-GFP and survivin_{NLS(SV40)}-GFP were retained in the nucleus (see Figures 2B and C). Lines were also generated that expressed GFP or GFP_{NLS}-GFP, for use as controls (data not shown). Importantly, the presence of an NLS on survivin did not hamper its localisation in mitosis, where both constructs were found at the same locations as survivin-GFP: the centromeres, midzone, and midbody, during prometaphase, anaphase and cytokinesis respectively, (Figures 2D-F).

Survivin_{NLS}-GFP is degraded more rapidly than Survivin-GFP.

From our low magnification fluorescence data in Figure 2 (right panels A-C), we noted that the level of expression of survivin_{NLS}-GFP in both lines appeared lower than for the line expressing survivin-GFP. By RT-PCR, we confirmed that the transcripts to these forms were present (Figure 3A), and therefore mRNA was still being expressed. We also ascertained that these forms did not have different rates of protein synthesis (see Figure 3G). Thus we reasoned that the differential expression was due to differences in the

rates of protein turnover. To examine this we immunoblotted whole cell extracts from asynchronous populations of cells expressing survivin-GFP or survivin_{NLS}-GFP. Strikingly, while survivin-GFP was abundantly present in untreated asynchronous cells, survivin_{NLS(LANA)}-GFP was barely detectable (Figure 3B). Furthermore, while 6 h treatment with MG132 caused only a modest (1.08 fold) increase in survivin-GFP expression (Figure 3B and C), survivin_{NLS}-GFP levels rose at steady rate to 3.9 fold (Figure 3B and C), demonstrating that the stability of survivin_{NLS}-GFP is proteasome dependent, as is the case for the endogenous protein (Figure 1A). Similar results were obtained with survivin_{NLS(SV40)}-GFP. As a control we also compared GFP expression in cells expressing GFP and GFP_{NLS}-GFP. No differences in GFP expression were observed in these lines, indicating that the increased rate of turnover was not an artefact of the tag (data not shown).

To determine the relative stability of these versions of survivin, cells were treated with the translational inhibitor, cycloheximide (Figures 3D and E). Due to the rapid clearance of the nuclear forms of survivin, this experiment had to be carried out after pre-treatment with MG132 (see lanes 1 and 2, Figure 3E). Note, 16 h treatment with MG132 did not affect cell cycle stage as assessed by FACS analysis (data not shown). Over a 16 h time course survivin_{NLS(LANA)}-GFP was degraded much more rapidly than survivin-GFP (Figure 3D and E), as is evident by the quantitation in Figure 3F. We also noted that the addition of an NLS to GFP itself did not decrease the stability of GFP (data not shown).

To exclude the possibility that the level of survivin expression was due to changes in the rate of protein synthesis, we next pulse labelled survivin-GFP and survivin_{NLS}-GFP cells with ³⁵S methionine. First, cells were treated with MG132 for 4 h, then exposed to ³⁵S methionine and incubated for a further 2 h (Figure 3G). Lysates were then prepared from each

population and survivin-GFP or survivin_{NLS}-GFP immunoprecipitated from the extracts using anti-survivin antibodies. As shown in Figure 3G survivin-GFP and survivin_{NLS}-GFP incorporated ³⁵S-methionine to similar levels as quantified using a Phosphorimager (pixel intensities of bands 245246 and 246678 respectively). Next, we followed the pulse labelling with a cold chase after the removal of MG132 and addition of cycloheximide. In accordance with our immunoblotting experiments in Figures 3D-F, the rate of survivin_{NLS}-GFP turnover was more rapid than survivin-GFP (Figure 3H). Thus these data further indicate that survivin is less stable in the nucleus than in the cytoplasm.

Survivin is preferentially degraded in the nucleus.

Next we made nuclear and cytoplasmic extracts from asynchronous cultures of the stable cell lines of interest and loaded equivalent numbers of cells per lane (Figure 4). Using tubulin and XRCC1 as markers of cytoplasmic and nuclear fractions respectively, we found that survivin-GFP, like endogenous survivin, was predominantly cytoplasmic (Figure 4A), but, consistent with our fluorescent imaging, expression of the NLS fused versions, survivin_{NLS(LANA)}-GFP (Figure 4B), and survivin_{NLS(SV40)}-GFP (Figure 4C), was extremely low. Moreover, there appeared to be little difference in expression between the two compartments which was surprising given that survivin_{NLS(LANA)}-GFP and survivin_{NLS(SV40)}-GFP were specifically sent to the nucleus. However, upon 6h MG132 treatment, the levels of all versions of survivin, wild type and NLS-fused, rose dramatically in the nucleus, further suggesting that survivin is less stable in the nuclear versus cytoplasmic compartment. We noted that the levels of the NLS-fused forms of survivin also increased in the cytoplasmic fraction upon treatment with MG132 (Figures 4B and C), illustrating the nucleo-cytoplasmic shuttling activity of the protein.

Survivin is degraded in the nucleus in G1.

Survivin expression is normally limited to the G2/M stages of the cycle, with a rapid decline in its levels as cells exit mitosis and enter G1. A combination of factors contributes to the reduction in survivin during G1, including transcriptional repression, externalisation of the midbody at the end of mitosis, and proteolysis. This prompted us to further investigate survivin degradation during G1, using cells expressing the survivin-GFP, which is not subject to transcriptional repression. Cells were synchronised in G1 using mimosine (Figure 4D) then treated with 20 μ M MG132 (Figure 4E) and fractionated (Figure 4F) to assess exogenous levels of survivin-GFP in the cytoplasm versus the nucleus. As with the asynchronous population following MG132 treatment (Figure 4A), survivin-GFP was selectively up regulated in the nucleus after 6 h MG132 treatment in G1 arrested cells (Figure 4F).

Degradation of nuclear survivin is mediated by Cdh1.

Expression of survivin, and its partner protein, aurora-B kinase, is known to be regulated by proteolysis as cells exit mitosis (17,15). Degradation of aurora-B has been demonstrated to be mediated by the APC activated by cdc20 and cdh1 (15), however, how survivin degradation is regulated has not been addressed. Thus to test whether survivin degradation was cdh1 or cdc20 dependent, we transiently over expressed myc-cdh1 or myc-cdc20 (gifts from Dr. K. Ravid), in cells expressing the survivin constructs of interest. Immunoblotting analysis 24 h post-transfection revealed that cells expressing either myc-cdh1 or myc-cdc20 decreased the abundance of survivin-GFP, survivin_{NLS(LANA)}-GFP and endogenous survivin in asynchronous cells (Figures 5A-C). This decrease in survivin levels was prevented by addition of MG132 for 1.5 h post-transfection (Figure 5D). (Note also, however, that the transfection

efficiency with cdc20 was always lower than for cdh1).

Quantitation of survivin expression from Figures 5A-D is shown in Figure 5E and plotted as a fraction of the expression in control cells.

Cdh1 is a nuclear protein (12), while cdc20, whose level is low in G1, is more membranous/cytoplasmic (13). Thus, having established that survivin is degraded preferentially in the nucleus, we next asked whether depletion of cdh1 could increase survivin levels. Cdh1 was depleted by siRNA from asynchronous HeLa cells, protein lysates were prepared 24 h post-transfection and analysed for survivin expression by immunoblotting. Despite an incomplete knock down of cdh1 (54%), survivin expression doubled under these conditions (Figure 5F).

Nuclear survivin does not protect cells against apoptosis.

To assess whether nuclear survivin is able to inhibit apoptosis, the cell lines indicated were subjected to a clonogenic survival assay after exposure to increasing doses of ionising X-irradiation. Consistent with our previous data (4), expression of survivin-GFP conferred resistance to X-irradiation, compared with cells expressing GFP (Figure 6A) or GFP_{NLS}-GFP (data not shown). By contrast no resistance to radiation was conferred by the lines expressing survivin_{NLS(LANA)}-GFP and survivin_{NLS(SV40)}-GFP, indeed, these lines exhibited some increased sensitivity to this treatment, although less so than cells expressing survivin_{L98A}-GFP.

Next we induced apoptosis by treatment with recombinant TRAIL, and measured caspase activity in a fluorogenic tetrapeptide cleavage assay using the caspase-3 specific substrate, DEVD-AMC (Figure 6B). Lysates were prepared from cells expressing GFP, GFP-NLS-GFP, survivin-GFP, survivin_{NLS}-GFP, or survivin_{L98A}-GFP (as indicated) 0 or 60 minutes post-treatment with TRAIL, and incubated for 1 h with DEVD-AMC. In these assays survivin-GFP conferred protection against TRAIL mediated

apoptosis, but cells expressing survivin_{NLS}-GFP exhibited similar and sometimes elevated levels of caspase-3 activity to GFP and GFP_{NLS}-GFP controls. The kinetics of the induction of caspase activity was most rapid in cells expressing the mutant version, survivin_{L98A}-GFP, which we previously showed was nuclear and pro-apoptotic (4).

As survivin is rapidly degraded in the nucleus, we asked whether inhibiting proteolysis could restore survivin's ability to inhibit apoptosis. The cell lines indicated were exposed to 5 Gy X-rays in the absence and presence of MG132. MG132 was removed after 6 h and cell viability analysed 7 days later using an MTT assay (Figure 6C). In this assay inhibiting proteolysis rescued survivin's anti-apoptotic activity possibly due to the increased cytoplasmic pool that accumulates under these conditions (see Figure 4C). To ascertain specifically whether the nuclear pool of survivin can be cytoprotective we repeated the TRAIL assay (Figure 6B) in the absence and presence of MG132, or MG132 and LMB. In this assay, MG132 treatment caused a decrease in caspase activity in controls and experimental samples making it difficult to assess whether increased stability of the exogenously expressed protein specifically contributes to the reduced caspase activity. However, the additional treatment of LMB caused an increase in apoptosis in survivin-GFP cells, while the level of caspase 3 activity in survivin_{NLS}-GFP cells appeared to be unaffected by either treatment. These data indicate that when stabilised and completely nuclear, survivin cannot inhibit apoptosis. Taken together our present data suggest that forced expression of survivin in the nucleus is sufficient to prevent it from inhibiting apoptosis in cultured human cells.

Discussion.

Survivin is a nucleo-cytoplasmic shuttling protein that is predominantly cytoplasmic when over expressed in cultured cells (2,9). We, and others,

have recently shown that this sub cellular localisation is dependent upon CRM1 (beta-exportin) and a rev-like NES in survivin's linker region, between its BIR domain and C-terminal alpha-helix (2-4,6-8).

Here we demonstrate that survivin is preferentially degraded in the nucleus in a cdh1/APC-dependent manner. These findings are consistent with cdh1 mediated degradation of survivin *in vitro* (14), and the nuclear localisation of cdh1 in G1(12). Somewhat paradoxically, survivin appears to be devoid of destruction motifs recognised by the APC/C. However, survivin's mitotic partner protein aurora-B has three putative D-boxes, a KEN box and an A-box, and mutation of the cdh1 specific KEN and A-boxes stabilise aurora-B suggesting that its destruction is mediated preferentially by cdh1 (15,16). In addition, aurora-B also exhibits accelerated clearance upon over expression of cdh1, increased stability upon cdh1 depletion and co-immunoprecipitates with cdh1 in mitotic extracts (15,16). Thus, as survivin and aurora-B are both destroyed at the end of mitosis (14-17), it is formally possible that survivin relies on aurora-B's consensus sequences for destruction. We are currently testing this hypothesis.

In a previous report we found that a mutant form of survivin that accumulates in the nucleus could no longer protect cells against ionising radiation or TRAIL-induced apoptosis (4). Corroborating data were recently presented by Stauber and co-workers (6-8). However, these experiments raised the question as to whether sub cellular relocalisation alone was responsible for abrogating survivin's anti-apoptotic activity, or whether the effect was mutant specific. Here, we have artificially forced wild type human survivin expression in the nucleus and observed that this relocation prevented survivin from acting as an inhibitor of apoptosis. Furthermore, in some cases we actually noted an increase in sensitivity to apoptotic stimuli, the reason for which is unclear. One

possibility may be that the sub cellular localisation of the exogenous protein influences the localisation of the endogenous protein. In a recent study Temme and co-workers also found that cells were more sensitive to apoptosis when they forced survivin expression in the nucleus, and interestingly they linked this observation to enhanced transcription of p53, and the pro-apoptotic genes, Bad and Bax (5).

Our present data appear to contradict the recent work by Stauber and co-workers (6), who reported that nuclear sequestration of murine survivin via deletion of the NES increased the stability of the protein, thus suggesting that it is preferentially degraded in the cytoplasm. However, it is possible that deletion of these residues could have affected the folding or stability of survivin specifically, rather than increased its stability as a result of its sub cellular relocalisation. (Note also that our experiments used stable cell lines rather than transiently transfected cells, which could have contributed to the different results). Differential stability due to sub cellular compartmentalisation has been noted for a number of proteins including p53 whose localisation and stability is altered upon DNA damage (18). Furthermore, the survivin isoform Delta-Ex3, which is nuclear when over expressed, (19,20), is also cleared from the cell more rapidly than wild type survivin (21), and may explain why endogenous survivin DeX3 is difficult to detect at the protein level (20,22). Interestingly, it has recently been reported that survivin degradation can also be facilitated by the XIAP association factor, XAF-1, in a proteasome dependent manner, which suggests that multiple pathways for ensuring the removal of survivin from interphase cells exist (24).

Finally, survivin has a functional NES, but no NLS. Thus, one outstanding question is how is survivin gaining access to the nucleus? Although the endogenous protein is small enough to enter the nucleus by diffusion even if dimerised, this is unlikely given the

behaviour of the GFP tagged form. Of survivin's known binding partners aurora-B has sequences that correspond to NLSs but appear non-functional in a nuclear targeting assay, and INCENP has three functional NLSs (3). However, when over expressed in MCF cells, neither aurora-B nor INCENP was able to influence survivin localisation, which remained predominantly cytoplasmic (3). Another candidate for nuclear targeting is TD60, an RCC1-like protein, which has a putative NLS, and co localises with the chromosomal passenger proteins (23). However, it should be noted that chromosomal passenger proteins have a cell cycle dependent expression, and whether they are present in interphase cells when survivin is over expressed is unknown.

In conclusion, we have demonstrated that relocating survivin to the nucleus accelerates its degradation and prevents it from protecting cells against IR and inhibiting apoptosis. We have also shown that the presence of an NLS on survivin does not affect its mitotic function. Thus sequestering survivin in the nucleus could be very helpful in cancer therapy as it would resensitise cells to radiation without affecting proliferation of non-cancerous cells.

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Figure Legends.

Figure 1: Endogenous survivin is preferentially degraded in the nucleus. (A) Nuclear (N) and cytoplasmic (C) fractionation was carried out on HeLa cells that had been incubated in the absence (-) or presence (+) of MG132 (50 μ M, 6 h). An increase in endogenous survivin was apparent upon proteasome inhibition in the nuclear (compare lanes 2 and 4), but not the cytoplasmic fraction (lanes 1 and 3). (B) HeLa cells were incubated in the absence (-) or presence (+) 6 ng/ml LMB for 12 h to inhibit exportation of survivin from the nucleus. This treatment alone caused a 30% reduction in survivin expression.

Figure 2: Expression of survivin-GFP and survivin_{NLS}-GFP constructs in HeLa cells. (A-C) Interphase cells stably expressing the constructs indicated were probed with anti-lamin B antibodies (red) to show the nuclear margins, and DAPI to visualise the DNA (blue). Right panels show a representative field of cells from each population (D-F). Mitotic cells as above were probed with anti-tubulin antibodies (red) and DAPI (blue). NLS-fusion caused nuclear sequestration of survivin-GFP in interphase but did not alter localisation during mitosis.

Figure 3: Survivin_{NLS}-GFP is degraded more rapidly than survivin-GFP. (A) RT-PCR was performed on cells expressing survivin-GFP (lane 1), survivin_{NLS(LANA)}-GFP (lane 2), and survivin_{NLS(SV40)}-GFP (lane 3) and confirmed that mRNA was expressed in each line. (B) Lysates were prepared from cell lines expressing survivin-GFP or survivin_{NLS}-GFP after the indicated times post treatment with 50 μ M MG132, and immunoblots probed using anti-GFP antibodies. To detect survivin_{NLS}-GFP at adequate levels for quantitation a 6 h treatment with MG132 was required. (C) Quantification of ECL signals in (B): Survivin-GFP expression is represented by circles, and survivin_{NLS}-GFP by triangles. (D and E) Treatment overnight with 20 μ M MG132 (lanes 2) followed by subsequent release into cycloheximide (50 μ g/ml) to inhibit protein translation, (lanes 3-6) revealed that survivin_{NLS(LANA)}-GFP degraded more rapidly than survivin-GFP. This experiment was performed twice with similar results. (F) Quantitation of immunoblots shown in D and E. (G and H) To assess the synthesis rate of survivin-GFP (time 0), or survivin_{NLS}-GFP (time 0), cells were pulse labelled with ³⁵S-methionine for 2 h and immunoprecipitation carried out with anti-survivin antibodies (Novus). Pixel intensities of bands (time 0) were similar: 255246 and 246678, assigned 100% in (H). Cells were then subjected to a cold chase before immunoprecipitation as above at 4, 8 or 16 h. Consistent with the immunoblotting experiments, survivin_{NLS}-GFP turned over more rapidly than survivin-GFP. In (H) expression at time 0 is taken as 100%. Data graphed is the mean and standard deviation from two independent experiments.

Figure 4: Survivin is preferentially degraded in the nucleus. Nuclear (N) and cytoplasmic (C) extracts were prepared from asynchronous HeLa cells expressing (A) survivin-GFP, (B) survivin_{NLS(LANA)}-GFP, or (C) survivin_{NLS(SV40)}-GFP. Immunoblots were probed with anti-tubulin and anti-XRCC1 antibodies to indicate cytoplasmic and nuclear fractions respectively. MG132 stabilised survivin-GFP in the nucleus, but did not alter its expression in the cytoplasmic fraction suggesting that it is preferentially degraded in the nucleus. MG132 caused increased expression of survivin_{NLS}-GFP lines in both compartments, which may reflect the nuclear-cytoplasmic shuttling activity of these proteins. (D) To arrest cells in G1, cells were treated for 16 h with mimosine and DNA content analysed by FACS. (E) No change in G1 distribution was observed upon addition of MG132. (F) Nuclear-cytoplasmic fractionation was carried out as in (A) on a G1 enriched population of survivin-GFP cells. As in (A), survivin-GFP was preferentially stabilised in the nuclear fraction upon MG132 treatment.

Figure 5: Cdh1 mediates survivin degradation in the nucleus. Asynchronous HeLa cells expressing (A) survivin GFP, (B) survivin_{NLS(LANA)}-GFP (C) or not expressing any construct were transfected with pcDNA3 constructs containing cDNA to cdh1-myc or cdc20-myc and whole cell lysates prepared 24 h later. To determine the level of survivin-GFP expression and

cdh1-myc or cdc20-myc expression, immunoblots were probed with anti-survivin and anti-myc antibodies respectively. Note that, due to the similarity in size between the tubulin and cdh1-myc/cdc20-myc, two separate gels were run: tubulin indicates the loading for survivin blots. Overexpression of both cdh-myc and cdc20-myc decreased the expression of all forms of survivin, exogenous and endogenous. **(D)** The decrease in survivin expression observed upon over expression of cdh1 or cdc20 was reversed when cells were treated with MG132 (50 μ M for 1.5 h). **(E)** Quantification of blots in A-D, showing the level of survivin as a fraction of the control. Data is representative of a minimum of two independent experiments. **(F)** Cdh1 was depleted from HeLa cells using predesigned siRNA oligos. Immunoblot analysis revealed a 54% decrease in cdh1 expression. This decrease was accompanied by a 200% increase in survivin levels 24 h post-transfection, when compared with the control (C) population treated with a scrambled oligo. Tubulin indicates equality in loading.

Figure 6: Survivin_{NLS}-GFP is not cytoprotective. **(A)** HeLa cells stably expressing the constructs indicated were seeded at low density, exposed to X-irradiation, and colonies of 50 or more cells counted 7 days post-irradiation. Surviving fraction was plotted in logarithmic scale. Overexpression of survivin-GFP, but not survivin_{NLS}-GFP, protected cells against irradiation. Note that neither survivin_{NLS}-GFP line was as sensitive to irradiation as survivin_{L98A}-GFP. Data is representative of three independent experiments. Each experiment was performed in triplicate and error bars show standard deviation from the mean. Paired T-test analysis revealed that at 2.5 Gy irradiation there was a significant difference between survivin-GFP expressing populations and those expressing survivin_{NLS(LANA)}-GFP ($P=0.031$), survivin_{NLS(SV40)}-GFP ($P=0.047$), or survivin_{L98A}-GFP ($P=0.037$). The differences were not significant at higher doses of irradiation. **(B)** Caspase-3 activity assay. Apoptosis was induced by the addition of recombinant TRAIL. Cell lysates were analysed for their ability to cleave the caspase-3 specific substrate (DEVD-AMC), and relative fluorescence release measured spectroscopically. Overexpression of survivin-GFP inhibited caspase-3 activity, but activity remained high in survivin_{NLS}-GFP lines. A paired T-test comparing TRAIL-treated survivin-GFP cells with those expressing the NLS-fused survivin-GFP constructs or survivin_{L98A}-GFP, revealed a significant difference in each case. *P-values (above control and survivin-GFP samples) were obtained by comparison with HeLa cells.* We noted also that cells expressing GFP_{-NLS(LANA)}-GFP also showed a significant difference in caspase-3 activity compared with HeLa cells alone, thus we compared GFP_{-NLS(LANA)}-GFP and survivin_{NLS(LANA)}-GFP samples. In this case the difference was also significant ($P=0.046$). **(C)** MTT Assay indicating mean survival with error bars indicating standard deviation. Cell lines indicated were exposed to 5 Gy irradiation in the absence or presence of 50 μ M MG132. MG132 was maintained in the medium for 6h post-irradiation. Cell viability, assessed 7 days post-irradiation using an MTT assay, revealed that the expression of survivin-GFP prevented cell death induced by 5 Gy irradiation, and was unaffected by MG132 treatment (compare grey and white bars). A paired T-test (5 Gy v. 5 Gy + MG132 samples) revealed no significant difference in GFP and survivin-GFP expressing populations, but, a significant difference ($P=0.03$) in the survivin_{NLS}-GFP cells. By contrast, the decreased viability observed with survivin_{NLS}-GFP with exposure to 5 Gy (survivin_{NLS}-GFP, grey bars) was restored upon stabilisation of the protein with MG132 (survivin_{NLS}-GFP, white bars). **(D)** A caspase-3 activity assay, as described for (B), was performed on cells that had been pretreated with no MG132 50 μ M MG132 (6 h), or 50 μ M MG132 and 10 ng/ml LMB (6 h) Time indicated is the duration of exposure to TRAIL; the mean and standard deviation from one experiment performed in triplicate is shown and is representative of two independent experiments. MG132 treatment alone reduced the extent of apoptosis in each sample, but interestingly, co-treatment with LMB increased the caspase activity in survivin-GFP expressing cells, and had no significant effect ($P>0.05$) on cells expressing survivin_{NLS}-GFP. These data indicate that nuclear survivin cannot inhibit apoptosis.

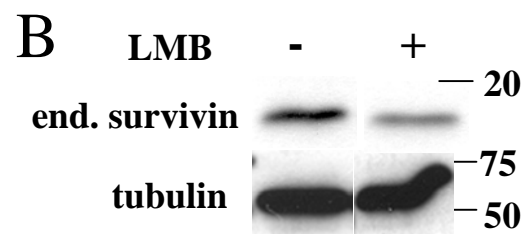
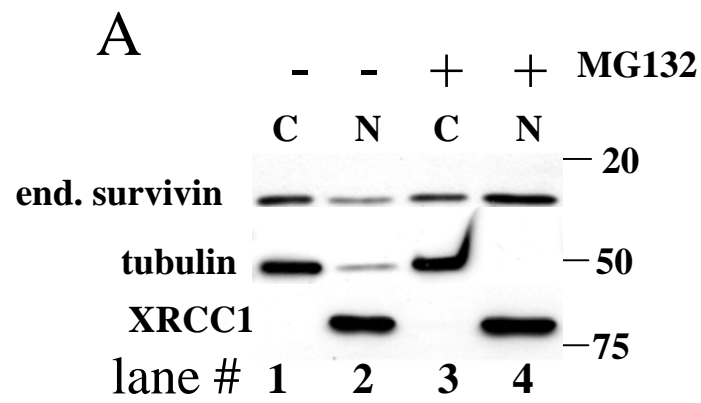


Figure 1

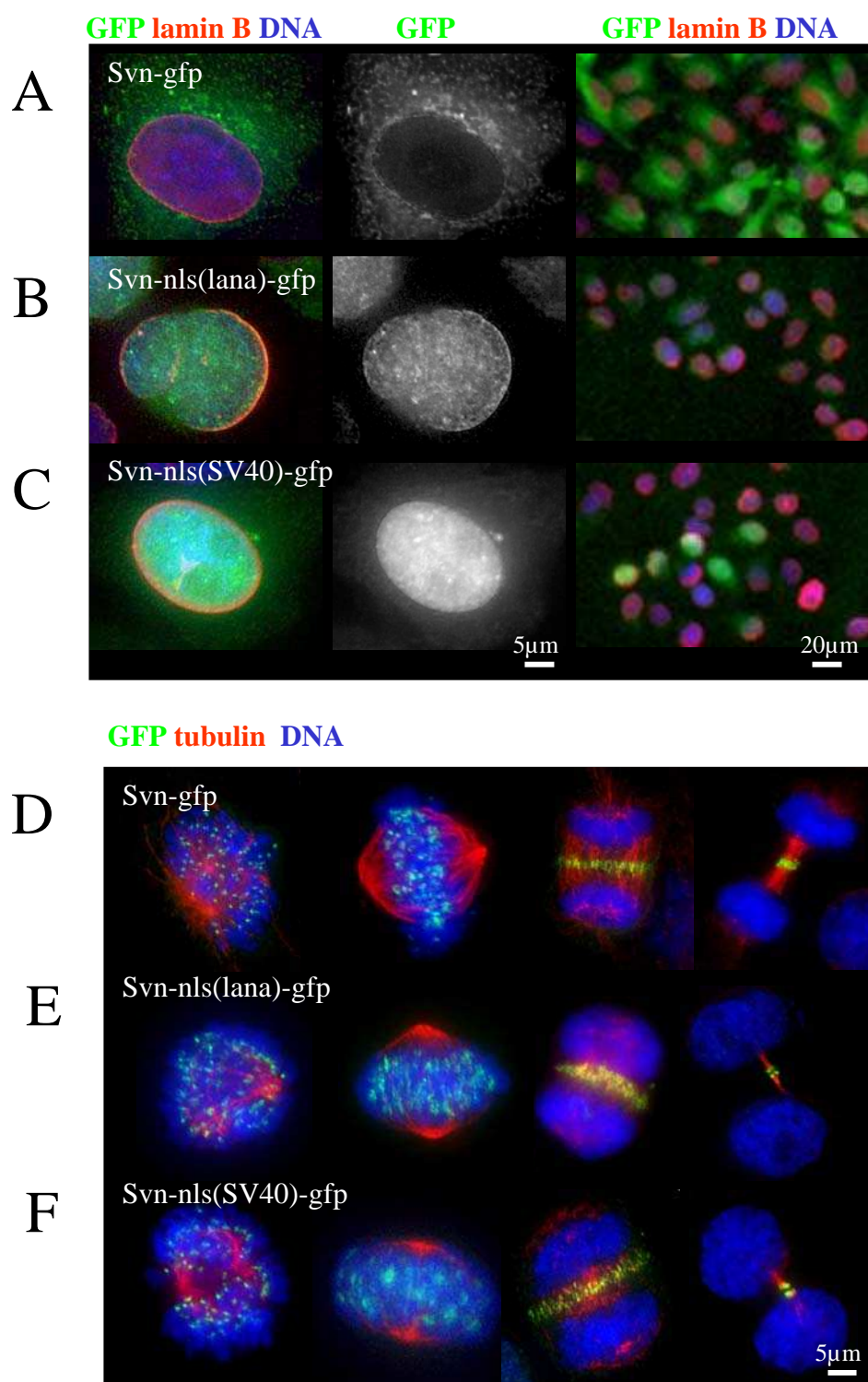


Figure 2

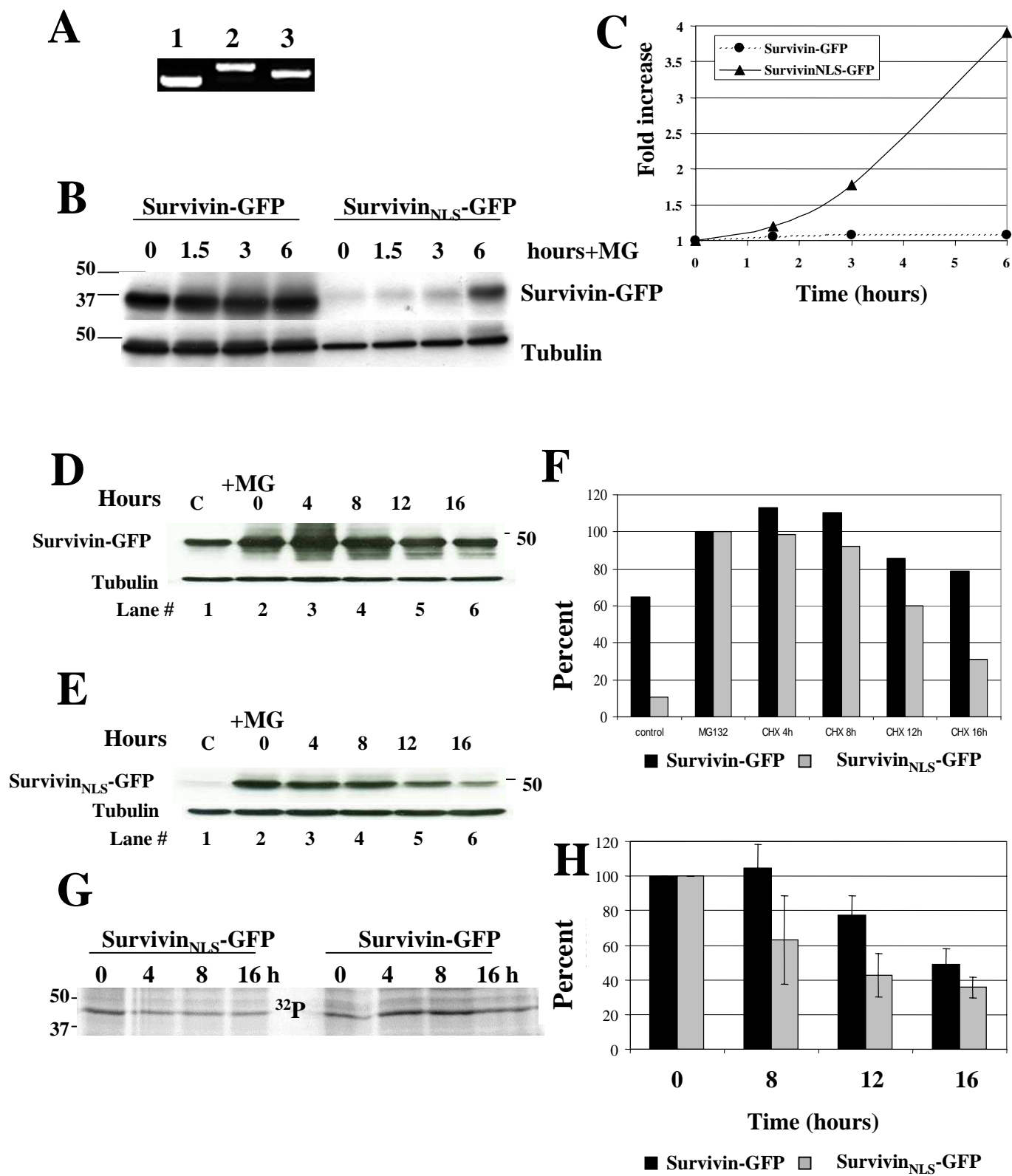


Figure 3

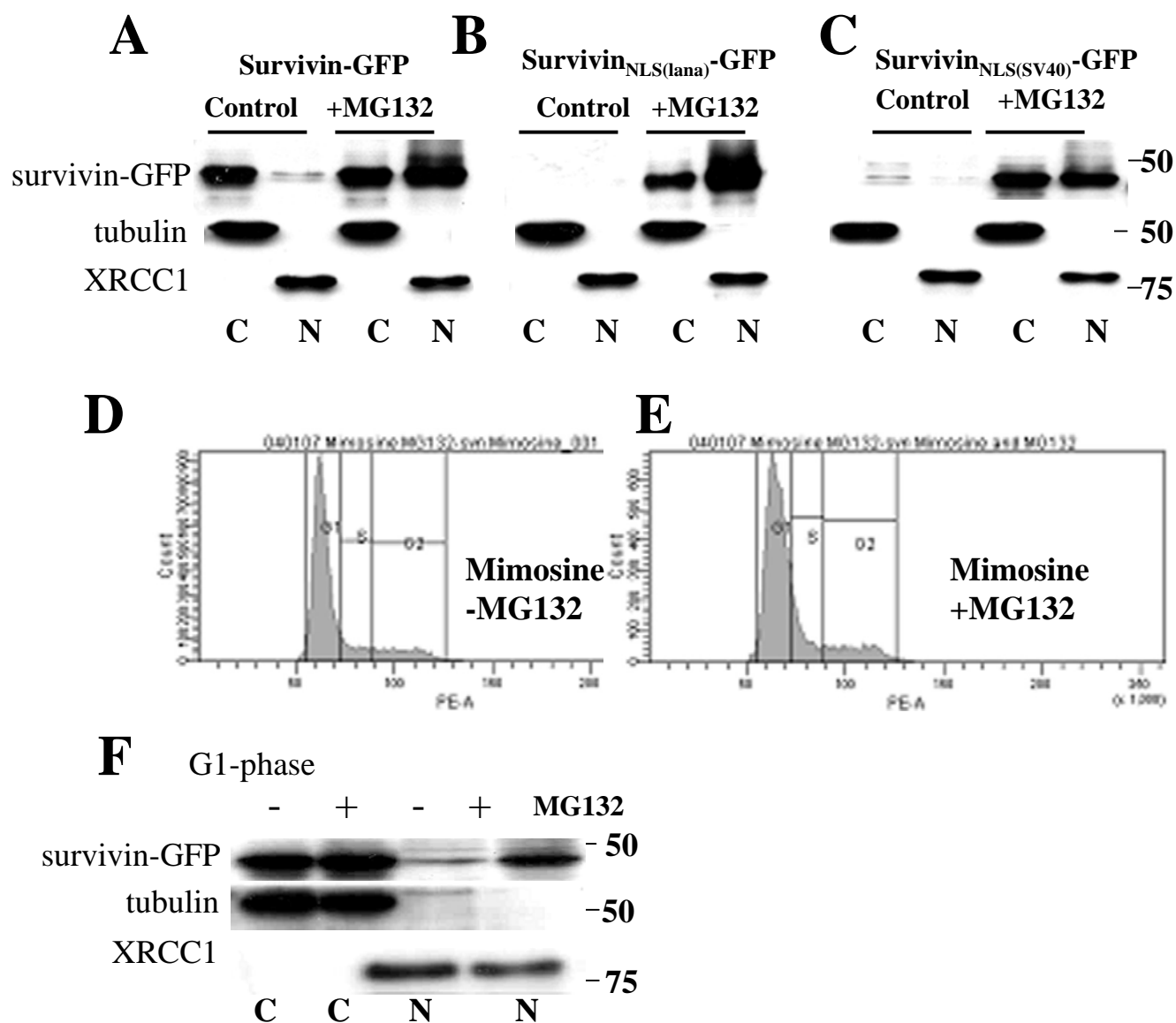


Figure 4

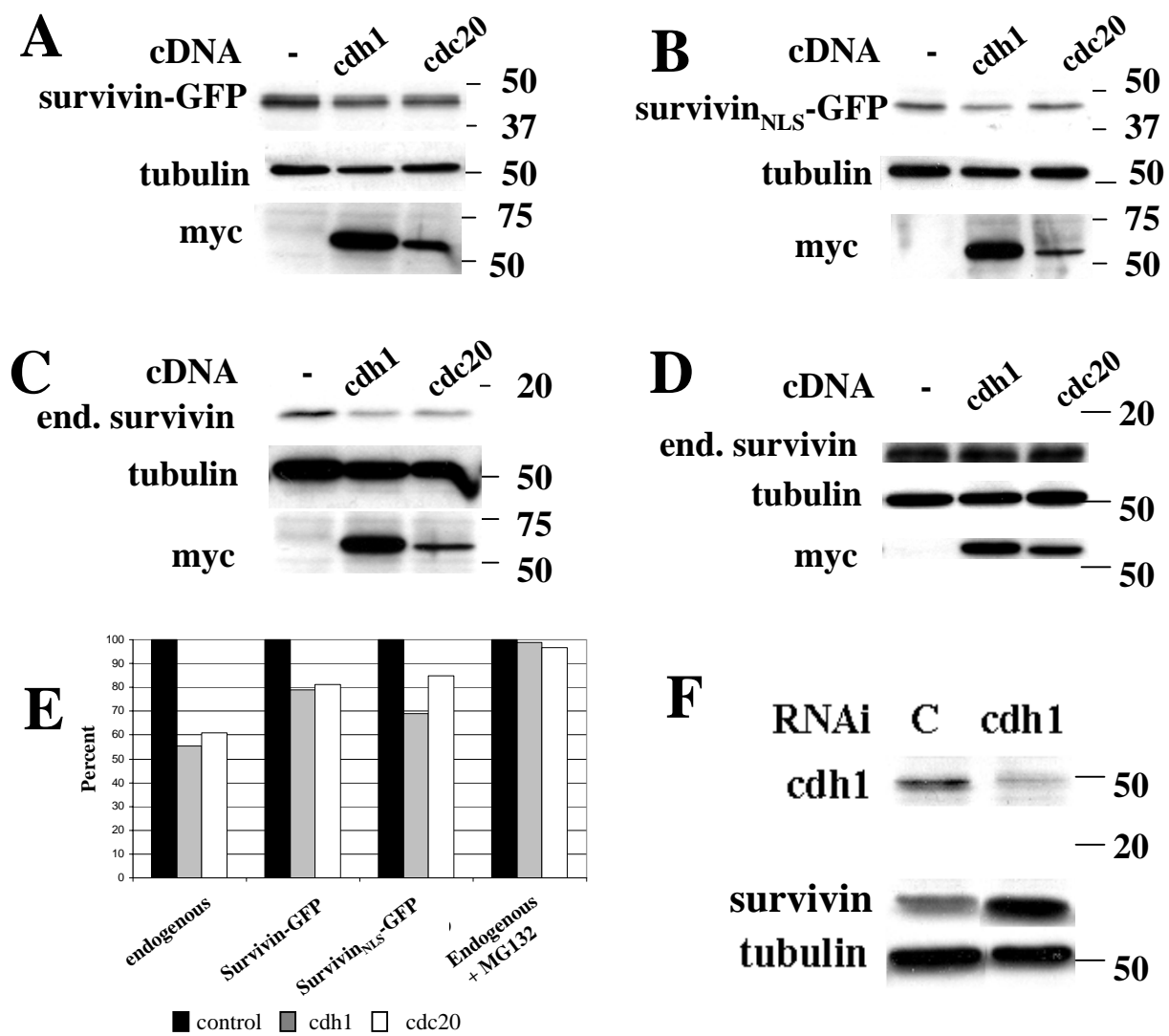


Figure 5

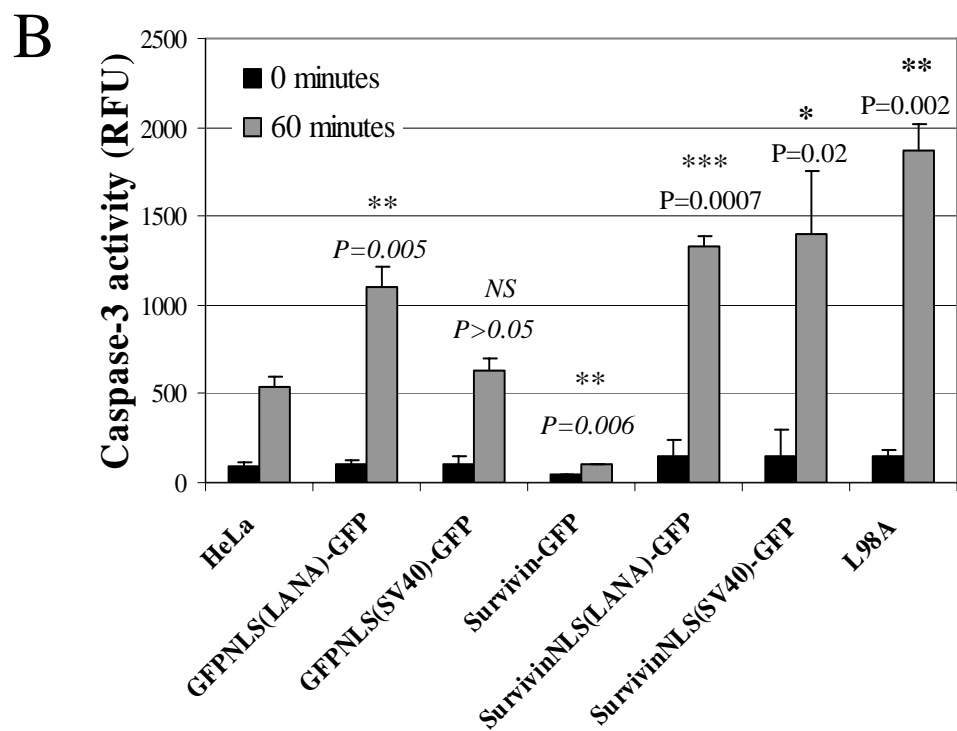
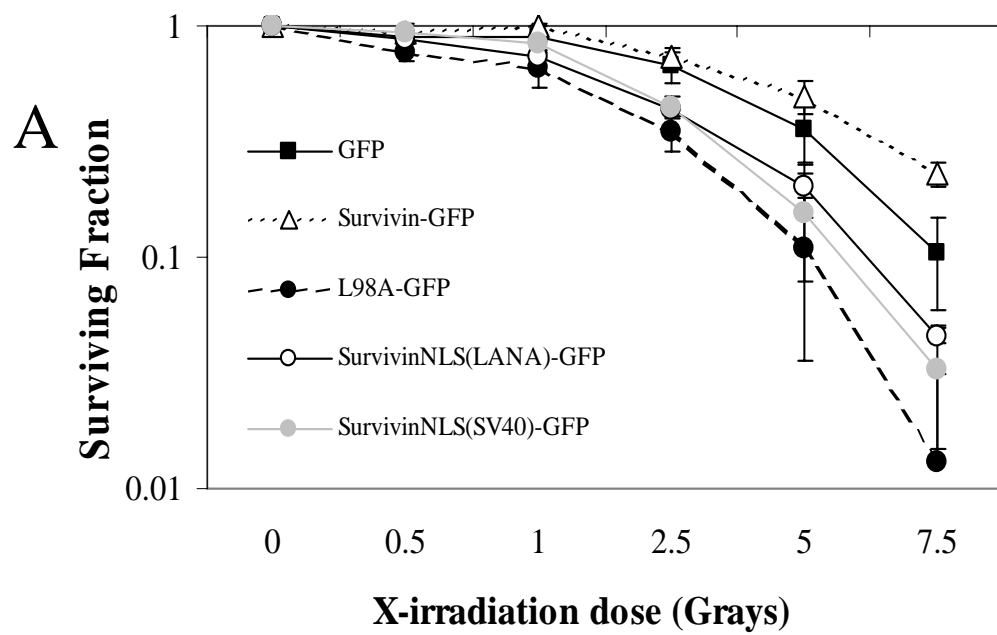


Figure 6

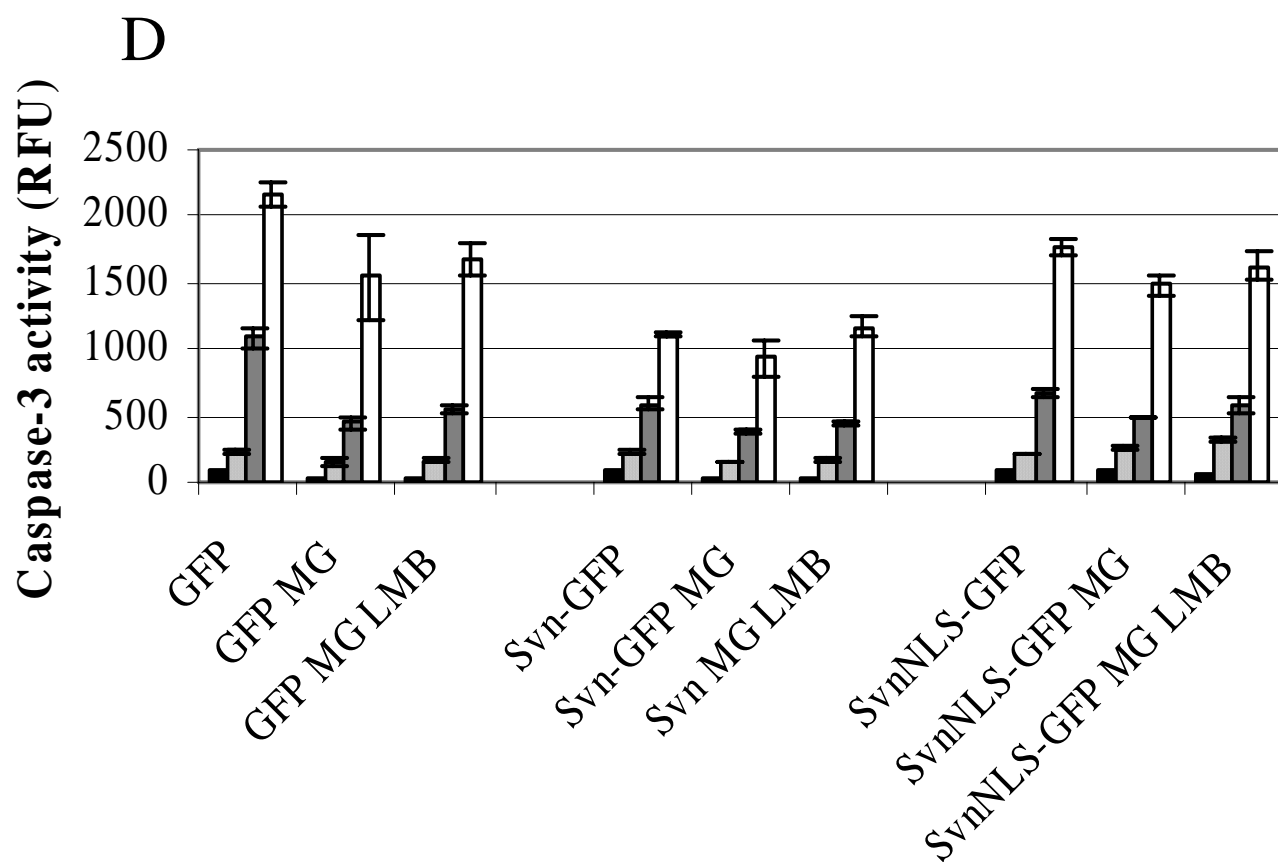
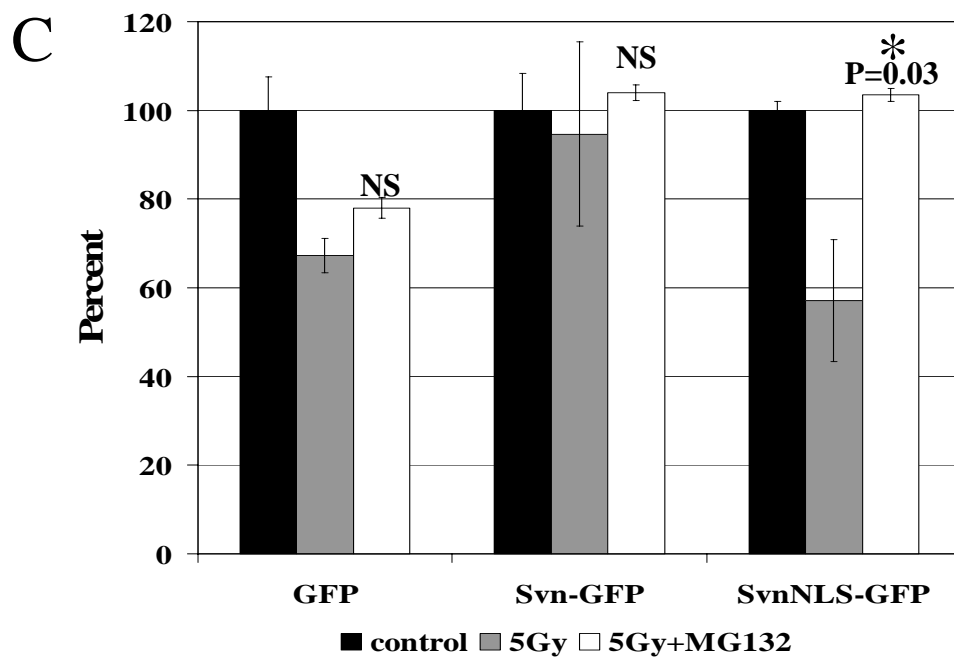


Figure 6 (continued)