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The Role of Apoptosis in HeLa Cells Expressing HIV-1 Rev

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The Role of Apoptosis in HeLa Cells Expressing HIV-1 Rev

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By

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I HEREBY RECOMMEND THAT THE THEIS PREPARED UNDER MY SUPERVISION BY Elizabeth S. Page ENTITLED The Role of Apoptosis in HeLa Cells Expressing HIV-1 Rev BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

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The HIV protein Rev is a nucleolar protein that regulates late gene expression in infected cells by promoting the export of under-spliced viral RNAs (Pollard and Malim, 1998). Its over-expression can also inhibit progression through mitosis (Miyazaki *et al.*, 1995), possibly through its ability to depolymerize microtubules (Watts *et al.*, 2000). Consequently, Rev may activate the spindle assembly checkpoint in mitotic cells and increase the frequency of apoptosis. Rev also binds the nucleolar protein B23 involved in ribosome maturation and centrosome duplication. Because loss of B23 function stimulates apoptosis (Ahn *et al.*, 2005), Rev expression may promote apoptosis by inhibiting B23.

Regardless of its mechanism, it is plausible to hypothesize that Rev expression stimulates apoptosis. To test this hypothesis, Rev and three mutants defective in multimerization, nuclear import, and nuclear export (M4, M6, and M10, respectively) and are known to cause defects in mitosis were transiently and stably over-expressed in HeLa cells. Three separate assays were then used to assay for apoptotic cell death. Since apoptosis is characterized by cell shrinkage, cell rounding, chromatin condensation and fragmentation (Kerr *et al.*, 1993; Lawen, 2003), Rev expressing cells were fixed with formaldehyde, stained with DAPI and examined for overt signs of apoptosis. In cells transiently expressing Rev-GFP, there was a two-fold increase in apoptosis compared to YFP expressing controls. Transient expression of Rev mutants M4, M6, and M10

resulted in comparable increased frequencies of apoptosis. This visual assay is limited by the apparent lack of sensitivity as demonstrated by the inability to detect apoptosis induced by treatment with actinomycin D.

To clarify these results, two additional biochemical assays were used. The first, an ELISA assay that quantifies chromatin fragmentation by measuring the release of mono- and oligo-nucleosomes, showed there was no statistical difference in apoptotic frequencies between Rev-YFP and YFP expressing cells ($p=0.43$). These results were confirmed by an immunoblot assay that was unable to detect the presence of activated Caspase 3 in Rev expressing cells. Thus it appears that the cell cycle defects induced by Rev expression are either corrected before mitosis is completed or not detected by the spindle assembly checkpoint.

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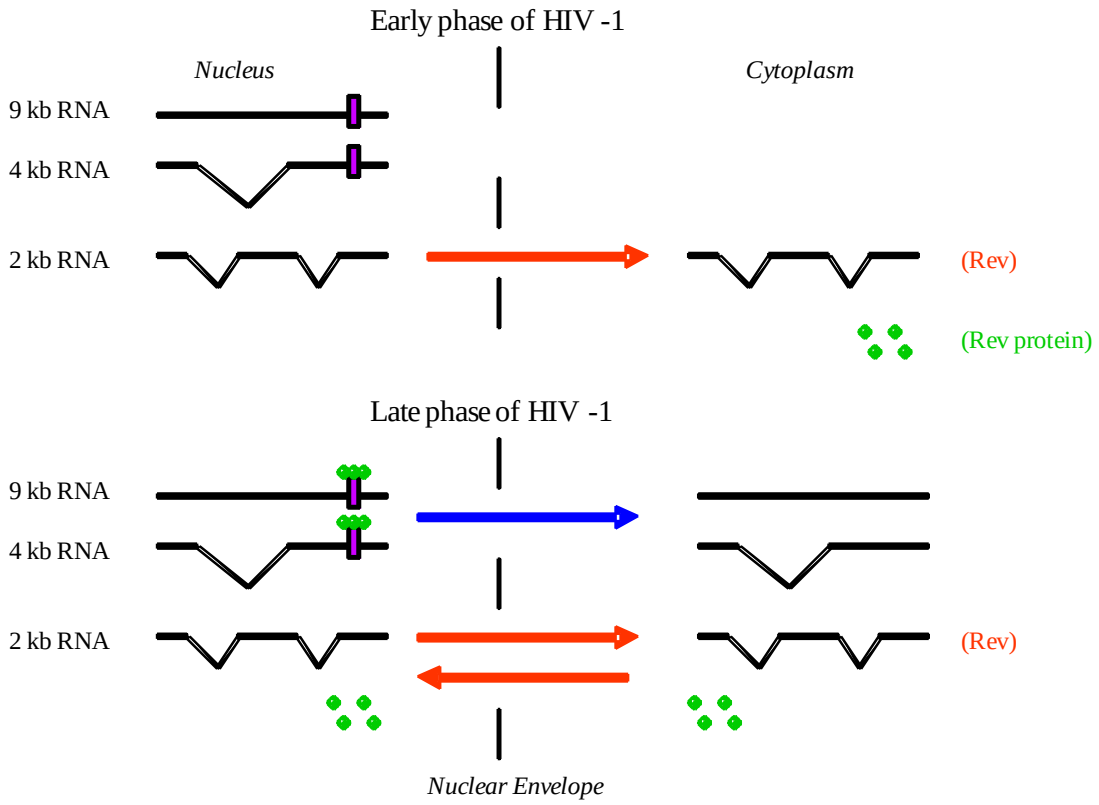
Introduction

HIV is the causative agent of acquired immunodeficiency syndrome (AIDS) (Fauci *et al.*, 1988). It is a retrovirus that encodes three regulatory genes expressed early in infection as well as six additional proteins including Gag, Pol, and Env that are expressed later (Pollard and Malim, 1998). Rev (Regulator of Expression of Virion proteins) is a regulatory gene product essential for viral expression of HIV (Pollard and Malim, 1998). It regulates late gene expression and a loss of Rev function through mutation inhibits HIV infection (Pollard and Malim, 1998). While many Rev mutations are recessive and inhibit virion production, *trans*-dominant mutations such as M10 have been identified that are capable of inhibiting infections by wild-type virus (Malim *et al.*, 1989; Pollard and Malim, 1998; Cochrane, 2004). Such mutations provide proof of the concept that Rev is an important potential target for antiviral strategies (Pollard and Malim, 1998; Cochrane, 2004).

The Rev gene encodes a 116 amino acid, 13 kDa protein that predominantly localizes in the nucleoli of cells (Pollard and Malim, 1998). There it promotes nuclear export of under-spliced viral mRNAs (Hope, 1999). Early in infection, three classes of viral RNA are expressed: full-length unspliced 9 kb RNAs, partially spliced 4 kb RNAs and fully spliced 2 kb RNAs (Pollard and Malim, 1998). In the absence of Rev function, only the 2 kb RNAs, one of which encodes Rev, are exported to the cytoplasm. The 9 kb and 4 kb RNAs are degraded (Pollard and Malim, 1998). However, when cellular concentrations reach a certain threshold, Rev promotes the export of the under-spliced RNAs (Figure 1) (Pollard and Malim, 1998; Hope, 1999). The ability to export intron-containing RNA requires four separate activities mediated by Rev: nuclear import, RNA

Figure 1. Rev function early and late infection. Early during infection, three classes of viral RNAs accumulate in the nucleus: full length unspliced 9 kb RNAs, partially spliced 4 kb RNAs and fully spliced 2 kb RNAs. In the absence of Rev, only 2 kb RNAs, one of which encodes Rev, are exported to the cytoplasm. The 9 kb and 4 kb RNAs are degraded. However, when cellular concentrations reach a threshold, Rev binds the Rev Response Element (purple box) in the 3' intron, multimerizes and promotes nuclear export (Adapted from Pollard and Malim, 1998).

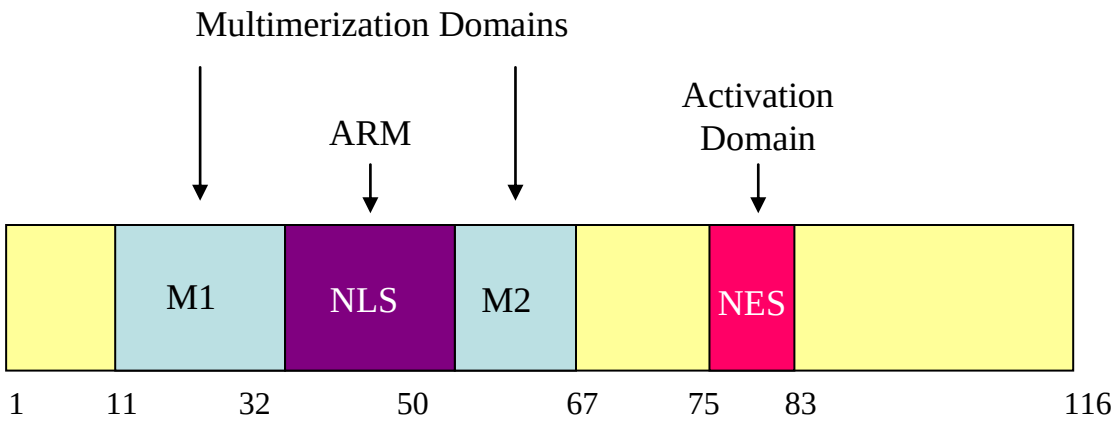


binding, multimerization, and nuclear export (Pollard and Malim, 1998; Hope, 1999). The amino acids important for each activity is known and summarized in Figure 2.

The competence for nuclear import is conferred by a nuclear localization sequence (NLS) located in an arginine rich motif (amino acids 34-50) (Hope, 1999). Whereas many nuclear localization signals are recognized by importin α transport receptor which then recruits importin β, Rev is targeted to nuclear pore complexes by directly binding importin β (Truant and Cullen, 1999). The importin β binding sites of Rev and importin α overlap and therefore compete with each other (Truant and Cullen, 1999; Hope, 1999). Rev is released into the nucleoplasm from nuclear pore complexes upon binding Ran loaded with GTP. Ran-GTP inhibits the simultaneous binding of importin β and Rev's NLS (Truant and Cullen, 1999). One mutation, M6 (RRRR→DL, amino acids 41-44), inhibits the ability of importin β to bind and inhibits RRE binding (Truant and Cullen, 1999).

The arginine rich region is also responsible for binding viral RNA. Rev binds these RNAs at the Rev Response Element (RRE) rich with stems and loops (Pollard and Malim, 1998; Hope, 1999). The RRE is approximately 300-350 nucleotides long and is located in the 3' intron (Pollard and Malim, 1998; Hope, 1999). Using NMR spectroscopy, Battiste *et al.* (1996) found the arginine rich α helix of Rev binds the major groove of the RRE (Battiste *et al.*, 1996). Curiously, the NLS and the RRE binding motif overlap one another which apparently prevents re-import of Rev bound to the RRE.

Figure 2. Rev domain Structure. The relative locations of the two multimerization domains (M1 and M2), the arginine-rich motif (ARM) important for RRE binding and its nuclear localization signal (NLS) and the nuclear export signal (NES) are depicted relative to amino acid location 1-116 (modified from Pollard and Malim, 1998; Hope, 1999; and Watts *et al.*, 2000).



The RRE has one high affinity binding site for Rev but the binding of one Rev monomer is insufficient to stimulate RRE export (Pollard and Malim, 1998). Once one Rev monomer binds, multiple Rev molecules bind with partial cooperativity (Pollard and Malim, 1998). Malim *et al.* (1989) identified two domains important for Rev multimerization by mutagenesis (amino acids 11-32 and 52-67). Many mutations including M4 (YSN→DDL, amino acids 23, 25, and 26, respectively) could bind the RRE but none multimerized on the RRE nor were able to trans-activate HIV gene expression and produce progeny virions (Brice *et al.*, 1999; Trikha and Brighty, 2005). M4 inhibits the formation of high molecular weight complexes on the RRE (Malim and Cullen, 1991; Auer *et al.*, 1994; Thomas *et al.*, 1997). The M4 mutation is recessive and is unable to inhibit wild type Rev activity.

A third mutation denoted M10 (LE→DL, amino acids 78 and 79) is a trans-dominant inhibitor of HIV infection (Malim *et al.*, 1989). The M10 mutation inactivates a nuclear export signal (NES) located at positions 75-83 towards the C-terminus (Pollard and Malim, 1998; Suhasini and Reddy, 2009). The NES is leucine rich and interacts with CRM-1, also called exportin 1 (Hope, 1999; Suhasini and Reddy, 2009). CRM-1 is a member of the importin β family of transport factors and accumulates in the nucleoli of cells when Rev is present (Hope, 1999; Cao *et al.*, 2009). In the presence of Ran-GTP, CRM-1 binds to the NES of Rev laden with RRE-containing messages and targets this transport complex to nuclear pore complexes triggering export (Fornerad *et al.*, 1997; Hope, 1999; Suhasini and Reddy, 2009; Cao *et al.*, 2009). Rev with its bound RNA is released into the cytoplasm following the hydrolysis of GTP. Multiple Rev monomers

bound to the RRE are required for export such that Rev multimerization is required for the trans-dominant effects (Malim *et al.,* 1989).

Given the importance of Rev to HIV infection, many labs have attempted to determine the three-dimensional structure of Rev. Auer *et al.* (1994) used circular dichroism (CD) spectroscopy of deletion mutants and found α helices at amino acids 8-26 and 35-50. Both of these regions contain residues important for multimerization and many are hydrophobic. These regions are separated from each other by a short loop rich in proline, an amino acid known to disrupt α-helices. Thomas *et al.* (1997) used functional assays to measure the functionality of point mutations to develop a model for Rev structure. Their data suggested that the two helices folded back upon each other to create a hydrophobic patch that was important for multimerization. This model was supported by the work of Blanco *et al.* (2001) who used NMR to detect resonances between amino acids between these α helices within the intact Rev protein. Overall, 50% of Rev assumes an α helical conformation.

Rev/Microtubule Interactions

Early structural studies were confounded by Rev's propensity to form fibrils that associate laterally and form insoluble aggregates (Wingfield *et al.,* 1991; Watts *et al.,* 1998). In an attempt to find solution conditions suitable for crystallography, Watts *et al.* (2000) discovered a unique interaction between Rev and the cytoskeletal protein tubulin. Knowing that Rev filaments could be depolymerized by polyanions such as polyglutamate (Wingfield *et al.,* 1991; Watts *et al.,* 1998), they reasoned that the polyglutamate tails of α and β tubulin would interact with the arginine rich region of Rev and promote filament disassembly. However, upon mixing tubulin with Rev, an

entirely new complex was formed: Rev-tubulin toroids (RTTs) (Watts *et al.*, 2000). RTTs are large 3.8 MDa, bilayered rings with tubulin exposed circumferentially and Rev lining the inner ring. RTTs result from specific interactions between Rev and tubulin. Their formation requires Mg^{2+} and is not inhibited by moderate NaCl concentrations, changes in pH, or the microtubule drugs Taxol or colchicine. RTTs do not form however when the anti-mitotic drug maytansine is added indicating Rev must bind at or near the vinca site. Surprisingly, RTT formation does not require tubulin's polyglutamic acid tails.

Based on a limited sequence similarity shared with kinesin-13 proteins, (Figure 3) Watts *et al.* (2000) posited that Rev binds and depolymerizes microtubules by a mechanism shared with these proteins. The vast majority of the proteins in the kinesin superfamily are microtubule motor proteins (Lawrence *et al.*, 2004; Wozniak *et al.*, 2004). However, kinesin-13 proteins are distinguished by their lack of microtubule motility and their potent ability to depolymerize microtubules. Kinesin 13 family members include orthologs of MCAK (Mitotic Centromeric-Associated Kinesin) (Desai *et al.*, 1999; Maney *et al.*, 2001; Hertzer *et al.*, 2003). MCAK abundantly localizes to mitotic spindles but also accumulates to kinetochores via an *N*-terminal targeting sequence (Walczak *et al.*, 1996; Desai *et al.*, 1999; and Maney *et al.*, 2001). Walczak *et al.* (1996) found that MCAK destabilizes microtubules and causes depolymerization of the spindle during mitosis. Desai *et al.* (1999) confirmed that MCAK depolymerizes microtubules and suggested these kinesins bind microtubule ends and cause a conformational change leading to catastrophes. Catastrophes are the rapid transitions from microtubule growth to shrinkage (Desai *et al.*, 1999; Tournebize *et al.*, 2000). MCAK was also found to

depolymerize microtubules. Maney *et al.* (2001) found that overexpression of MCAK *in vivo* resulted in fewer and shorter mitotic spindle microtubules in mitosis.

Like MCAK, Rev triggers microtubule depolymerization from both ends (Watts *et al.*, 2000; Maney *et al.*, 2001). Part of the Rev sequence, in the second α helix, amino acids 34-70 were compared to MCAK sequence 506-543, in the loop 11-α4-loop12 region, and some of the amino acids were identical while still others were similar (Watts *et al.*, 2000). This is the region important for microtubule binding in motile kinesins (Woelke *et al.*, 1997). However, residues present in MCAK and absent from motile kinesins are thought to be important for microtubule depolymerization. Intriguingly, Rev possesses many amino acids that are present in MCAK and absent in motile kinesins (Watts *et al.*, 2000, Figure 3).

Cellular Effects of Rev Expression

Because the experimental and essentially artificial conditions in which Rev-tubulin interactions were discovered, it became important to determine whether Rev could bind tubulin in living cells. Immunoprecipitation experiments that can detect physical interactions between proteins provide supportive evidence. Rev is precipitated from cell extracts prepared from HeLa cells transiently or stably expressing Rev using tubulin-specific antisera (Middaugh, 2005; Kotha, 2010). Tubulin is reciprocally precipitated using Rev-specific antibodies (Kotha, 2010). Co-immunoprecipitations are not affected by mutations in the NLS, NES and multimerization motifs (M6, M10, and M4 respectively) (Kotha, 2010).

Figure 3. Sequence similarities between Rev and MCAK, a Kinesin 13 family member, and KIF1A (a motile kinesin). Identical amino acids are colored in red and conservative substitutions are in green. Adapted from Ogawa *et al.*, 2004 and Watts *et al.*, 2000.

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Rev    34  TRQARRNRRRRWRERQRQIHSISERILSTYLGRSAEP
MCAK  506 TASADRITRMEGAEINRSLLALKECIRALGQNKSHTP
KIF1A 260    AKGTRLKEGANINKSLTTLGKVISALAEMDSGPN
```

Substantial evidence suggests cellular expression of Rev can interfere with microtubule behavior. Rev inhibits the formation of mitotic asters formed *in vitro* from *Xenopus* egg extracts that faithfully recapitulate microtubule dynamics seen during cell cycle progression (Watts *et al.*, 2000). This shows that Rev can perturb microtubule function in cellular environments. Transient over-expression of Rev in Cos7 cells also leads to an accumulation of cells in G2/M (Miyazaki *et al.*, 1995). Many of these cells had an abnormal DNA content and mitotic abnormalities were common. HeLa cells transiently or stably expressing Rev similarly exhibit slower doubling times in culture, have altered mitotic indices and there is an accumulation of mitotic cells before the spindle assembly checkpoint (N. Smith, personal communication; Lore, 2005). These same defects are also seen when Rev mutants M4, M6 and M10 are expressed. Since purified M6 and M4 can bind tubulin heterodimers *in vitro* (Sharma, 2009) but are unable to depolymerize microtubules, the cell cycle defects resulting from Rev expression are hypothesized to be due to Rev sequestering tubulin heterodimers.

Taken together, these data suggest that Rev expression may activate the spindle assembly checkpoint, a biochemical pathway that monitors the movement and ultimate positioning of chromosomes at the metaphase plate. The checkpoint is often activated when chromosomes are misaligned and progression into anaphase is prevented. Proteins such as Bub and BubR1 are localized to the kinetochores where the spindle assembly checkpoint is located. Bub and BubR1 bind to other proteins in the kinetochore to inhibit the anaphase-promoting complex (APC) which halts the progression of mitosis. If there is a weakened checkpoint and misalignment still occurs, apoptosis can ensue (Musacchio

and Hardwick, 2002; Weaver and Cleveland, 2005; Tanaka and Hirota, 2009). Thus, it is formally possible that Rev expression may activate apoptosis.

The exact pathway from the spindle assembly checkpoint leading to apoptosis is unclear (Weaver and Cleveland, 2005). However, when the spindle assembly checkpoint is activated, mitotic progression is stopped and the cell will either correct the defect or die. There are three possible outcomes that lead to apoptosis when mitotic cells are treated with antimitotic drugs (Weaver and Cleveland, 2005). The first is that the cell can exit to G1 from mitosis without dividing and can go into senescence or become apoptotic. The second is that with a weakened mitotic checkpoint the cell can exit from mitosis into G1 and is apoptotic. Third, without the cell exiting into G1 and staying in mitosis, the cell becomes apoptotic (Weaver and Cleveland, 2005).

Since antimitotic drugs such as taxol and paclitaxel activate the spindle assembly checkpoint by interfering with microtubules and because Rev also has the ability to interfere with microtubles and disrupt mitosis, Rev may activate the spindle assembly checkpoint. If the checkpoint is activated, it is possible the cell becomes apoptotic by one of these three possible ways.

There are other explanations for the cell cycle defects that result from Rev expression. Since the work of Miyazaki *et al.* (1995) predated the discovery of Rev-tubulin interactions, these authors suggested that Rev was exerting its cell cycle effects through its interactions with ribosomal protein B23. B23 is a member of the nucleoplasmin protein family (Okuwaki, 2007) and is involved in ribosome biogenesis possibly acting either as a chaperone (Okuwaki *et al.*, 2001) or facilitating ribosome transport (Borer *et al.*, 1989). Because Rev is able to bind B23 through its arginine rich

motif (Fankhauser *et al.,* 1991) and because Rev over-expression deforms nucleoli (Nosaka *et al.,* 1993), Miyazaki *et al.* speculated that the cell cycle defects they observed following Rev expression was due to an inhibition of ribosome synthesis.

More recently, B23 has been shown to be involved in centrosome duplication (Okuwaki, 2007). Centrosomes consist of two centrioles and promote microtubule nucleation during interphase of the cell cycle. During G1, single centrosomes duplicate under the control of B23. B23 acts as a licensing factor that allows duplication. However, when it is phosphorylated by the cyclin-dependent protein kinase, CDK2/cyclin E, B23 dissociates and duplication ensues (Shinmura *et al.,* 2005). Duplicated centrosomes remain associated with each other until the onset of mitosis where they separate and nucleate microtubule spindles. B23 has also been shown to inhibit apoptosis in cells and prevent DNA fragmentation (Han *et al.*, 2008; Choi *et al.,* 2008). When B23 levels are knocked down by RNA_i, synthesis of ribosomal DNA is inhibited and this leads to cell death (Ahn *et al.,* 2005). If the ribosomal defects lead to cell cycle defects and Rev is forming a complex with B23 (Fankhauser *et al.,* 1991), by Rev inhibiting B23's function of centrosomal duplication and causing cell cycle defects, this could ultimately lead to apoptosis. Thus, Rev expression may stimulate mitotic defects by interfering with centrosome duplication, inhibiting ribosome synthesis, or by altering microtubule dynamics.

Apoptosis

Apoptosis is a type of cell death characterized by cell shrinkage, cell rounding, chromatin condensation and fragmentation (Kerr *et al.,* 1993; Lawen, 2003). In particular, the first overt sign of apoptosis is when the cells start getting smaller and

rounder and start to lose contact with other cells. Chromatin then condenses fragments due to endonucleolytic activity (Kerr *et al*,. 1993; Lawen, 2003). The plasma membrane then becomes convoluted and swells and the cell dies (Lawen, 2003).

Apoptosis can be triggered extrinsically and intrinsically (Okada and Mak, 2004). The extrinsic pathway occurs when receptors on the cell membrane are activated by extracellular ligands such as TNF and FAS which initiate signals to the cell to undergo apoptosis (Lawen, 2003; Okada and Mak, 2004; Riedl and Shi, 2004). When TNF and FAS bind to specific receptors on the cell formation of a Death Inducing Signaling Complex or DISC ensues (Grütter, 2000; Lawen, 2003; Riedl and Shi, 2004). DISC leads to activation of the initiator caspases 8 or 10. Caspases are proteases specific to apoptosis. Once the initiator caspases are activated, they activate the effector caspases 3, 7, and/or 6 by a conformational change of the active site produced by proteolytic cleavage (Grütter, 2000; Lawen, 2003; Riedl and Shi, 2004). Activated effector caspases stimulate protein release from the mitochondria. These proteins are degraded and the chromatin starts to fragment which ultimately leads to apoptosis (Grütter, 2000; Riedl and Shi, 2004).

The intrinsic pathway occurs when extracellular and intracellular stresses such as hypoxia and DNA damage trigger cytochrome c release from mitochondria (Lawen, 2003; Okada and Mak, 2004). The intrinsic pathway is also called the mitochondrial pathway (Lawen, 2003). The outer mitochondrial membrane becomes permeabilized and molecules like cytochrome c are released and bind to the Apoptotic Protease Activating Factor-1 (Apaf-1) (Green and Kroemer, 2004). Apaf-1 stimulates formation of the apoptosome. The apoptosome is a complex made of multiple monomers of cytochrome c,

and Apaf-1 and requires ATP (Riedl and Shi, 2004). The apoptosome recruits the initiator caspase Procaspase 9 to the complex and becomes activated (Okada and Mak, 2004). Activated caspase 9 activates effector caspases such as caspase 3 by cleaving a peptide from the N-terminal region of the caspase. In turn, activated caspase 3 cleaves a variety of substrates including proteins involved in cytoskeleton, nucleus, and DNA repair which accounts for the DNA fragmentation and cell swelling seen during the terminal stages of death (Grütter, 2000; Lawen, 2003; Riedl and Shi, 2004).

Specific Aims

Because of Rev's ability to depolymerize microtubules, alter microtubule expression and create mitotic defects in cells, it is possible Rev may activate the spindle checkpoint and promote apoptosis. It is possible that Rev inhibiting B23 function alters ribosome synthesis and centromere duplication. **To test the hypothesis that Rev expression induces apoptosis, I will search for signs of apoptosis in Rev-expressing cells. Specifically, I will look for DNA fragmentation by visual inspection, measure the presence of mono- and oligonucleosomes using an ELISA-based assay, and use western analyses to detect activated caspases.**

Materials and Methods

Plasmids:

Overnight cultures were grown using LB broth, kanomycin (10 mg/mL), and a plasmid of choice (pYFP, pRevEYFP, pRevEGFP, pRevM4EYFP, pRevM6EYFP, pRevM10EYFP). Plasmids were perpetuated in DH5α cells and purified using Qiagen maxiprep kits per manufacturer's recommendations.

Cell culture:

HeLa cells were grown in 100 mm plastic dishes with DMEM, 10% fetal bovine serum at 37°C with 5% CO_2 in a humidified incubator. Cells were split when cultures were 80-90% confluent using trypsin:EDTA and typically reseeded at 1:5 dilutions. A stable cell line, Rev GFP, was also used and grown according to the same conditions.

Transfection:

Polyfect transfection reagent (Qiagen, Valencia, CA) was used to transiently transfect HeLa cells according to manufacturer's recommendations. Briefly, HeLa cells were split and reseeded at 500,000 cells/ml. The following day, 3 or 6µg of plasmid DNA were mixed with 20,100µl Polyfect transfection reagent and incubated at room temperature for 10 min. After 10 min, 1 ml of DMEM was added to the mixture and added then applied to Hela cells present in 7 ml of serum-free DMEM. After 24-48 hrs of incubation, the transfection media was replaced with DMEM-10% FBS.

Detection of apoptosis:

Elisa Method:

The Cell Death detection ELISA^{PLUS} kit (Roche, Indianapolis, IN) was used to detect apoptotic cells. Adherent HeLa cells were treated with trypsin:EDTA and

collected by 200 × g centrifugation for 10 min. Cell pellets were resuspended in a proprietary lysis buffer, incubated at room temperature for 30 min, and subjected to 200 × g centrifugation for 10 min. Aliquots of the supernatant were put into the streptavidin-coated microtiter plate. Biotin-conjugated antibodies specific for histones and nucleosomal DNA were then added and incubated at room temperature for 2 hours. Wells were rinsed with a proprietary incubation buffer and the amount of bound histones were detected using a 10 min incubation using the ABTS solution (used for color development). The reaction was stopped using ABTS stop solution. Optical densities of each well were measured at 405 nm and 490 nm.

Immunofluoresence method:

HeLa cells were seeded at 500,000 cells/ml on coverslips placed in 100 mm dishes and transfected with plasmid DNA the following day as described above. Two days after transfection, cells were fixed with 4% formaldehyde in 1x PBS for 20 min, rinsed with TBSTx (TBS: 20 mM Tris, 150 mM NaCl, pH 7.5; 0.1% Triton-X100) and treated with Abdil (2% donkey serum, 0.1% NaN_3, TBSTx) for 30 min. Coverslips were rinsed with TBSTx and immunostained sequentially using 1:2000 diluted DM1a and 1:500 diluted rhodamine-conjugated goat anti-mouse antibodies. After washes with TBSTx, coverslips were stained with DAPI (1:20,000 concentration) and mounted on glass slides using p-phenylenediamine antifade mounting media.

SDS-PAGE and Immunodetection

Cell lysis:

HeLa cells were washed with PBS and scraped off the plates 48 hours after transfection using a rubber policeman. Cells were collected by 5,000 × g centrifugation

for 5 min. Pellets were washed twice with PBS and cells were collected by centrifugation at 13,000 rpm for 5 min. Cells were lysed in 300 mM NaCl, 100 mM Tris, 0.2 mM EDTA, 1% NP40, 10% glycerol, protease inhibitors (80 µM aprotinin, 100 mM AEBSF, 5 mM Bestatin, 1.5 mM E-64, 2 mM leupeptin, 1 mM pepstatin A) by end-over end mixing for 45 min. Samples were spun at 2,000 × g for 10 min; the supernatant was collected and frozen at -80°C.

SDS-PAGE:

Samples were prepared by mixing sample and 2x sample buffer and boiling for 5 min. Samples were loaded in a 15% polyacrylamide gel and run for 1 hour 5 min at 35 mA.

Immunodetection:

Gels were transferred to nitrocellulose membranes for 2 hours at 150mA. Blots were then treated with blocking buffer (5% donkey serum in 1x TBST) for 40 min, washed with 1x TBST 3 times, 5 min each, and incubated with antibodies specific for Rev, α tubulin, or caspase 3. Rev was detected using sequential incubations of 1:2000 diluted sheep ant-Rev sera obtained from US Biologicals (Swampscott, MA) and 1:2000 HRP-conjugated donkey-anti-sheep IgG antibodies. Tubulin was detected using 1:2000 diluted DM1a and detected with 1:100,000 diluted HRP-conjugated donkey-anti-mouse IgG antibodies. Caspase 3 was detected using 1:1000 diluted rabbit antibodies gifted by Dr. T. Brown followed by incubation with 1:20,000 diluted donkey-anti-rabbit IgG antibodies. All secondary antibodies were obtained from Jackson Immunolabs (Westgrove, PA) and blots were incubated in antibody containing solutions for 40 min.

Blots were washed with 1x TBST and developed using Pico detect kit (Pierce, Rockford, IL).

Results

Apoptosis can be measured by a variety of assays each subjected to different biases and artifacts. Therefore, any attempt to determine whether Rev expression induces apoptosis, multiple assays should be used. Three assays were selected for this study. The first assay involved visual inspection looking for nuclear fragmentation, the canonical sign of apoptotic death. Parental HeLa cells and HeLa cells stably expressing Rev fused to GFP were fixed two days after passage, stained with DAPI and the fraction of cells with fragmented nuclei were scored. Stable cell lines were used because they are easier to work with and the levels of Rev expression are identical from cell to cell. The average fraction of apoptotic control cells was 0.34% whereas the average of Rev-expressing apoptotic cells was 0.40% (Table 1a). A one tailed paired T-test confirms that there is no statistical difference between the parental HeLa cells and cells stably expressing Rev-GFP, $p=0.25$. This data is inconsistent with the hypothesis that Rev induces apoptosis.

Since the creation of stable cell lines selects for viable cells that compensate for lethal effects due to Rev expression, cells transiently expressing Rev were also examined. HeLa cells were transfected with equal amounts of either a control YFP plasmid or Rev-GFP for 48 hours before fixation and visualization. Wild-type Rev is normally found in the nucleus of cells, specifically the nucleoli whereas YFP is dispersed throughout the entire cell (Figure 4a). Under these conditions, Rev expression leads to an approximately two-fold increase in apoptosis relative to YFP controls (means equal to 1.35% and 0.61% respectively, $n=2$, Table 1a). Examples of apoptotic cells can be seen in Figure 4b. A one tailed paired T-test shows a statistically significant difference ($p=0.011$) between cells expressing YFP and Rev-GFP. These data are consistent with the hypothesis that

Table 1. Frequency of Apoptosis in Rev expressing cells: the visual assay. A. Average frequencies of apoptotic cells (± standard deviations) of 2 trials comparing HeLa control cells with HeLa cells transiently (t) expressing wild-type Rev as scored by the visual assay.

B. Average frequencies of apoptotic cells (± standard deviations) of 3 trials comparing HeLa control cells with HeLa cells stably (s) or transiently (t) expressing wild-type Rev or Rev mutants as scored by the visual assay.

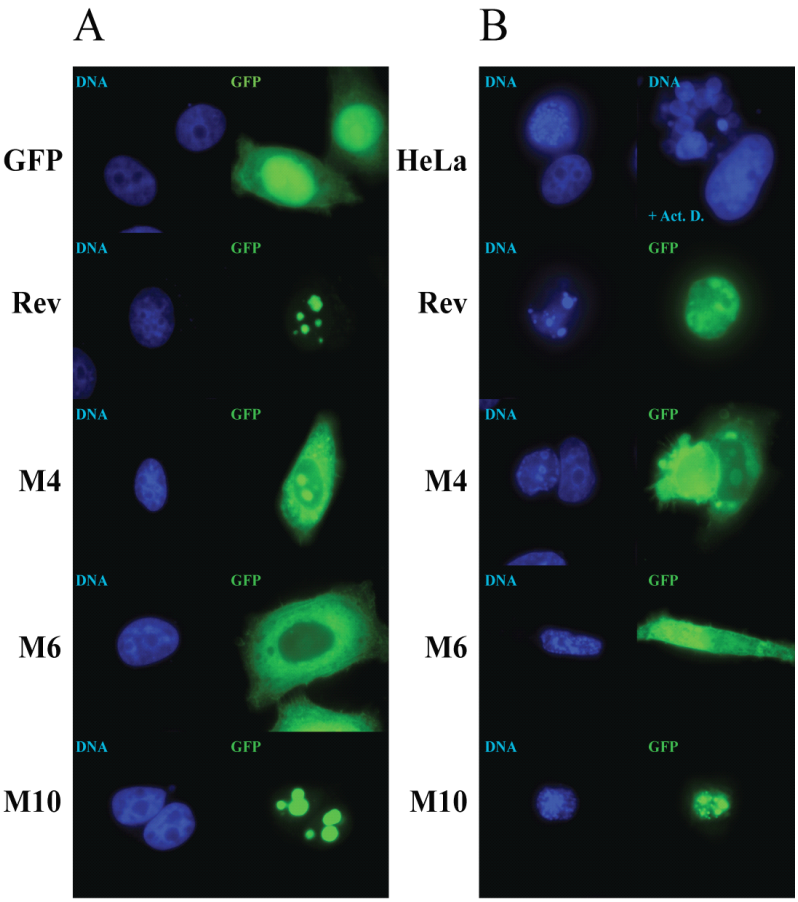
A.

Rev (t)	YFP (t)
1.35% ± 0.07	0.61% ± 0.03

B.

HeLa	Rev (s)	Rev (t)	M4 (t)	M6 (t)	M10 (t)
0.34% ± 0.12	0.40% ± 0.11	3.3% ± 3.2	1.34% ± 0.61	1.38% ± 0.36	0.99% ± 0.15

Figure 4. Subcellular localization of Rev and mutants and apoptotic cells. A. Normal HeLa cells showing wild-type Rev in the nucleoli and YFP in the cytoplasm. M10 is also localized to the nucleoli while M6 is localized to the cytoplasm and M4 is found in both the nucleoli and cytoplasm. B. Examples of apoptotic HeLa cells and HeLa cells expressing wild-type Rev, M4, M6, and M10.



Rev stimulates apoptosis. This increase was not due to an increase in the amount of fluorescent protein that was expressed. As seen in Figure 5, the levels of transgene expression are similar. Similarly transfection frequencies of both plasmids were similar, with YFP having an average of 35.5% and Rev-GFP having an average of 26.0% frequency (Table 2 a,b). A two tailed paired T-test reveals no statistical difference when comparing the transfection rates of YFP and Rev-GFP, $p=0.17$.

The effects of M4, M6, and M10 expression were also examined for their ability to stimulate apoptosis owing to the observations that they also affect cell cycle expression (N. Smith, personal communication). M10 is found in the nucleoli of cells while M6 is found mainly in the cytoplasm and M4 is in both the nucleoli and the cytoplasm (Figure 4a). DNA from each construct as well as DNA from YFP were transfected and 48 hours later were scored for visual signs of apoptosis. Examples of apoptotic cells can be seen in Figure 4b. When using the visual assay to compare the effects of Rev-GFP expression with M4-GFP, M6-GFP, and M10-GFP expression, no statistical differences were observed using an ANOVA, $p=0.57$ (Table 1b). These mutants are not statistically different from wild-type Rev and by inference are greater than YFP. These data are consistent with the hypothesis that Rev induces apoptosis.

As seen in Table 1b, the average percent of apoptotic Rev-GFP cells is larger than the mutants M4-GFP, M6-GFP, and M10-GFP. This is because one trial of Rev had a very high number of apoptotic cells. Although there was no reason for removing this trial, if that number was removed, an average of $1.45\% \pm 0.07$ was found for Rev-GFP. This result is comparable with the Rev-GFP and the mutants M4-GFP, M6-GFP, and M10-

GFP. By inference, Rev and the mutants are the same which is greater than YFP. These results are still consistent with the hypothesis that Rev induces apoptosis.

To determine whether these data were biased by differences in transgene expression, transfection frequencies were measured in these experiments and an ANOVA was performed. The transfection frequencies of Rev-GFP, M4-YFP, M6-YFP and M10-YFP are shown in Table 2. ANOVA showed there were differences in transfection frequencies ($p=0.018$) and post hoc testing using Tukey's HSD showed that M10-YFP transfection frequencies were significantly greater than wild-type control ($p<0.01$).

Figure 5. Rev expression in HeLa cells 48 hr after transient transfection. Twenty five micrograms of whole cell extracts prepared from cells transfected with 6 µg of YFP, YFP Rev, M4, M6, and M10 were resolved by SDS-PAGE and blotted onto nitrocellulose. Rev was detected using Rev specific antibody as described in the "Materials and Methods." The positions migrated by molecular weight standards as shown at the left and an arrow points to the Rev-GFP fusion protein.

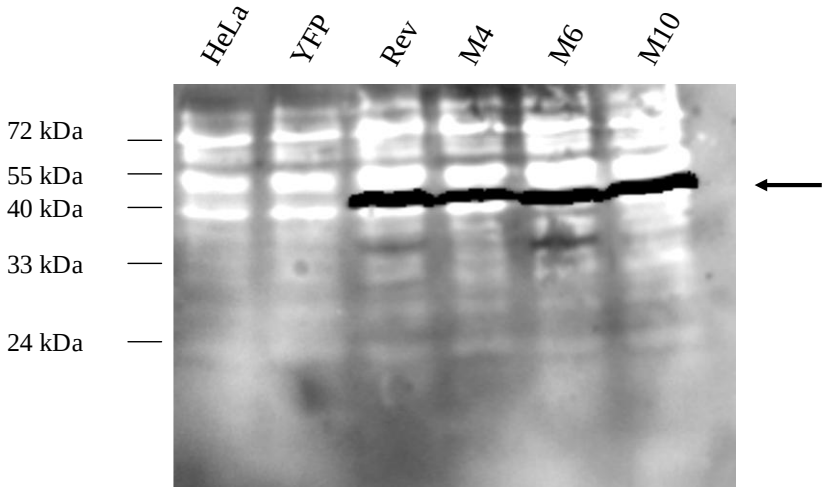


Table 2. Transfection frequencies. A. Averages and standard deviations of the transfection frequencies of wild-type Rev and YFP. B. Averages and standard deviations of the transfection frequencies of wild-type Rev, and Rev mutants M4, M6, and M10.

A.

Rev	YFP
26.0% ± 0.07	35.5% ± 3.9

B.

Rev	M4	M6	M10
23.5% ± 5.1	22.5% ± 6.5	24.9% ± 3.2	36.4% ± 1.8

Transfection frequencies between Rev-GFP, M4-YFP and M6-YFP were not statistically different from each other ($p>0.05$). It is unclear why M10 has a higher transfection frequency as all the plasmid DNAs used in these experiments were isolated in parallel at the same time. Differences may be due to the fact that M10 is slightly less toxic than the other mutants or that the subcellular localization plays a role in this phenomenology. M10 preferentially accumulates in the nucleolus which, because a lot of Rev accumulates in a small localized region, M10 transfectants may be easier to score. Of course, it is formally possible that transfection frequencies are higher because the other transgenes are toxic. Additionally, levels of protein expression were also monitored (Figure 5). Levels of Rev and mutant expression were equal when gels were loaded with the same amount of protein.

In an attempt to validate the sensitivity of this assay, HeLa cells were treated with 0.1 µg/ml of Actinomycin D, a known apoptosis stimulator. When the assay was repeated, the percentage of apoptotic cells was surprisingly low, only 0.69%. The fraction was low, not because Actinomycin D was not inducing cell death but instead, because most apoptotic cells were seen floating off the coverslip before fixation. It is clear that visual inspection is not an overly sensitive assay because a large fraction of apoptotic cells are omitted from the sample. Thus, the true percentage of apoptotic cells induced by Rev expression may actually be higher than what was measured. Therefore another assay was needed.

To this end, a commercially available ELISA based assay capable of detecting apoptosis was used. This assay detects apoptosis by measuring the amount of mono- and oligo-nucleosomes that are released during nuclear fragmentation. After cells were

collected and lysed, a mixture of anti-histone antibody conjugated to biotin and anti-DNA antibody conjugated to peroxidase were incubated with cell extracts. The anti-histone antibody binds to the nucleosomes and its covalently bound biotin promotes attachment of nucleosomes to the streptavidin coated microtiter plate. The anti-DNA antibody will bind to the DNA present in these nucleosomes and the amount of immobilized histones/DNA is proportional to the amount of peroxidase activity.

In this test, transient YFP and Rev-YFP were again compared. YFP expressing control cells had an average net optical density of 0.86 while YFP Rev had an average net optical density of 0.87 (Table 3). An ANOVA was performed and it confirms that YFP and Rev-YFP expression did not have statistically significant different amounts of apoptosis ($p= 0.37$). This data is inconsistent with the hypothesis that Rev induces apoptosis. For comparative purposes, parental HeLa cells and Actinomycin D-treated HeLa cells were used as negative and positive controls, respectively. Parental cells had a low average value of 0.48 and the Actinomycin D treated cells had a high average value of ≈ 2 (Table 3). These data demonstrate that the assay is working as expected.

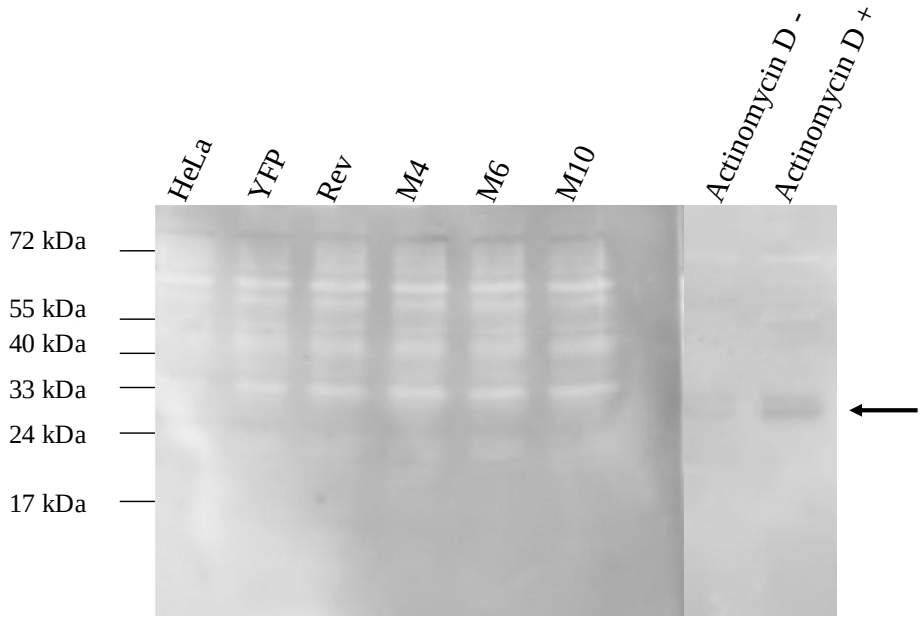
Despite the fact that there was no significant difference between YFP and Rev-YFP, a pilot experiment was done with the mutants (Table 3). Although there were not sufficient replicates to make a definitive conclusion, the results suggest that the mutants M4, M6, and M10 did not stimulate apoptosis.

Since the first two assays provided conflicting results, western blots were performed with whole cell extracts looking for activation of caspase 3 (Figure 6). Caspase 3 is an effector caspase that is activated during apoptosis. If Rev expression stimulates cell death, then caspase 3 should be activated. Activated caspase 3 is readily

Table 3. **Fractions of apoptotic cells: The ELISA assay.** The fraction of apoptotic cells following expression of wild-type Rev, M4, M6, and M10 or treatment with 0.1µg/ml actinomycin D (ActD) compared to untreated HeLa controls. Tabulated values represent average optical densities (±standard deviations) obtained using the ELISA assay. The standard deviations for the mutants M4, M6, and M10 are based on replicates of one trial. The standard deviations for HeLa, ActD, YFP, and Rev are based on averages between trials. Individual standard deviations between each trial were comparable to the mutants.

HeLa	ActD	YFP	Rev	M4	M6	M10
0.37 ±0.54	2.01 ±0.49	0.86 ±0.45	0.87 ±0.32	0.60 ±0.04	0.56 ±0.01	0.39 ±0.03

Figure 6. Caspase 3 is not activated following Rev expression. Whole cell extracts prepared from cells transfected with Rev, Rev mutants, and YFP were resolved by SDS-PAGE, and blotted onto a nitrocellulose membrane. Caspase 3 was detected using specific antibodies. Activated Caspase 3 is present in extacts prepared from HeLa cells treated with actinomycin D (+) but is absent (-) in untreated HeLa cell extracts. (gift of Dr. T. Brown)



distinguished by its molecular size which is smaller than procaspase 3 that has not been proteolytically cleaved. No activated caspase 3 is detected in Rev and YFP expressing cells. As seen in Figure 6, apoptosis is only detected in the positive control (Figure 6, lane 9). Because caspase 3 is only activating the positive control cells, these results suggest that Rev expression is not triggering apoptosis. These results are inconsistent with the hypothesis that Rev induces apoptosis.

Since there was only one assay testing the mutants with Rev-GFP, the caspase 3 assay was used to determine if Rev mutants stimulated apoptosis. As with YFP and YFP Rev, there was no detection of any apoptosis in M4, M6, or M10 lanes 3-7 in Figure 6. This is inconsistent with the hypothesis that Rev induces apoptosis.

Discussion

The goal of this research was to determine whether Rev expression stimulates apoptosis. Three separate assays commonly used to detect apoptosis were used and collectively, the combined results suggest Rev expression that does not stimulate cell death. No evidence of apoptosis was detected in Rev expressing cells using either the ELISA-based assay that detects chromatin fragmentation or the western blot analysis that detects activated caspase 3. Even the visual assay showed that the cell line stably expressing Rev-GFP did not have elevated rates of apoptotic cells than HeLa controls. This latter observation is not surprising in that creating stably expressing cell lines involves selecting for viable cells. The only significant signs of apoptosis were detected following transient expression of Rev and Rev mutants M4, M6, and M10 using the visual assay.

There are several possible explanations for the discrepant results provided by the visual assay. The most likely explanation is that the visual signs of apoptosis were confused for the combined stresses incurred by transfection and/or Rev expression. Certainly the ELISA and western assays that detect specific biochemical markers of apoptosis should not detect necrosis or other forms of cell stresses. Alternatively, the visual assay was clearly less sensitive than the other two assays in that it was poor at detecting apoptosis stimulated by Actinomycin D. Lastly, it is possible that investigator bias interfered with the visual assay. In retrospect, it may have been prudent to conduct these assays in a blinded manner.

Even though no apoptosis believed to result from Rev expression, it does not mean that Rev is not toxic to cells. Indeed, Rev expression alters cell cycle progression

and leads to an accumulation of cells in mitosis prior to the spindle assembly checkpoint (N. Smith, personal communication; Miyazaki *et al.*, 1995). These observations have been explained variously. Rev may be affecting the function of the nucleolar protein B23 that is involved in centrosome duplication and ribosome biogenesis (Miyazaki *et al.*, 1995; Zatsepina *et al.*, 1999). It is well established that B23 function is critical for cell proliferation although there is contradictory evidence whether its depletion leads to apoptosis (Korgaonkar *et al.*, 2005; Okada *et al.*, 2007). Even if B23 acts as a suppressor of apoptosis, the results presented here suggest Rev binding B23 is not sufficient to trigger cell death.

Rev may also affect cell cycle progression by altering the polymerization state of microtubules thereby affecting spindle function, activating the spindle assembly checkpoint which ultimately triggers apoptotic signaling (Watts *et al.*, 2000; Kotha, 2010). In such a model, Rev inhibits microtubule polymerization or promotes microtubule depolymerization such that spindle formation and chromosomal congression that occurs in prophase and prometaphase are slowed. In one model, Rev is hypothesized to inhibit microtubule polymerization by sequestering tubulin heterodimers. If correct, because cellular concentrations of tubulin far exceed Rev concentrations, it is probable that Rev expression will only slow spindle function and not activate apoptotic signaling. In another model, Rev may be inhibiting chromatin-mediated nucleation of microtubules by interfering with Ran activity (Quimby and Dasso, 2003; Kalab and Heald, 2008; Kotha, 2010). If this is correct, spindle assembly should be slowed but not completely blocked because redundant spindle microtubules are populated by those polymerized from centrosomes. In a final model, if Rev is depolymerizing existing microtubules, then

apoptosis may be achieved providing that the extent of depolymerization is high. However, it is not and there are no obvious spindle defects seen in Rev expressing cells (Kotha, 2010).

Since Rev does not induce apoptosis in cells and cells may correct for spindle defects, future experiments should determine whether Rev expression activates the spindle assembly checkpoint. This can be achieved by determining if checkpoint proteins such as Bub1 and BubR1 accumulate on kinetochores more frequently in Rev expressing cells. This can also be quantified by measuring the ability of Rev expressing cells to recover from activation the checkpoint by taxanes. If Rev expression alters microtubule behavior, then Rev expressing cells should recover poorly after taxane removal. Similarly, the nature of the cell cycle defect due to Rev expression should be better characterized. This includes time-lapse videography looking for defects in chromosomal congression and spindle tension. Some research has been done toward this direction, but not enough to form a conclusion.

After this work was completed, Levin *et al.* (2010) found that Rev expression stimulated expression of TNF and caused cell death in non-dividing cells. Dividing cells were viable. Because TNF is involved in both necrosis and apoptosis (REF?), they were unable to determine the nature of death. Necrosis is a type of cell death that is cell destruction by the release of intracellular components. Necrosis occurs due to cellular trauma such as inflammation or infection whereas apoptosis is a type of programmed cell death and can be initiated by internal and external cell factors (Okada and Mak, 2004.) Since apoptosis is not detected here, it is likely the cell death observed in the visual assays may be due to necrotic cell death.

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