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Characterization of HIV-1 Rev Mutants

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Characterization of HIV-1 Rev mutants

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jennifer Lynn Chang ENTITLED Characterization of HIV-1 Rev mutants 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 Rev protein has the ability to bind tubulin heterodimers and depolymerize microtubules (MTs) *in vitro* (Watts et al. 2000). These interactions may account for MT defects observed in HIV infected cells or cells that over-express Rev. Watts et al. hypothesized Rev interacts with MTs by a mechanism shared with Kinesin-13 (Kin13) proteins owing to the presence of a shared amino acid sequence. Kin13 proteins are potent MT depolymerizing agents affecting MT behavior during mitosis.

To test this hypothesis, point mutations were introduced into Rev substituting amino acids shared with Kin13. In this study, eight mutant Rev proteins (T34A, A37D, R39A, R42A, E47A, R50A, E57A, and E47A/E57A) were fused to YFP and over-expressed in HeLa cells. The ability of these cells to grow in culture was measured. Previous results show over-expression of wild-type Rev slows growth in culture and alters cell cycle progression. If these defects are due to Rev-MT interactions, mutation of residues critical for these interactions should mitigate these defects. T34A and R50A have the most restorative effects in cell morphology and growth in culture suggesting the affected amino acids are important for Rev function. R50A is expected to be an important mutation, as this residue is necessary for Kin13 function. Consequently, if the Rev-Kin13 hypothesis were correct, an amino acid substitution in Rev would be necessary for Rev function. T34A is a surprising result suggesting that this shared amino acid might be important for Kin13 function as well and merits investigation. Curiously, over-expression of Rev mutants R39A, R42A, and the double mutant E47A/E57A also alter doubling times and increase the frequency of multinucleated cells. Taken together, Rev over-expression can lead to defects not directly attributable to Rev-MT interactions.

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Table of Contents

List of Figures

List of Tables

Introduction

The human immunodeficiency virus (HIV) causes Acquired Immune Deficiency Syndrome or AIDS (Weiss 1993). HIV infected cells exhibit disruption of mRNA processing, interruptions in cellular differentiation patterns, and morphological changes in cellular structure (Marin et al. 2008, Delezay et al. 1997). Infection also disrupts the cell's microtubule cytoskeleton, which is critical for the organization of cellular organelles, in addition to separation of chromatin during mitosis, and the movement of materials through the cytosol (Malorni et al. 1997, Cenacchi et al. 1996, Macreadie et al. 1995, Karczewski et al. 1996). Watts et al. (2000) suggested these cytoskeletal defects might be caused by the viral protein Rev, owing to the observation that it is a potent microtubule (MT) depolymerizing agent. Because Rev is essential for HIV infection, Rev-MT interactions would make an attractive target for the development of antiviral drugs (Pomerantz et al. 1992, Pollard and Malim 1998, Zhang et al. 2008). Rev also might be able to serve as an antimitotic given its ability to affect MTs *in vitro* (Watts et al. 2000).

Rev and HIV

HIV is a single stranded RNA retrovirus. After virion entry, a double stranded DNA provirus is synthesized and integrates into the host genome (Luciw 1996). Three classes of RNAs are initially transcribed but only the fully processed 2 kb RNAs are exported into the cytoplasm and translated. Rev is one of several proteins encoded by these 2 kb transcripts. Larger 4 and 9 kb transcripts, which encode Gag, Pol, and Env, proteins that are essential for viral infection, are retained and degraded in the nucleus owing to the presence of unspliced introns (Pollard and Malim 1998).

Rev protein is a small (13 kDa), 116 amino acid best recognized for its role in regulating the shift from early-to-late gene expression during infection (Emerman et al. 1989, Hammarskjöld et al. 1989, Pollard and Malim 1998). How Rev achieves this regulation is well understood.

Cytosolic Rev enters the nucleus, binds 4 and 9 kb viral RNAs trapped in the nucleus, and promotes their export into the cytoplasm where they are expressed [Figure 1] (Pollard and Malim 1998).

Although Rev is small enough to diffuse into and out of the nucleus, it possesses separate nuclear localization (NLS) and export (NES) signals. The NLS maps to a highly basic, arginine-rich region located between amino acids 34-50 located at the *N*-terminus [Figure 2] (Pollard and Malim 1998). Nuclear import depends on importin β that docks Rev to nuclear pore complexes and a Ran-GTP gradient, high in the nucleus and low in the cytosol, that transports the protein into the nucleus (Truant and Cullen 1999, Arnaoutov and Dasso, 2005). Unlike many nuclear proteins, importin β binds Rev's NLS without the aid of other transport factors such as importin α (Truant and Cullen 1999). In fact, importin α binding inhibits importin β-Rev binding, indicating that they both compete for the same binding site on Rev (Truant and Cullen 1999). Mutations of arginine residues in the NLS inhibit importin β binding and Rev accumulates in the cytoplasm (Malim et al. 1989).

The NES, consisting of a *C*-terminal leucine-rich region (residues 75-83), provides competence for nuclear export [Figure 2]. The NES recruits Exportin1, whose binding is stabilized by Ran-GTP, forming a transport competent complex (Pollard and Malim 1998, Groom et al. 2009, Suhasini and Reddy 2009). Once exported out into the cytoplasm, GTP hydrolysis stimulated by cytosolic RanGAP disrupts the Rev-RRE Exportin 1-Ran complex, and releases viral mRNA into the cytoplasm (Pollard and Malim 1998, Azuma and Dasso 2000, Dayton 2004).

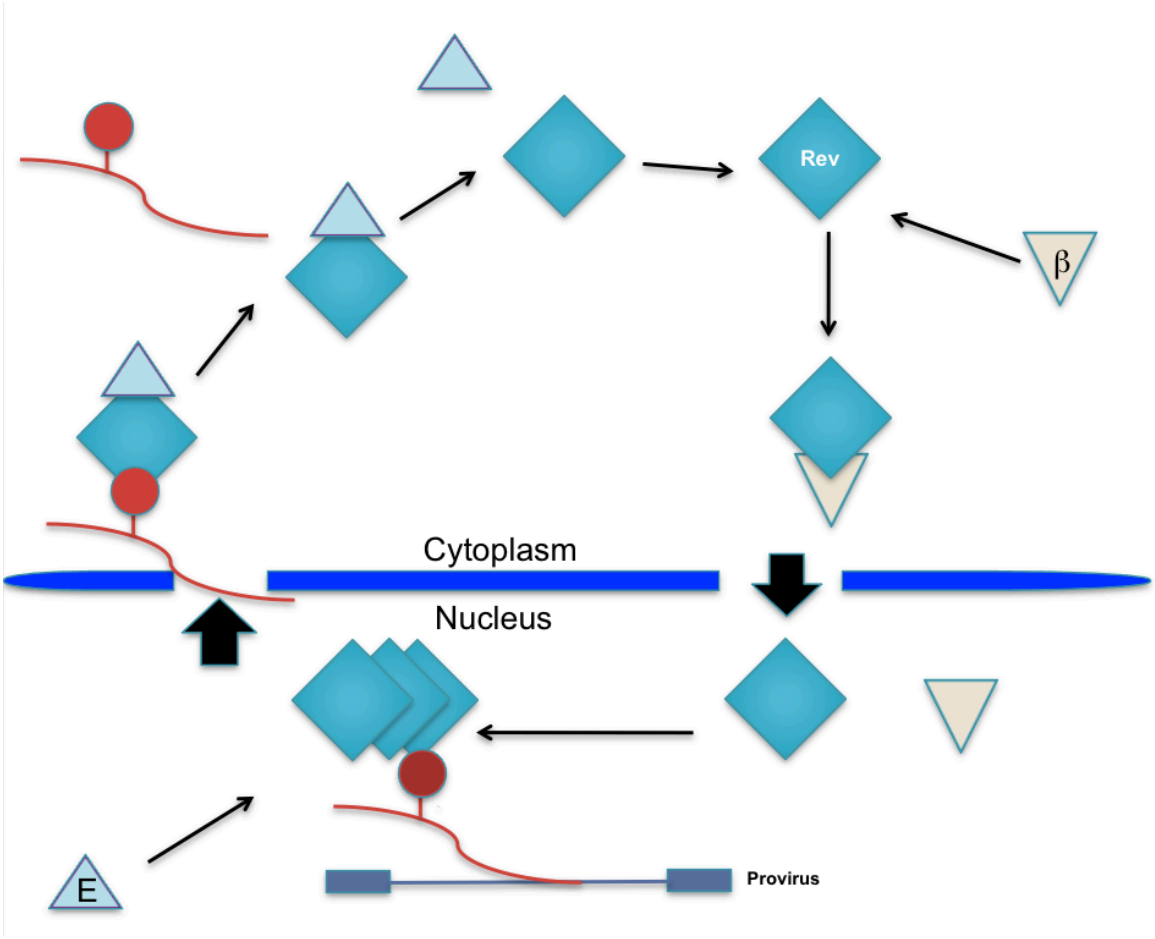
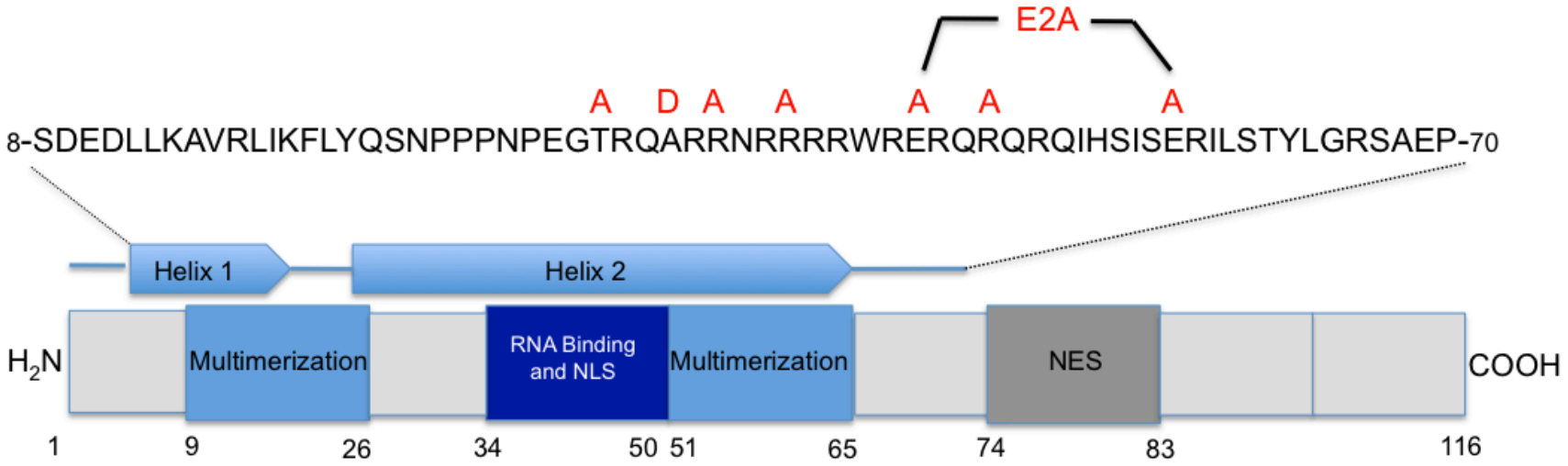


Figure 1: Cellular distribution of Rev during the HIV infection cycle.
Cytosolic Rev (teal diamonds) first binds Importin β (white diamonds) and is imported into the nucleus in a Ran-dependent manner. Upon nuclear entry, Rev and Importin β dissociate and Rev binds the RRE (red lollipop) found in viral mRNA transcribed from the proviral template. Multimerization of Rev monomers occurs, and with the help of Exportin (light blue triangles), the Rev-viral mRNA complex is exported out of the nucleus. The viral mRNA dissociates from Rev-Exportin and is free to bind the cell's translation machinery to make viral proteins. Rev dissociates from Exportin and is thus allowed to continue the cycle.

Figure 2: Rev Domain Structure

Areas for multimerization (lighter blue boxes) straddle the RNA binding/NLS domain, which is shown in a darkest blue box. The NES is shown in the darkest gray box towards the *C*-terminus. The numbers below the boxes show the location of each domain according to amino acid number in the sequence. The schematic above the boxes depicts the secondary structure of the Rev protein. Rev forms two α-helices and the position of these α-helices is shown in their proper position above the boxes. Mutated residues are shown in red above the sequence demonstrating amino acids 8-70 of the Rev protein. Mutant E2A represents a double mutant of E47A/E57A. The primary amino acid sequence is shown above both these cartoons in black. Point mutations are represented above this sequence with red letters demonstrating the amino acid substituted.


E2A
A D A A A A A
8-SDEDLLKAVRLIKFLYQSNPPPNPEGTRQARRNRRRRWRERQRQRQIHSISERILSTYLGRSAEP-70
Helix 1
Helix 2
H2N
Multimerization
RNA Binding and NLS
Multimerization
NES
COOH
1
9
26
34
50
51
65
74
83
116

When in the nucleus, Rev binds a high affinity site called the Rev response element (RRE) located in the 3' intron of the 4 and 9 kb transcripts. The RRE consists of a ≈351 nucleotide sequence that forms a complex series of intramolecular double stranded stems and single stranded loops. The folded RRE possesses a high affinity binding site ($K_d \approx 1$ nM) that allows Rev to distinguish between viral and host cell RNAs (Brice et al 1999, Malim and Cullen 1991). The binding of a single Rev monomer is insufficient to activate its export machinery required for the expression of the 4 and 9 kb transcripts (Daly et al. 1993, Zemmel et al. 1996). The RRE binding promotes Rev multimerization, which stabilizes Rev-RRE complexes and ultimately exposes the NES (Malim et al. 1989, Pollard and Malim 1998).

Multimerization is thought to stabilize Rev-RRE complexes and ultimately expose the nuclear export sequence (Pollard and Malim 1998, Malim and Cullen 1991, Daugherty et al. 2010, Fernandes et al. 2012). Amino acids 9-26 and 51-65 that straddle the arginine rich motif are important for multimerization [Figure 2] (DiMattia et al. 2010). These motifs promote dimerization via hydrophobic interactions and stabilize a helix-loop-helix motif (amino acids 34~60) (Pollard and Malim 1998, DiMattia et al. 2010). Mutation of hydrophobic residues impairs multimerization and reduces Rev affinity for the RRE (DiMattia et al. 2010). Mutations at these residues also reduce the affinity of Rev for RRE binding at stem-loop IIB (DiMattia et al. 2010).

Rev Interactions in vitro and in vivo

In vitro: Rev-MT interactions

In an attempt to solve the structure of Rev, Watts et al. (2000) discovered Rev has the ability to bind tubulin and depolymerize MTs *in vitro*. Specifically, highly purified Rev depolymerizes taxol-stabilized MTs from both ends. The curious end products of depolymerization are ≈3-4 MDa, bilayered rings consisting of both Rev and tubulin. These rings called Rev-tubulin toroids (RTTs) are significant because many anti-mitotic and anti-cancer drugs

that depolymerize MTs also produce curved tubulin structures such as rings, spirals, and bracelets (Watts et al. 2000, Davis and Wordeman 2007, reviewed by Jordan and Kamath 2007). The ability to bend tubulin heterodimers out of the plane of MT polymers is thought to be an important event initiating MT depolymerization (Hertzer et al. 2003, Davis and Wordeman 2007, Hertzer et al 2003, Desai and Mitchison 1997).

Rev is a basic protein (pI≈9.2) with 12 arginine residues that is predicted to interact electrostatically with most negatively charged proteins. Tubulin would be a natural binding partner since it is an acidic protein with a pI ≈ 4.6, in large part due to the highly acidic *C*-termini that function as binding sites for MT associated proteins (Nogales et al. 1999). However, *in vitro* interactions between Rev and MTs are more specific than can be explained by simple electrostatic interactions (Watts et al. 2000). RTTs form despite either the removal of tubulin's acidic *C*-termini or the addition of the MT poisons Taxol and colchicine (Watts et al. 2000). This is surprising given that Rev is a basic protein and would most likely bind tubulin at a negatively charged location. These C-terminal tails, also called "E-hooks", are the primary binding sites for MAPs and other tubulin associated proteins (Nogales 2001). Curiously, Rev can also form RTTs when mixed with unpolymerized tubulin heterodimers suggesting that Rev does not bind tubulin interfaces involved in MT polymerization. The anti-MT drug maytansine, however, blocks RTT formation suggesting that Rev binds MTs at or near the exposed groove formed between tubulin heterodimers on the outer tubule surface (Watts et al. 2000). This observation is supported by *in silico* modeling (D. Sweeney and M.W. Miller unpublished).

One hypothesis for how Rev is able to accomplish MT depolymerization is that it behaves in a manner similar to the Kinesin 13 (Kin13) family of kinesins, a family of kinesin motor proteins. Unlike most kinesins that bind tubulin and translocate along MTs, the Kin13s are potent MT depolymerases critical for the assembly of the mitotic spindle (Ems-McClung et al.

2007, Shipley et al. 2004, Hertzer et al. 2003, Desai et al. 1999). The best studied of these kinesins is the Kin13 MCAK (mitosis centromere-associated kinesin).

MCAK is the prototypical Kin13. It is a MT catastrophe factor and regulates microtubule dynamics *in vivo* (Maney et al. 2001, Rizk et al. 2009, Desai and Mitchison 1997). Over- and under-expression of MCAK produces striking spindle defects in cell extracts (Ems-McClung et al. 2007). For example, depletion of MCAK from *Xenopus* egg extracts suppresses formation of bipolar spindles. The resulting monopolar asters, however, are much larger due to loss of depolymerization activity (Ems-McClung et al. 2007). There are four primary domains that comprise MCAK: the *C*-terminal domain, the neck, the motor region, and the *N*-terminal domain.

Mutational analyses of MCAK have identified the neck region and the motor domain of MCAK are important for MT depolymerization (Ems-McClung et al. 2007). When motile kinesins walk, ATP hydrolysis drives conformational changes in the motor domain. When the motor domain is treated with non-hydrolyzable ATP (AMP-PNP), MCAK still bends and depolymerizes MT protofilaments (Desai et al. 1999, Maney et al. 2001). ATP hydrolysis is required for efficient depolymerization as it is required for recycling MCAK off of the released tubulin heterodimer where it is then free to depolymerize more MTs (Desai et al. 1999, Maney et al. 2001). Thus, it is possible to lock MCAK and bent MT protofilaments into and rings and "bracelets" of curved filaments. Deletion of the neck region showed diminished MT depolymerization but MT binding ability was improved (Ems-McClung et al. 2007). This region consists of ≈60 amino acids rich in arginines and lysines. The positively charged amino acid residues of the neck are conserved throughout the kinesin family (Ogawa et al. 2004). This observation coupled with *in silico* modeling suggests the neck region of the mouse MCAK ortholog Kif2C sterically hinders MT lattice binding allowing the protein to preferentially bind the ends of MTs (Shipley et al. 2004, Mulder et al. 2009).

In vivo Interactions

There are several means by which Rev can perturb MTs and MT function in living cells. Watts et al. (2000) showed Rev also binds free tubulin heterodimers *in vitro*. It could be possible that Rev can sequester tubulin heterodimers in the cytoplasm, thus pushing equilibrium to favor depolymerization. However, it could also be interacting with several other cellular factors such as the nucleolar protein B23, which has been shown to bind Rev, and the Ran/importin β axis of MT nucleation occurring during mitosis (Miyazaki et al. 1996). These interactions could also account for the mitotic defects seen in cells over-expressing Rev.

Patients with advanced AIDS exhibit MT rearrangements with a loss of cell-cell adhesions (Malorni et al. 1997). Whether these defects are due to Rev-MT interactions or caused by other viral proteins is unclear. Since Rev is primarily a nuclear protein that is enriched in the nucleolus, it is more likely that Rev interacts with MTs during cell division after nuclear envelope breakdown and nucleolar dissolution. Significantly, COS7 cells over-expressing Rev exhibit cell cycle defects, most notably an accumulation of cells in G2/M phase and a disproportionate number of cells in prophase (Miyazaki et al. 1996). During prophase, prometaphase, and metaphase, Rev concentrates around the periphery of chromosomes where MTs that comprise the spindle apparatus are known to nucleate owing to the Ran-GTP gradient [Figure 3] (Dundr et al 1996). Consequently, Rev is temporally and spatially well positioned to perturb the mitotic spindle (P. Narayan unpublished, A. Sharma unpublished).

"Rev-B23 interactions"

Another possible explanation of the effects seen in cells over-expressing Rev could be due to one of Rev's binding partners: the B23 protein (Szebeni et al. 1997). The interaction between Rev and B23 was thought to be the cause behind the cell cycle disturbances seen in COS7 cells (Miyazaki et al. 1996). B23 is associated with preribosomal ribonucleoproteins and is thought to be apart of the ribosomal maturation process (Dundr et al. 1997). It also plays a role in

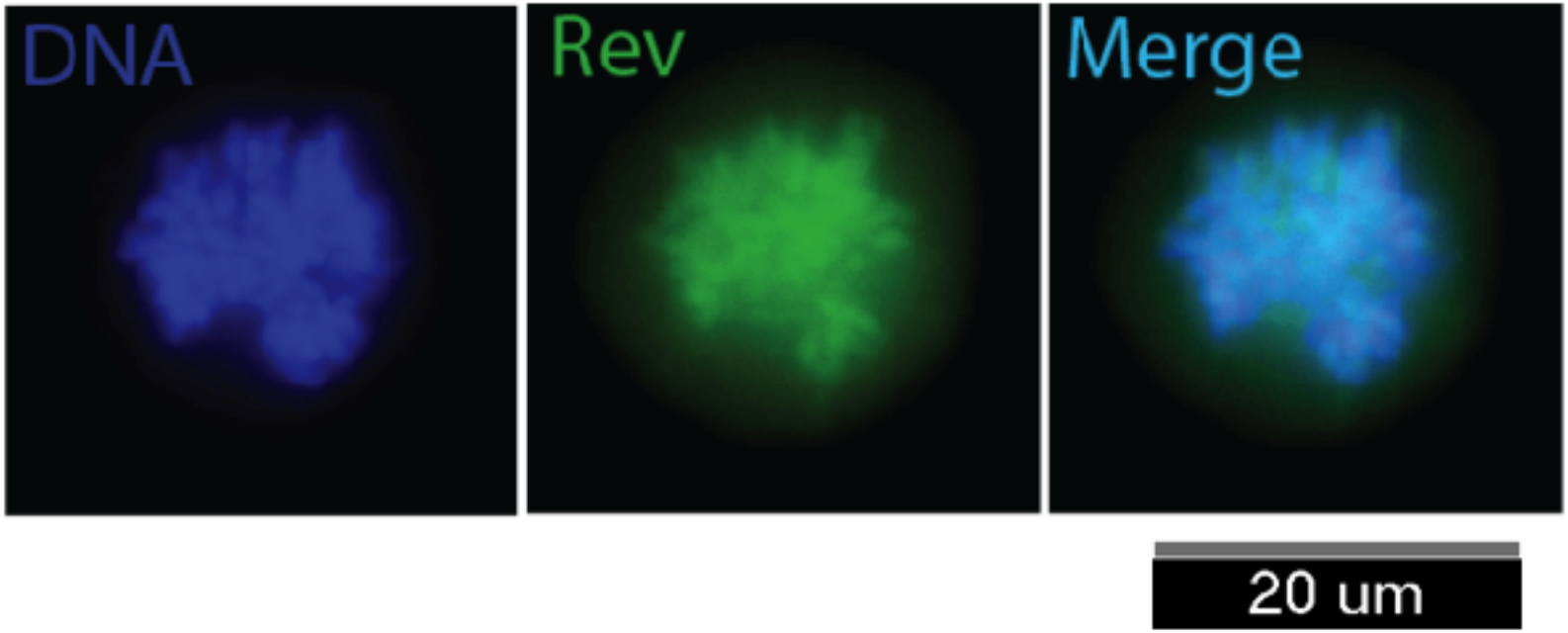
centrosome duplication (Lindstrom 2011). B23 is found primarily in the nucleoli of cells and is found in significantly higher concentrations in the nucleoli of Rev infected cells (Miyazaki et al. 1996). Miyazaki et al. (1996) hypothesized that Rev affected cell cycle dynamics by inhibiting ribosomal transport from the nucleus to the cytoplasm via B23 interactions.

"Rev-Importin β/Ran interactions"

During mitosis is one of the few times Rev can be located in the cytoplasm. During this time, Rev is localized perichromosomally [Figure 3] (Dundr et al 1996). Mitotic chromosomes, in addition to centrosomes, are sites of spindle MT nucleation (Reviewed by Dasso 2002). It has also been suggested that given Rev's location in the cytoplasm during mitosis, it may interact with two proteins: Ran and importin β. Ran is a small GTPase that is located perichromosomally and promotes spindle assembly. It is also responsible for generating a GTP/GDP gradient that aids in nuclear import and export (Kalab et al. 2002). Importin β is a nuclear import receptor that acts to inhibit spindle assembly during mitosis (Kalab et al. 2002). As stated previously, Rev docks to NPCs upon importin β binding (Truant and Cullen 1999, Henderson and Percipalle 1997). Importin β also acts in opposition to spindle assembly. It is thought to act by binding molecules that are needed for MT polymerization and organization (Kalab et al. 2002).

Figure 3: Rev localizes close to mitotic chromosomes.

Blue color represents DAPI staining. Green color represents Rev localization. Merge of the two panels on the left is shown in the far right panel. Weak cytoplasmic localization of Rev can be seen in the panel in the center. Scale bar represents 20 μm.


DNA
Rev
Merge
20 um

As many of these proteins are located around the periphery of chromosomes during mitosis, and since Rev is also found closely located to nuclear entities during mitosis, it could be possible that Rev interacts with these different proteins at this time.

Many MT-stabilizing proteins important for spindle formation are inhibited during interphase by the nuclear transport factors importin α and β. During mitosis after nuclear envelope breakdown, this inhibition is relieved by Ran-GTP, which is locally high near chromosomes owing to RCC1, a chromatin-bound guanine-nucleotide exchange factor (Ohtsubo 1989). Since Rev has the ability to bind importin β and Ran-GTP, Rev may also impact spindle MT dynamics without having direct contact with MTs themselves (Henderson and Percipalle 1997).

Rev Similarities to Kin13s

Rev not only behaves in a similar manner to MCAK, but also shares an amino acid sequence similarity with MCAK/Kin13s in its RNA-binding domain of its second helix in its *N*-terminal domain helix-loop-helix motif [Figures 2, 4]. This motif is located between residues 9 and 65 and comprises the RRE-binding arginine-rich motif (Malim et al. 1989, Malim and Cullen 1993). This area corresponds to the motor/catalytic domain in MCAK, analogous to the neck and KEC helix [Figure 4] (Watts et al. 2000). Rev multimerizes via hydrophobic patches on the surface interface. This region interacts with the corresponding region on other Rev molecules to allow for symmetrical interactions between Rev monomers (DiMattia et al. 2010).

Both MCAK and Rev can depolymerize MTs from its ends and create tubulin ring structures without the use of ATP hydrolysis for tubulin removal (Watts et al. 2000, Desai et al. 1999, Moores et al. 2002, Wagenbach et al. 2008). Both MCAK and Rev can depolymerize MTs treated with MT stabilizers such as GMPCPP and Taxol (Watts et al. 2000, Moores and Milligan

Figure 4: Sequence similarities between Rev and Kin13s.

Primary amino acid sequences of Rev and selected Kin13s are aligned. Identical amino acids are highlighted in red, conservative substitutions in green, and amino acids with similar properties are in blue. The first line represents Rev amino acids 34-70. The middle eight sequences represent various members of the Kin13 family in mice (Mm), nematodes (Cg), *Xenopus* (X), fruitflies (Dm), plasmodia (Pf), and sea snails (Cf). The last two sequences are present in motile kinesins. Key residues shared between Rev and Kin13s were mutated in this study.

2008, Miller unpublished). MCAK normally functions as a homodimer, but it is able to depolymerize MTs as a monomer indicating that multimerization is not necessary for MCAK function (Maney et al. 2001). Thus, there are enough binding sites on one MCAK alone to bind the MT and induce depolymerization. Given the size of Rev, multimerization of this protein may provide an explanation for how Rev is able to perform the same function as a Kin13 monomer (Miller and Sharma unpublished).

Structurally, both possess helices with a shared glutamic acid in their corresponding regions. This helix is critical for Kin13 activity and is believed to bind tubulin at the intradimer groove. Computer modeling predicts the "KEC helix" of Rev can bind tubulin's intradimer groove (D. Sweeney and M. Miller, unpublished). Moreover, the arginine-rich helix in Rev may double as the Kin13 neck as both are helices rich in positively charged amino acids. Finally, both proteins affect cell cycle progression in living cells. Overexpression of Rev alters cell cycle progression specifically during prophase and metaphase, which is consistent with the hypothesis that Rev is altering spindle dynamics (Watts et al. 2000, Miyazaki et al. 1996, Desai et al 1999, Sharma et al. 49th Annual ASCB Meeting Poster). Whereas the defects induced by Rev are not as nearly as potent as Kin13s, it is important to recall Rev is not an enzyme. In addition, MT depolymerization mediated by Kin13s is not dependent upon enzyme activity. The hydrolysis of ATP is required for recycling the enzyme, which increases its efficiency (Friel and Howard 2011).

The most striking similarity is the shared amino acid sequence between the motor region of MCAK, and thus the Kin13s, and the tubulin-binding domain of Rev [Figure 4]. This tubulin-binding domain contains predominantly negatively charged, acidic amino acids located predominantly on the same interface of the α-helix. It is this interface that comes in contact with the tubulin heterodimers of MTs (Watts et al. 2000). The sequence similarity between Rev and

Kin13s occurs in the catalytic/motor domain of the Kin13s, which is thought to be the area responsible for MT depolymerization (Shipley et al 2004, Ogawa et al 2004). In Rev, this region is responsible for RNA binding (Watts et al. 2000). This suggests that this region in Rev might also have the ability to depolymerize MTs because of this shared region. If this is the case, then mutating residues important for function which are shared between the Kin13s and Rev should see decreased depolymerization ability and alleviation of any defects caused by Rev in these experiments.

Critical Amino Acids for Rev/Kin13 Depolymerization

Rev amino acid residues 34-70 are shared by residues 506-543 in XMCAK, a *Xenopus* Kin13. These shared residues are exposed to the surface of the microtubule, suggesting that they may function in microtubule binding. Residues in this region must be exposed on Rev's surface since this region binds importin β and the RRE. It is predicted that this region also binds tubulin as shown by computer modeling (Miller unpublished).

Three amino acids in this region of Rev, T34, R39 and R42 are important amino acids located on this surface. This indicates that they may be available for binding. Residue T34 is conserved both in Rev from different HIV strains and Kin13. This residue also lies very close to the arginine rich region and could be important in Rev activity (Battiste et al 1996). R39 and R42 are located in Rev's arginine rich region and have been shown to inhibit RNA binding and nuclear protein import function in Rev when mutated (Malim et al. 1989, Hammerschmid et al 1994). These residues in the arginine rich region form an α-helix with the arginines along the outer rim of the helix, which is thought to be the area where RRE binding occurs. Residues R39 and R42 are widely conserved in the Kin13 family and are not found in the motile kinesins [Figure 4]. Amino acid A37 in the arginine-rich domain multimerization domain and might be essential for Rev function in HIV infection as well as MT depolymerization.

Glutamic acid amino acids 47 and 57 are two of the few acidic amino acids on an otherwise basic protein (pI = 9.2). These residues are found on the surface of the protein. Structural analyses of Rev suggest that these residues are involved with binding as they are located on the surface and not hidden within the protein in the "hydrophobic patch" (DiMattia et al 2010). E57 is modeled to fit in the edge between Rev and the MT based on sequence similarities in the Kin13s. These residues are conserved in the Kin13 family (Shipley et al 2004, Ogawa et al 2004). In mutation studies with corresponding amino acids in Kin13s, depolymerization was significantly decreased (Shipley et al. 2004). This suggests that as both residues are in fairly close proximity to each other that they may be important for MT destabilization.

Of these Rev mutants, R50A and E57A are expected to be important mutations in these microtubule-binding experiments. Ems-McClung et al. found corresponding Kin13 mutants R522A and E529A exhibited diminished MT depolymerization (Ems-McClung et al 2007). In sperm-induced spindle assembly assays, both mutants also rescued spindle formation; however, the extracts with the R522A mutant demonstrated a disruption in chromosome alignment and increased MT polymerization. The E529A mutant is located in the KEC region and was also found to cause chromosome misalignment and increased the number of MT polymers present (Ems-McClung et al 2007). As these are corresponding residues in the catalytic site on Rev, it is expected that similar results will be seen in spindle assembly assays. These residues chosen are not only important for MT interactions, but they also may be important for interactions with B23 and Ran/importin β.

Specific Aims

In my work, I intend to study these Rev mutants in order to discern which amino acids are critical for Rev activity, disrupting MT assembly, and changes in cell cycle progression. I aim to examine this in the following studies:

1. Using flow cytometry to distinguish mitotic changes that either increase the number of defects, or return to a normal G1/G2 distribution.
2. Performing doubling-time analyses to discern if cells over-expressing Rev and these mutants take a longer or shorter time through mitosis.
3. Investigating through immunofluorescence experiments Rev and mutant Rev interactions with MTs and other cellular entities, such as those named above.

These mutations will demonstrate the degree to which Rev activity is affected in comparison to WT Rev and a YFP-transfected control. Those that show similar characteristics to WT Rev are residues that do not appear to impact Rev activity, whereas those that resemble the YFP control are critical residues. If correct, this means that when mutations are introduced in critical amino acids in this shared sequence between Rev and Kin13s, the defects seen in WT Rev overexpressing cells should be alleviated. These studies will also identify any important interactions between Rev and other cellular proteins active during mitosis. The immunofluorescence experiments will provide further insight into the cellular localization and mitotic effects of Rev.

If Rev is perturbing mitosis due to Rev-MT interactions, it would be expected that the cytoskeletal structure of cells would be vastly different from that of the YFP-Control. MTs would appear shorter than normal and mitosis would be affected due to the absence of dynamic MTs needed in order to correctly segregate sister chromatids. However, as B23 affects

centrosome duplication, and Rev is a known binding partner of B23, Rev could be affecting mitosis through its binding of the B23 protein. Rev could also be producing mitotic defects through its interactions with Ran and importin β. As Ran and GDP/GTP gradients cause MT nucleation around the periphery of chromosomes, and since both Ran and importin β are responsible for spindle assembly during mitosis, Rev could affect these proteins. Therefore, if it does not directly affect MTs, it is possible that Rev affects MTs via other protein interactions such as seen through its binding B23 and/or importin β.

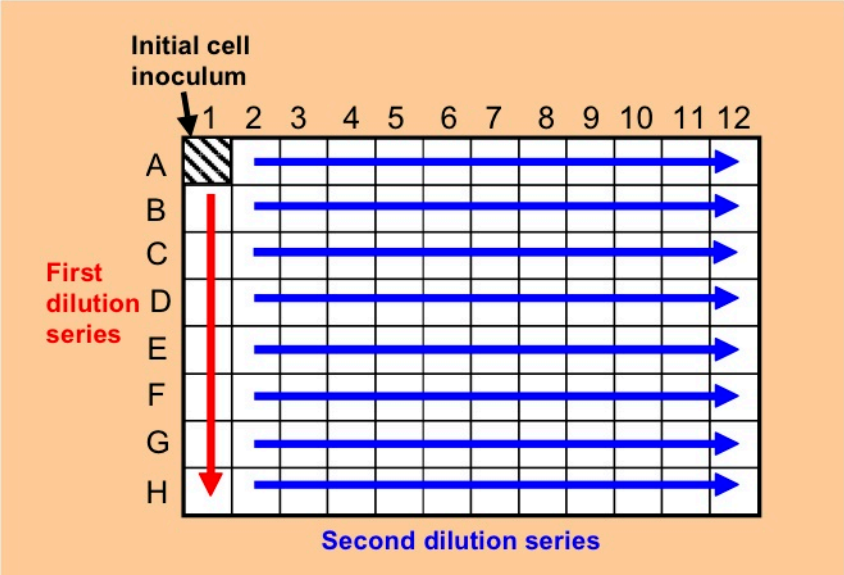
Materials and Methods

Cell Culture:

HeLa cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 µg/mL penicillin in a humidified incubator with 5% CO_2. Cell lines were grown in 100 mm x 20 mm polystyrene cell culture dishes. When confluent, media was aspirated, washed with PBS, and, cells were incubated for two minutes in 1 mL of 0.5 mM EDTA with 2.5% trypsin at 37°C. When cells rounded up, 9 mL of fresh DMEM media was added. Cells were split according to what was needed for experimentation. For regular maintenance, cells were split 1:10.

Cells were transfected with 6 µg of plasmid DNA encoding different Rev mutants subcloned into NheI and KpnI site of pEYFP-N1 (gift of M. Miller) using 100 µL of PolyFect Transfection Reagent (Qiagen, Valencia, CA) according to manufacturer's recommendation. DNA constructs used in this study included pEYFP, Rev-EYFP, RevT34A-EYFP, RevA37D-EYFP, RevR39A-EYFP, RevR42A-EYFP, RevE47A-EYFP, RevR50A-EYFP, RevE57A-EYFP, and RevE2A-EYFP DNA. Cells were either transiently transfected or subjected to selection to create stable cell lines.

Twenty-four hours after transformation, some cultures were subjected to a 1 mg/ml G418 selection to create stable cell lines. After 48 hours, cells were plated in a 96-multiwell plate in G418 media and diluted in a double dilution series to form single colonies expressing the appropriate Rev plasmid. The cells were diluted as such:



(Adapted from Cell Cloning by Serial Dilution in 96 Well Plates, Life Sciences, Lowell, MA)

A single innoculum (two hundred microliters) of cells were placed in well A1 and serially diluted by a factor of two down the first dilution series. Each serial dilution was subsequently diluted eleven times until single-cell colonies were formed (second dilution series). Once single, fluorescing colonies were formed, these cells were replated in 100 mm culture dishes and expanded (Life Sciences, Lowell, MA).

DNA Purification:

Rev-EYFP plasmids were transformed into competent E. coli (DH5α) and allowed to grow in an overnight LB/Amp broth culture (Invitrogen). Bacteria were pelleted at 6000 x G for 15 min at 4°C. A QIAfilter Midi Kit (Qiagen) was used to purify the plasmid DNA per manufacture's instruction.

Immunostaining:

Cells plated on glass coverslips were fixed with 4% paraformaldehyde in PBS solution for 20 min at room temperature. Fixation was followed with three 5 min washes with PBS. The cells were permeabilized with PBS supplemented with 1% Triton-X100 solution for 5 min.

Coverslips were then placed in a blocking buffer solution for 30 min. Coverslips were then incubated with primary antibody diluted in blocking buffer for 1 hr. Coverslips were washed for three times 5 min each in PBS. Texas Red or rhodamine-labeled secondary antibody [see Appendix 1] diluted in blocking buffer was then added and allowed to incubate for one hour on the bench. Coverslips were incubated PBS-1:20,000 DAPI for 5 min and mounted onto glass slides with PPD mounting media.

Slides were imaged using a Nikon Eclipse TE 2000-5 Microscope. Images were collected using a Roper Scientifics Photometrics Cool Snap ES monochrome camera. Images were then analyzed and processed using the Metamorph software program (Metamorph Meta Imaging Series 6.1).

Doubling Time Determination:

One hundred thousand cells from each cell line were plated in six well plates. One well from each cell line was collected in approximately 24 hr intervals by trypsinization. The cells were counted using a Beckman Coulter Vi-Cell automatic cell counter. The number of cells counted each day was plotted as a function of time. A growth curve was fitted by an exponential regression to obtain the equation:

$$\#cells_{t=n} = (\#cell_{t=0})e^{2(\text{growth rate})\,(t)}$$

where #cells is the number of cells counted, t equals time, and growth rate is the number of cell doublings that occur per unit of time. The doubling time for each cell line is equal to ln(2)/growth rate. Doubling times were determined using five trials.

Flow Cytometry:

5 X 10^5 cells were trypsinized and resuspended in 1 mL of PBS and collected by centrifugation. Two hundred microliters of PBS were added and pellets were resuspended. Cells

were fixed with drop-wise addition of 1 mL of ice-cold 100% ethanol with gentle vortexing and stored at -20^{o}C. The cells were collected by centrifugation as above, and resuspended in a 500 µL a 0.05% solution of RNaseA in PBS. Following 30 min incubation at 37^{o}C, propidium iodide was added to a final concentration of 2 mg/mL. A Becton-Dickinson FACScan cell sorter with Cell Quest Pro software was used to quantify the fraction of cells in G1, S, and G2 phases. For each run, 10,000 cells/events were counted, and any outliers to the data were gated out.

Data for the cell doubling times and flow cytometry were combined were used to calculate the approximate time spent in each phase of the cell cycle (Pozarowski and Darzynkiewicz 2004). This was calculated by taking the doubling time of each cell line and multiplying it by the percent of cells in G1, S, and G2/M phases as determined by flow cytometry experiments.

Protein Determination:

Cells were grown to confluency and washed with ice cold 1x PBS in 0.1 M PMSF. Five mL of fresh PBS/PMSF was added and cells were scraped off the dish using a rubber policeman and transferred to a 15 mL conical tube. Samples were then centrifuged for 5 min at full speed in an Eppendorf Centrifuge 5415 R at 4^{o}C. Supernatant was removed and the pellet was washed in RIPA buffer supplemented with a 1:1000 dilution of 10 mg/mL stock solutions of leupeptin and pepstatin A protease inhibitors.

Cells were then homogenized using a 1.0 ml tuberculin syringe with a 22 x 1" B level needle. Homogenized samples were centrifuged for 5 min at 12,400 rpm to collect the extracted protein. Protein determination was done using Pierce BCA Protein Assay Kit and run in triplicate according to the microassay instructions. Samples analyzed using a Spectra Max 190 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA) using SoftMax Pro 5.4.35 software for collecting the data.

Gel Electrophoresis and Western Blot Analysis:

Blots were stained with Ponceau, photographed, and incubated in blocking buffer for 30 min and incubated in primary antibody diluted in blocking buffer for 1 hr. Blots were washed in TBST solution for three 10-min incubations and then reincubated for 90 min in the appropriate secondary antibody diluted in blocking buffer. The blots were washed with TBST as before. Pierce Super Signal West Pico Chemiluminescent Substrate was then added to the surface of the blots and allowed to incubate for 2-3 min. Blots were imaged with LAS 4000 Fuji Chemiluminescence. Blots were stripped with stripping buffer (0.1 M glycine, 2% SDS in 1X TBS) and reprobed and developed as necessary.

Results

Previous work has shown that over-expression of Rev leads to cell cycle defects (Miyazaki et al. 1996, Sharma et al. unpublished). Mutations in amino acids that are critical to these defects should restore normal patterns of cell growth seen in control cells. With the identification of amino acids critical for these defects, it is then possible to differentiate between hypotheses attempting to explain Rev's effects on cell growth.

Rev Expression and Cellular Localization

Cell lines stably expressing wild-type (WT) and mutant Rev fused to YFP were created from the same founder HeLa cells as described in "Materials and Methods." Only early passage (< 20 passages) cells were used in all experiments. HeLa cells stably expressing either Rev, a YFP control, or Rev mutant express the recombinant proteins at comparable levels [Figure 5]. WT Rev predominantly accumulates in the nucleoli of cells although Rev is present in the nucleoplasm and cytoplasm, consistent with published observations (Dundr et al. 1995, Malim et al. 1989, Suhasini and Reddy 2009) [Figure 6]. In contrast, there is considerably more fluorescence in the cytoplasm of YFP control cells. Although there appears to be nuclear enrichment, this is only because z-stack normalization was not performed.

Mutants T34A, R42A, and the E47A/E57A double mutant, otherwise known as E2A, all appear to follow the WT phenotype during interphase. They show considerable nucleolar localization [Figure 6]. In contrast, mutants A37D, E47A, and E57A, have considerably more cytoplasmic fluorescence comparable to YFP controls. They exhibit only limited nucleolar enrichment. Like these mutants, R39A and R50A have substantial amount of cytoplasmic fluorescence; however, nucleolar accumulation is observed [Figure 6].

Figure 5: Rev expression Western Blot

Twenty micrograms of protein were resolved using a 15% SDS-polyacrylamide gel. Blots were probed for Rev, YFP, and actin using the appropriate primary and secondary antibodies. Blots were stripped and reprobed as needed. Mutant E2A was run on a separate gel due to lack of loading space.

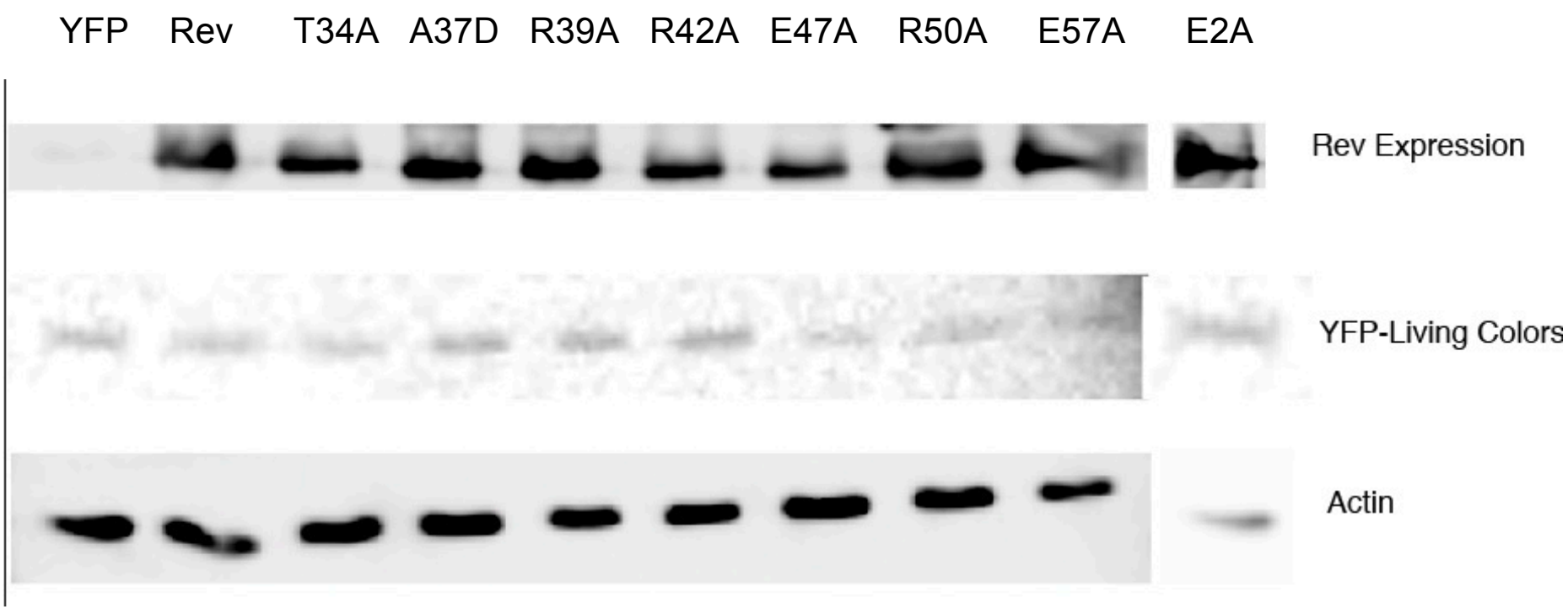
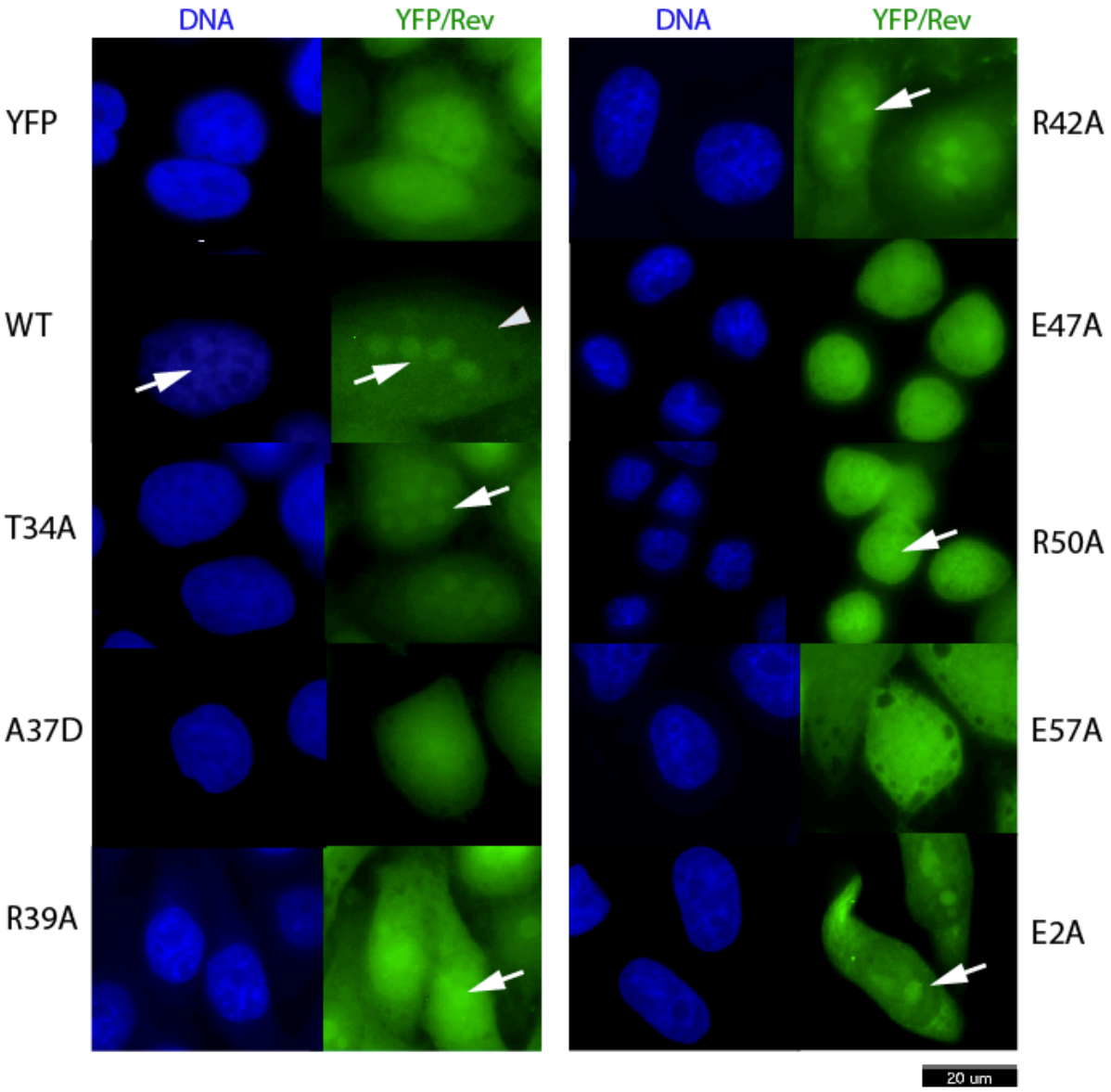

YFP
Rev
T34A
A37D
R39A
R42A
E47A
R50A
E57A
E2A
Rev Expression
YFP-Living Colors
Actin

Figure 6: Rev localization in cells expressing YFP, WT Rev, and various Rev mutants during interphase demonstrated a range of phenotypes.

HeLa stably expressing Rev fused to YFP (right) and the corresponding DAPI images (left) cells demonstrated a variety of phenotypes. The YFP expressing cells showed equal distribution of YFP between the cytoplasm and the nucleus. WT Rev expressing cells showed predominantly nucleolar localization (see white arrows) with some localization at the nuclear envelope (see white arrowhead). The eight mutants fell in between these two phenotypes. Nucleolar localization is noted with white arrows, similar to what is shown in the WT Rev images. T34A, R42A, and the double mutant E2A demonstrated distinct nucleolar localization similar to the WT control. R39A also appeared to localize to nucleoli; however, this mutant also had much more cytoplasmic localization than T34A, R42A, and E2A (see arrow in R39A). Mutant R50A had some localization to nucleoli; however, it was less than that of other mutants. Mutants A37D, E47A, and E57A demonstrated Rev localization that was similar to the YFP control. Scale bar represents 20 μm.


DNA
YFP/Rev
DNA
YFP/Rev
YFP
WT
T34A
A37D
R39A
R42A
E47A
R50A
E57A
E2A
20 um

During the early phases of mitosis (prophase through metaphase), WT Rev initially concentrates around chromosomes [Figure 7, 8]. In contrast, YFP cells exhibit uniform fluorescence throughout the cell and neither localizes to chromatin nor perichromosomally [Figure 8]. Mutants share mitotic accumulations exhibited in both YFP/WT Rev cells. Like WT Rev, mutant E2A localizes to chromosomes during prophase and retains chromosomal localization through mitosis. In contrast, R42A and E47A localize to chromosomes only during metaphase. Mutant A37D and E57A localize to chromosomes early in prophase but become perichromosomal during metaphase [Figures 7, 8]. Mutant T34A does not localize to chromosomes, instead localizing perichromosomally in both prophase and metaphase [Figure 7, 8]. The localization of mutants R39A and R50A resemble that of YFP controls. These mutants localize less to the nucleoli of cells in interphase, and during mitosis, they do not appear to localize around chromosomes [Figure 6, 7, 8].

The subcellular localization of WT Rev and Rev mutants could play an important role in affecting the abilities of cells to grow in culture. However, as is summarized Table 1, the subcellular localization of WT and mutant Rev is quite variable in interphase and early stages of mitosis. Overall, there is little correlation between the interphase localization of each Rev mutant and where it accumulates during mitosis. The phenotypic consequences of each mutation run the gamut from a wild-type appearance to a YFP control appearance. However, the localization of E2A most closely resembles WT Rev whereas R39A more closely resembles YFP controls.

	Nucleolar Localization	Nuclear (N) or cytoplasmic (C) localization	YFP Localization in Prophase	YFP Localization in Metaphase
YFP	no	N=C	equal	cytoplasm
WT Rev	yes	N>C	chromosomes	chromosomes
T34A	yes	N>C	cytoplasm	cytoplasm
A37D	no	N=C	chromosomes	cytoplasm
R39A	slight	N>C	cytoplasm	cytoplasm
R42A	yes	N>C	equal (with enrichment in nucleoli)	chromosomes
E47A	no	N=C	equal	chromosomes
R50A	slight	N>C	cytoplasm	cytoplasm
E57A	no	N=C	chromosomes	cytoplasm
E2A	yes	N≥C	equal (with enrichment in nucleoli)	chromosomes

TABLE 1 Summary of YFP, WT Rev, and Rev mutants' localizations in HeLa cells.

Figure 7: Rev localization in cells expressing YFP, WT Rev, and various Rev mutants during prophase/prometaphase also demonstrated a variety of localization patterns.

As in Figure 6, DAPI (DNA) images are featured on the left and YFP or Rev fused to YFP images are featured on the right. Again, the YFP expressing cells showed equal cytoplasmic and chromosomal localization. WT Rev expressing cells demonstrated enrichment around chromosomes (see white arrow). However, in this case, not all the mutants followed localization patterns seen in either controls. Mutants T34A, R39A, and R50A appeared to avoid chromosomes completely and seemed to localize primarily in the cytoplasm (see white arrowhead). Mutants A37D, E57A, and E2A demonstrated a different localization altogether. While these mutant versions of Rev appeared to localize around chromosomes as in the WT, it also appeared to localize in the cytoplasm. Only mutants R42A and E47A seemed to follow a localization pattern similar to the YFP control. The images shown in these figures demonstrate the most representative cells seen in these cell lines.

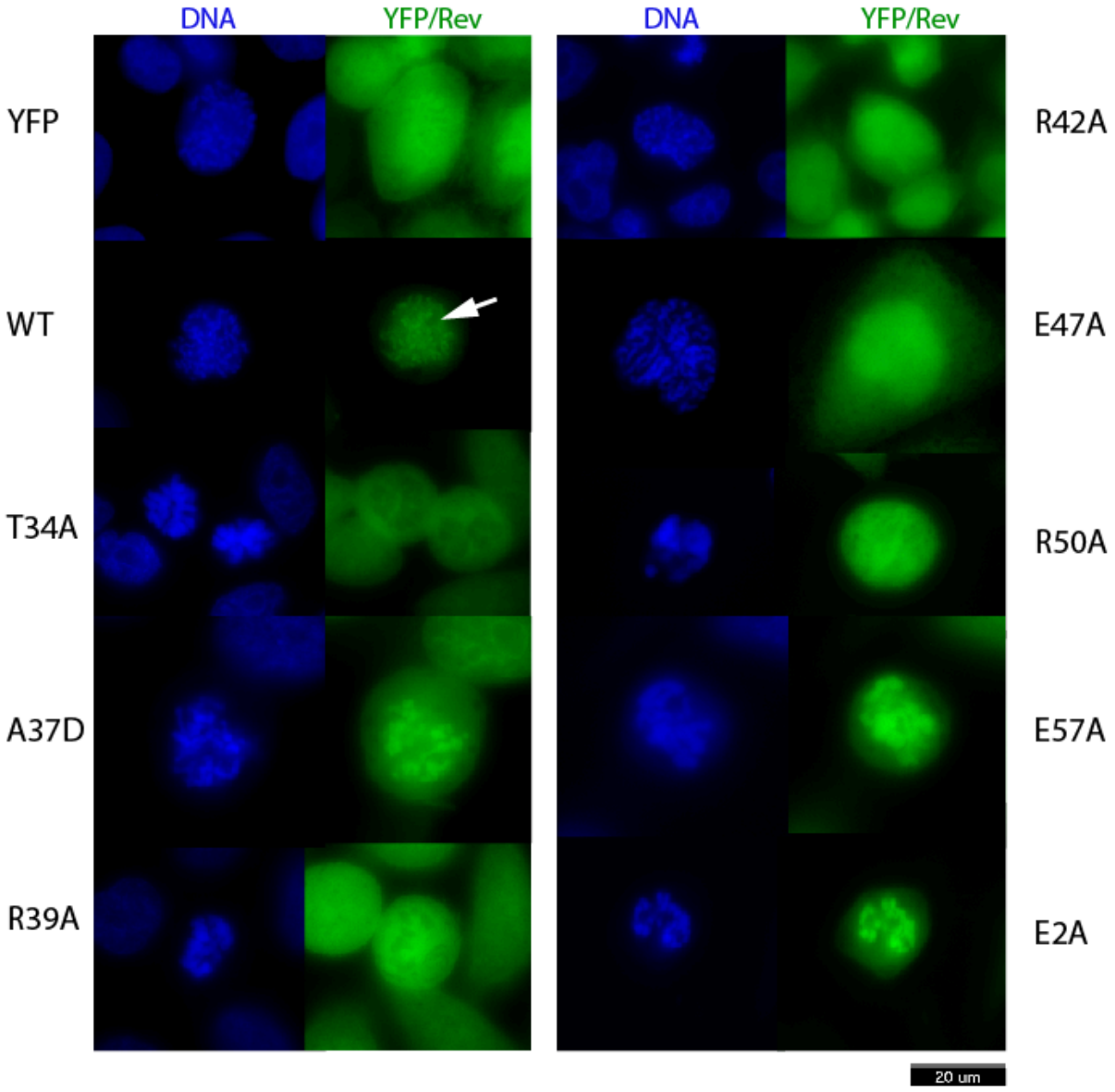
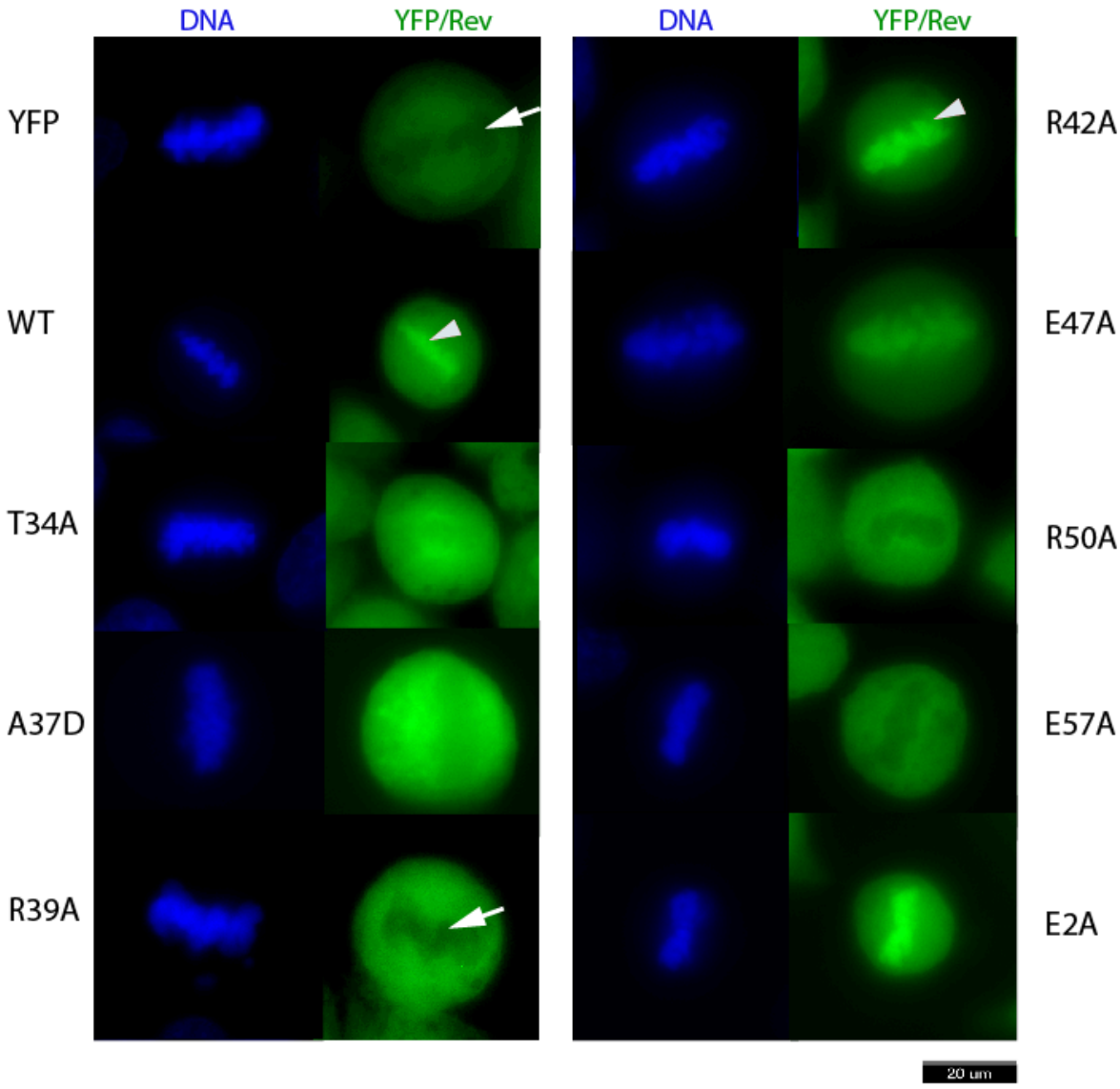

DNA
YFP/Rev
DNA
YFP/Rev
YFP
WT
T34A
A37D
R39A
R42A
E47A
R50A
E57A
E2A
20 um

Figure 8: Localization in cells expressing YFP, WT Rev, and various Rev mutants during metaphase.

Metaphase cells stably expressing YFP appeared to have YFP localizing perichromosomally (see white arrows). Mutants T34A, A37D, R39A, R50A, and E57A all appeared to follow this pattern. The WT Rev control showed enrichment around chromosomes with some localizing to the cytoplasm (see white arrowheads). Mutants R42A, E47A, and E2A demonstrated a similar pattern to this.


DNA
YFP/Rev
DNA
YFP/Rev
YFP
WT
T34A
A37D
R39A
R42A
E47A
R50A
E57A
E2A
20 um

Characterization of Cell Line Growth

Previous results have shown over-expression of wild-type Rev increases the number of G2/M phase cells while simultaneously suppressing the mitotic index in a statistically significant manner (Miller unpublished). To determine whether any of the Rev point mutations affected cell growth in culture, the fraction of cells at each stage of the cell cycle was measured by flow cytometry (Appendix 2). The doubling time for exponentially growing cells was also calculated (Appendix 3). Growth curves of the control cells are shown in Figure 9. As shown in this figure, the WT Rev control grows slower than the YFP control cells as demonstrated by the curves [Figure 9]. Using these data and the methods of Pozarowski and Darzynkiewicz (2004), the average length of each stage of the cell cycle was calculated for each cell line [Figure 10].

As is seen in Figure 10, Rev expressing cells spend more time in each stage of the cell cycle than control cells. Rev expressing cells exhibit a statistically significant increase in G2/M phase cells compared to controls ($p=0.012$, $n=3$, paired t-test). The proportion of cells in G1 and S phase were almost significantly different from control cells ($p=0.08$ and $p=0.07$, respectively). The total length of a single cycle was 64% longer when WT Rev was expressed. As predicted by the 'Kin-13' hypothesis, each point mutation partially restored the cell cycle to control levels. However, none of these differences are statistically significant (χ^2, $p>0.7468$), probably due to the small sample size ($n=4$).

Mitotic Indices of Cell Lines

Previous results have suggested that Rev expression altered changes in mitotic indices (Miller unpublished). Mitotic indices were measured as described in "Materials and Methods," and subjected to Chi Square testing (Table 2). Three cell lines were statistically significant from YFP expressing controls. Consistent with previous results, WT Rev had a lower mitotic index

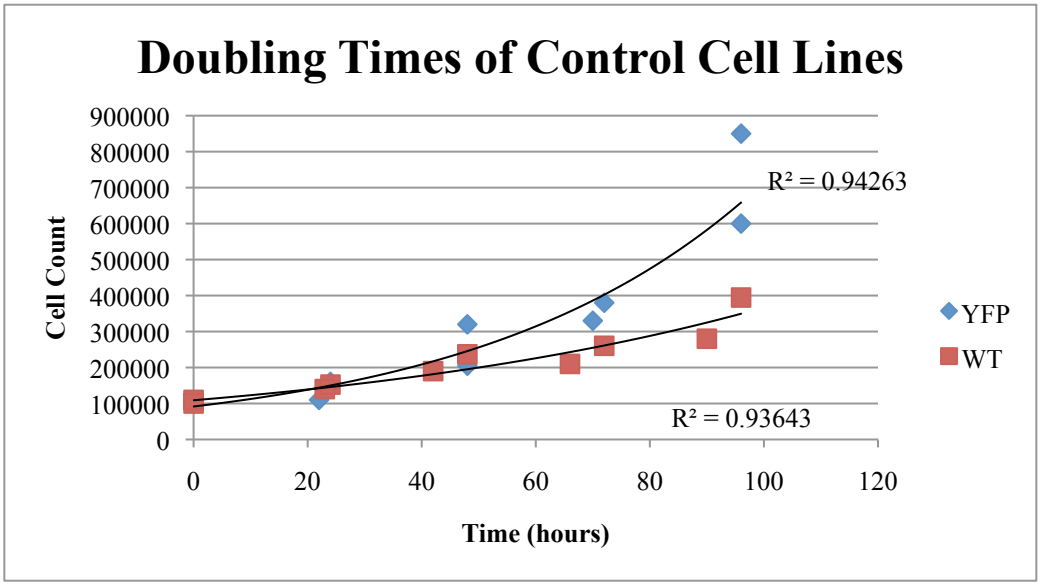


Figure 9: The WT Rev control has a longer doubling time than the YFP control cell line.

Approximately 1 X 10^5 cells were plated and counted every 24 hours for six days. This graph demonstrates a compilation of all the counting data for the control cell lines of this experiment. The YFP control growth curve grows faster than the WT control curve. This pattern is consistently shown for both cell lines.

0.002). The mitotic index of T34A was almost significantly lower than YFP controls (p-value ≤0.06). Surprisingly, mutant R39A had a statistically significant increase in the number of cells undergoing mitosis ($p < 0.0001$). The mitotic indices of the remaining cell lines, R42A, E47A, R50A, E57A, and E2A, were not statistically different from the YFP control ($p > 0.3$).

Analogous statistical comparisons between WT Rev and Rev mutants were made. All mutants except A37D were significantly different from WT. This indicates that a partial correction is occurring in these mutants. Mutant R39A is the only mutant that is significantly different from both control lines.

	Mitotic Index	**P-values: compared to YFP**	**P-values: compared to WT**	**% of Cells after/before checkpoint**	**P-valules: compared to YFP**	**P-values: compared to WT**
YFP	10.1%	-------	-------	0.32	-------	-------
WT Rev	6.0%	<0.0001	-------	0.02	<0.0001	-------
T34A	7.9%	0.0596	0.0427	0.14	0.03	0.0056
A37D	6.7%	0.0024	0.3791	0.15	0.05	0.0031
R39A	23.8%	<0.0001	<0.0001	0.08	0.0009	0.17
R42A	8.8%	0.3143	0.0036	0.18	0.11	0.0010
E47A	10.4%	0.8542	<0.0001	0.03	<0.0001	1.0000
R50A	11.3%	0.4093	<0.0001	0.11	0.0084	0.017
E57A	11.1%	0.4900	<0.0001	0.05	0.0001	0.44
E2A	10.5%	0.7754	<0.0001	0.35	0.74	<0.0001

TABLE 2 Mitotic Indices of YFP Control, WT Rev, and mutant Rev proteins. Also shown are proportions of cells before and after the metaphase checkpoint. Statistical values are demonstrated in the two right-hand columns next to each experiment.

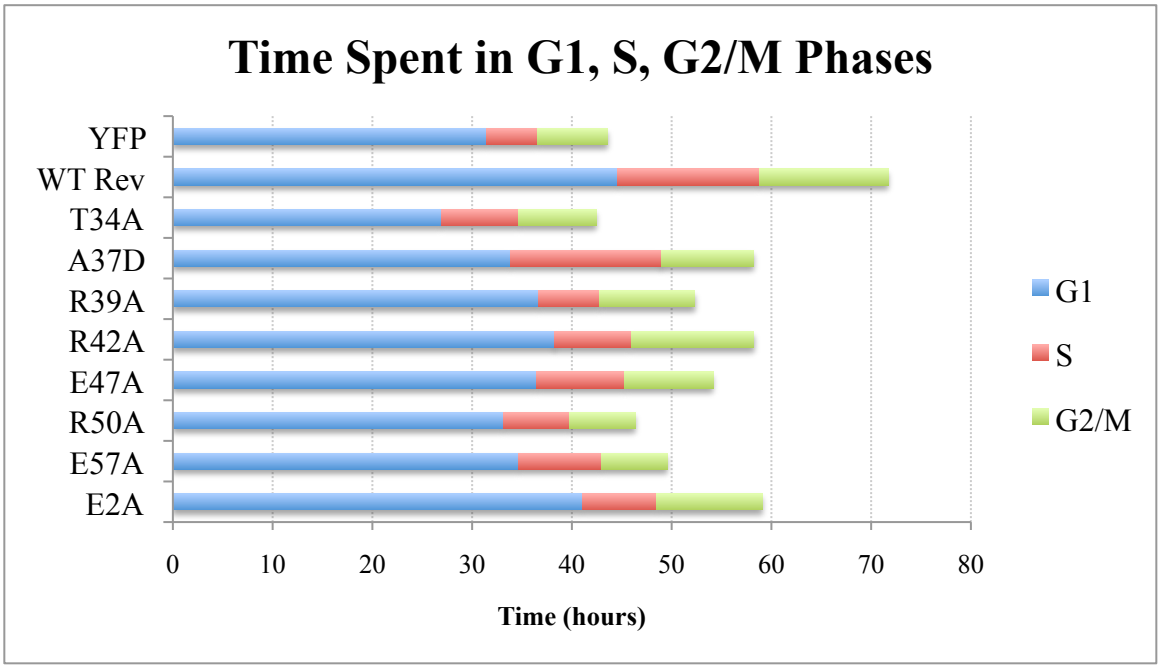


Figure 10: Graphical representation of time spent in G1, S, and G2/M phases.

This is a graphical depiction of the length of time that control and mutant cell lines spent in each phase of the cell cycle using the methods of Pozarowski and Darzynkiewicz (2004). WT Rev expressing cells appear to spend more time in each stage of the cell cycle overall. They also spend ten more hours in G1 in comparison to the YFP control. The double mutant E2A spent almost as long in G1 as the WT Rev control. Mutants A37D, R50A, and E57A restore the normal time period that cells stay in G1. T34A was the only mutant to show a decrease in time spent in G1. The remaining mutants demonstrated a prolonged period of time in G1. All cell lines show increased time spent in S phase in comparison to the YFP control. WT Rev and mutants A37D, R39A, R42A, E47A, and E2A all spent longer in G2/M. R42A spent roughly the same amount of time in G2/M as the WT Rev control. R50A and E57A had shorter G2/M phases in comparison to the other cell lines. T34A was the only cell line had a G2/M phase time that was similar to the YFP Control.

Multinucleated cells and other cellular oddities

In the course of these experiments, mutants A37D, R39A, R42A, the double mutant E2A, as well as the WT Rev control cell lines exhibited an increase in the number of multinucleated cells [Figure 11, 12]. HeLa cell lines normally possess a few multinucleated cells; however, these cells normally do not survive past the third cycling of mitosis (Krzywicka-Racka and Sluder 2011). It is therefore noteworthy that some cells had upwards of ten nuclei present in the aforementioned cell lines.

Several cell lines exhibited characteristics of cellular stress. The most severe effects were seen in WT Rev expressing cells [Figure 12]. Many had perturbed MT cytoskeletons and multinucleated cells were more common. Examples are depicted in Figure 13. These cells also divided into more than the normal number of daughter cells. Figure 13A depicts a tri-polar cell where MTs are shared between the three forming daughter cells. Figure 13B shows a cell that is dividing into four daughter cells, two of which are smaller than normal. MTs are disorganized and cell morphology was highly abnormal. Some cells were also much larger than normal HeLa cells [Figure 13, C]. The nuclei of these cells were abnormally larger and the MT cytoskeleton was highly perturbed. This was expected, as the cell has to accommodate the increased size of the cell [Figure 13, C]. Mutants E47A and E57A demonstrated the opposite phenotype from the WT control having a more rounded and smaller cell shape than other cell lines in culture [Figure 14]. Mitotic cells had certain cytoskeletal defects in the E2A over-expressing cells [Figure 13, E]. Multi-spindled cells were prevalent, which could account for the mitotic defects seen in these cells [Figure 13, E]. This mutant was the only mutant cell line to demonstrate this type of effect. The presence of rounded cells and the occurrence of multinucleated cells are consistent with the hypothesis that there could be a tubulin defect.

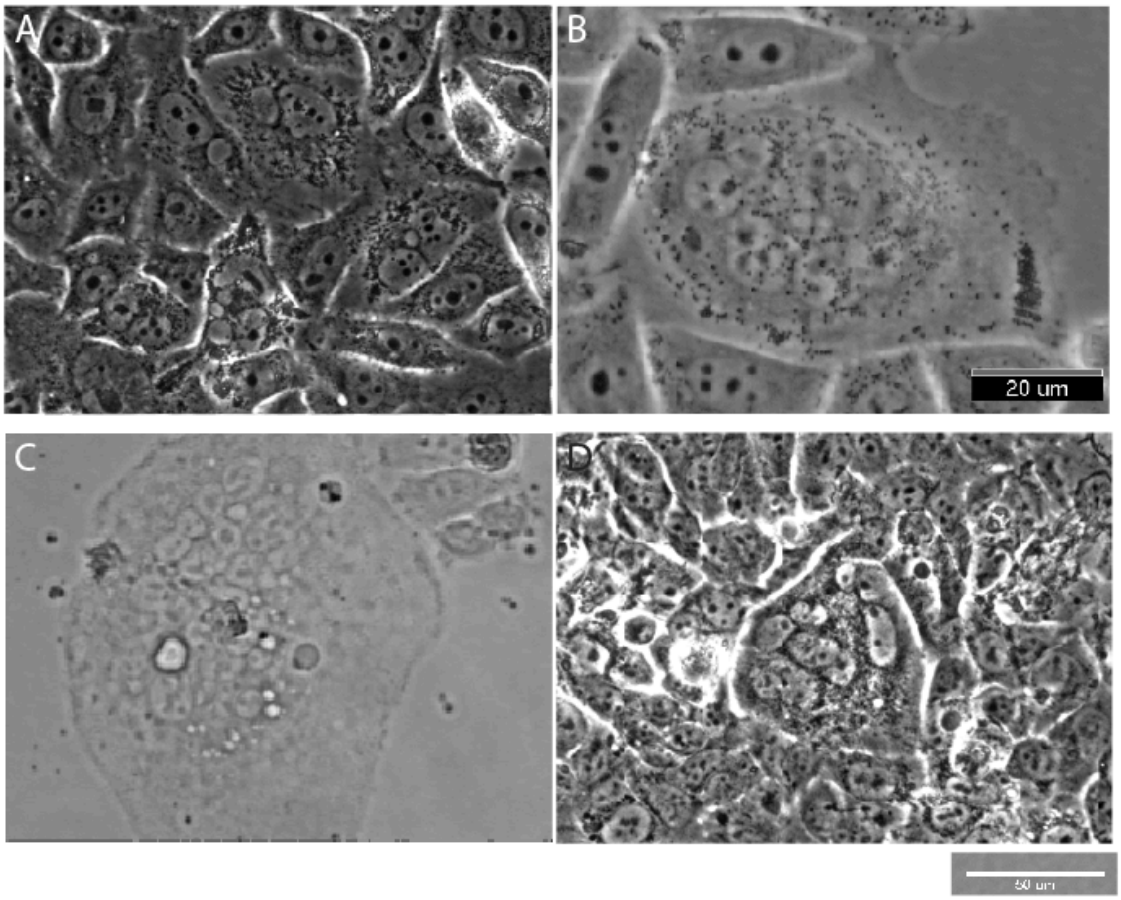


Figure 11: Multinucleated cells were also present in cells expressing mutants A37D, R39A, R42A, and E2A.

The degree of severity of multinucleated cells depended upon the particular Rev mutant. Some cells, like A37D (A), contained nuclei of different sizes present in one cell. Mutant R39A (B) demonstrated the most varieties of multinucleated cells. The sizes would range from relatively small multinucleated cells to large, fairly perturbed cells. Mutant R42A (C) also had the ability to create large cells that were multinucleated. The double mutant E2A (D) also produced multinucleated cells. Scale bar for A, C, and D represents 50 µm. Scale bar for B represents 20 µm.

Figure 12: Cells in the WT Rev control exhibited a variety of morphologies.
Examples of abnormal cells as seen in the WT Rev control in culture are demonstrated below. These cells often formed unusual cell shapes that differed from what was seen in the YFP control in culture (A). In addition to having abnormal cell morphologies, the WT control also formed multinucleated cells that exhibited various cell shapes. The cell in (B) appears to have hair-like protrusions emanating from the plasma membrane. A clear nucleus is also not present in this cell. Multinucleated cells were a common occurrence in culture in the WT Rev cell lines (C). These cells were of differing sizes and shapes in which the cell had an extensive cytoplasm in some cells (D). Scale bar for images A, B, and C represents 50 μm. (E) demonstrates the occurrence of multinucleated cells in this control cell line at 10X magnification. This type of cell was found to appear in ≈40% of the cell population. Scale bar for E represents 50 μm.

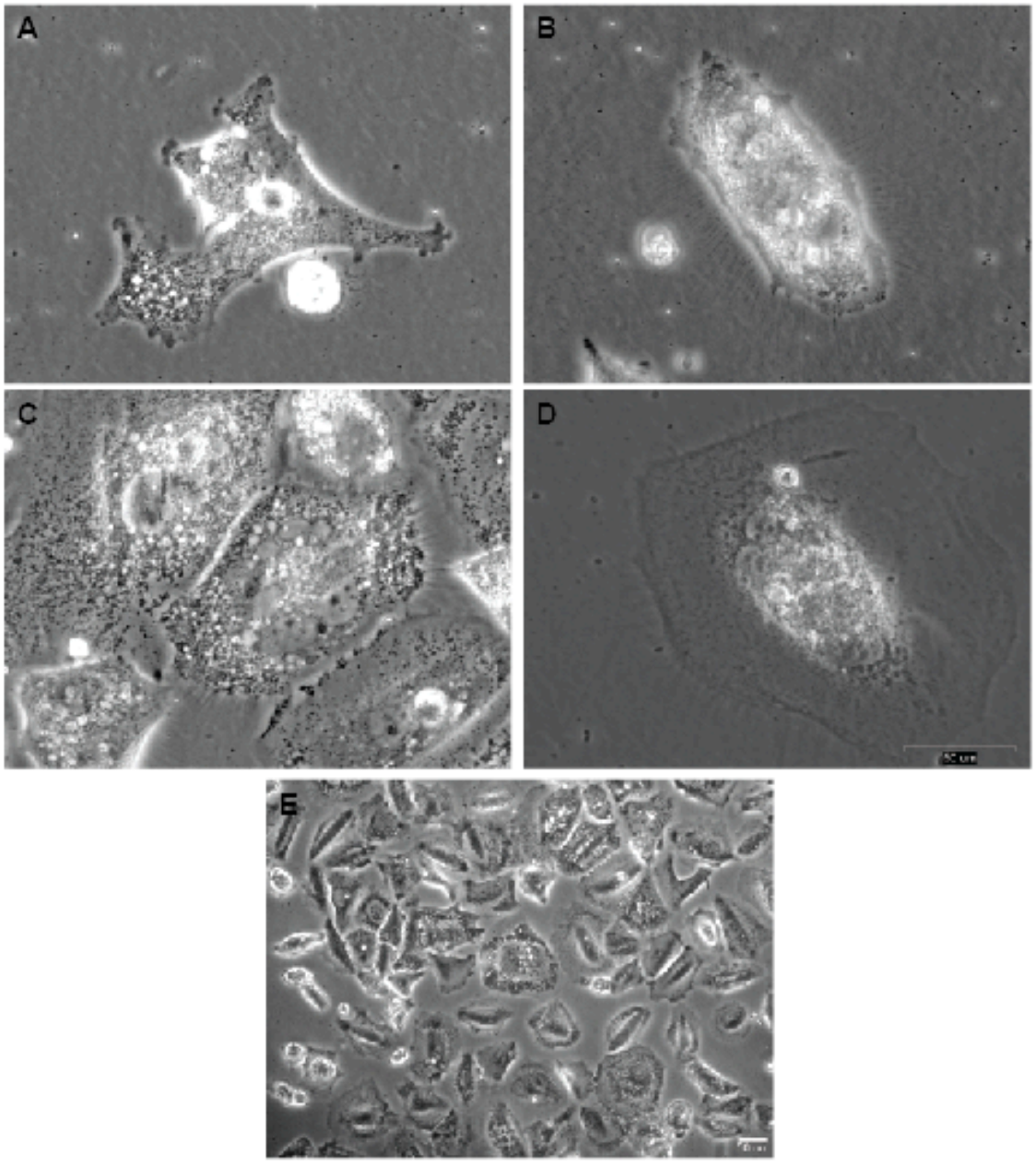

A
B
C
D
E

Figure 13: WT Rev and the E47A/E57A (E2A) double mutant appeared to create severely perturbed cytoskeletal phenotypes in cells.

(A) demonstrates a triply dividing cell in which the microtubule cytoskeleton appears to be dividing into three separate cells. (B) shows a cell that is attempting to divide into two large daughter cells and what appears to be two additional, smaller daughter cells. The cellular and microtubule structure of these cells does not appear normal. WT Rev also demonstrated the ability to create larger than normal cells (C). The nucleus of this cell is noticeably larger as well as the microtubule structure making up the cell. As multinucleated cells are possible in WT Rev-over expressing cells, the cytoskeleton of these cells is much more extensive to accommodate for the increased cell size (D). The E2A mutant is the only mutant cell line that demonstrates this phenotype. Rhodamine-labeled tubulin is shown in the right-hand column. DAPI staining on the far left-hand column shows the chromosomes taking on alternate configurations because of the multi-polar spindles (E). Scale bar represents 20 μm.

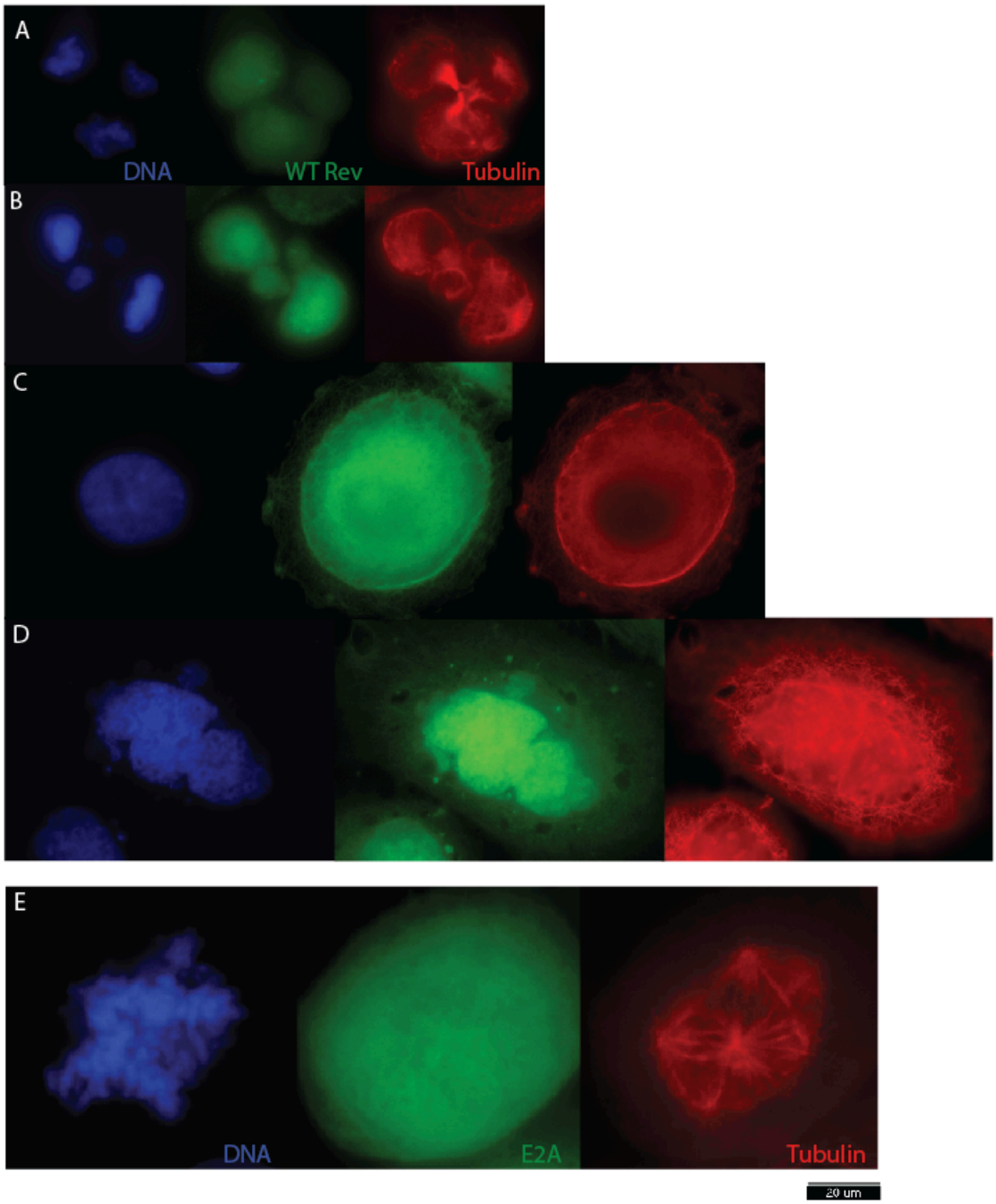
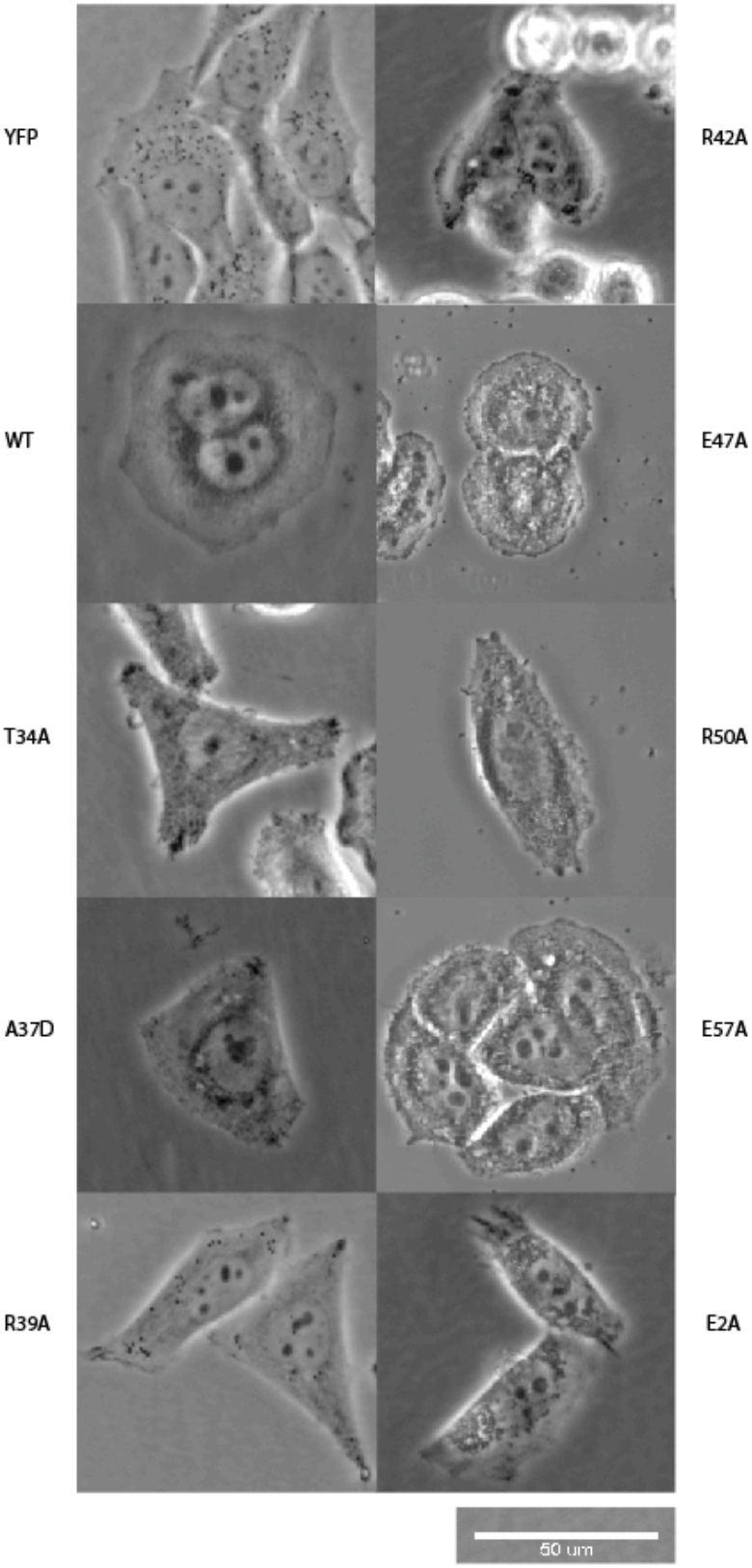

A
DNA
WT Rev
Tubulin
B
C
D
E
DNA
E2A
Tubulin
20 um

Figure 14: Cell morphologies differed between early passage mutant and control cell lines.

These are representative images of what the different cell lines appeared like in culture. Wild type Rev expressing cells early on in culture often exhibited multinucleated cells. Cells with single nuclei were also present, but these cells also were abnormal in morphology. T34A cells were similar in size and shape to the YFP expressing cells. Mutant A37D cells were typically larger than control cells. This cell line also was able to produce large multinucleated cells in culture. Mutants R39A and R42A both had similar morphologies that somewhat resembled the YFP control line. Both cell lines were also capable of producing large multinucleated cells in culture. Mutants E47A and E57A demonstrated similar cell shapes. These cells were rounder and smaller than the other cell lines. The nuclei of these cells were also smaller in comparison. R50A typically demonstrated elongated cells in culture that were oval shaped. The double mutant E2A usually most closely resembled cells in the WT Rev control cell line, and like the WT cells, E2A had the ability to create large, multinucleated cells. The scale bar represents 50 μm.


YFP
R42A
WT
E47A
T34A
R50A
A37D
E57A
R39A
E2A
50 um

Immunofluorescence

Differences between cell lines were immediately apparent in this study in examining the various cell lines' sizes and shapes in culture. Some cell lines had consistently different cell shapes. Mutant R50A demonstrated elongated, rounded, and smaller cell shapes. Mutants E47A and E57A were also rounded, and mutant R42A and the double mutant E2A demonstrated cells that were visibly stressed. Representative images of these cells in culture are depicted in Figure 14. It is possible that these defects are the result of tubulin depolymerization and/or imporper spindle nucleation in cells expressing Rev. MTs can also be perturbed indirectly via Rev through its interactions with the G-protein Ran or importin β. To differentiate between hypotheses, cells were immunostained to determine whether MT arrays or the Ran/importin β system were affected.

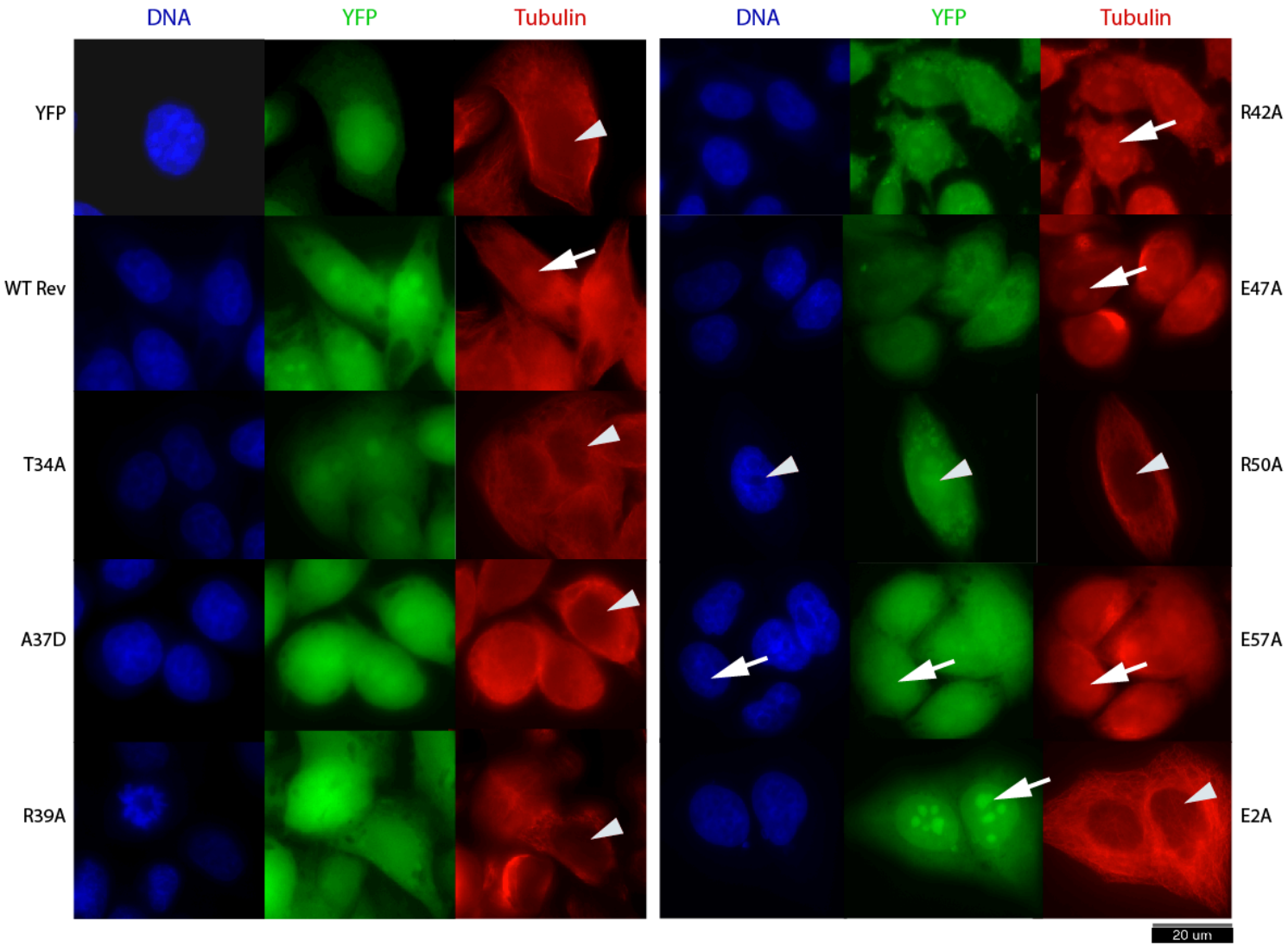
Tubulin

The presence of rounded cells and multinucleated cells is consistent with a MT cytoskeletal defect. Moreover, Rev is a potent MT depolymerizer *in vitro* (Watts et al. 2000), which suggests that MTs may be affected. YFP-control cells have a normal morphology and the MT cytoskeleton spreads throughout the cell [Figure 15]. Individual MTs are easily visible. In contrast, the cytoskeleton of the WT-Rev cell line was not often as well organized [Figure 15]. Curiously, nucleolar localization of alpha tubulin was seen in the WT cells. This observation is consistent with Rev-tubulin interactions in that Rev can bind tubulin and transport tubulin elsewhere in the cell [Figure 15].

Different mutants affected the cytoskeleton differently. Mutants A37D, E47A, and E57A fell into one group. These mutants demonstrated a more rounded cell shape and appeared to have a more tightly packed MT structure [Figure 15]. Mutants E47A and E57A both appeared to have tubulin in nucleoli of cells. They also had a MT structure that appeared compacted, which reflected their unusual shape. A37D mutants had a similar rounded shape and tightly packed MT structure, but did not have any nuclear staining whatsoever [Figure 15]. The cytoskeleton of these mutants was more compressed [Figure 15].

Figure 15: Tubulin immunostaining demonstrated differences in cell morphology and α tubulin localization between the YFP control, WT Rev control cells, and mutants.

In the YFP control cell line, α tubulin staining was cytoplasmic and avoided the nucleus completely (see white arrowhead). Mutants T34A, A37D, R39A, R50A, and the double mutant E2A appeared to demonstrate this phenotype as well. The WT Rev expressing cells seemed to have tubulin localization in nucleoli (see white arrow). Mutants R42A, E47A, and E57A appeared to follow this pattern as well. For cells expressing WT Rev and mutants R42A, E47A, and E57A, Rev appeared to colocalize with tubulin in nucleoli. But, there were instances in which mutant Rev localization did not correspond to tubulin localization. For example, in mutants T34A, R50A, and E2A, these mutant Rev proteins appeared to localize to nucleoli of cells, but their tubulin localization is similar to what is seen in the YFP expressing cells where tubulin appeared to avoid the nucleus (see white arrowheads in mutant R50A). There also existed a case in which the opposite is true. Mutant E57A Rev did not appear to localize to nucleoli, but tubulin was be found localized to nucleoli in these cells (see white arrows in E57A).


DNA
YFP
Tubulin
DNA
YFP
Tubulin
YFP
WT Rev
T34A
A37D
R39A
R42A
E47A
R50A
E57A
E2A
20 um

Mutants R39A, R42A, and R50A had abnormal but not identical cell morphologies. Mutant R39A cells appeared to be average-sized cells; however, the cytoskeleton was often disorganized [Figure 15]. Tubulin also appeared to be localized in the nucleoli of mutant R42A cells as well [Figure 15]. Immunolabeling of α tubulin was weak. Individual MTs of these two mutants were more difficult to discern than as in the YFP-control cells [Figure 15]. Mutant R50A cells were elongated and did not have the same physiology as normal HeLa cells [Figure 15]. However, MTs had a similar phenotype as the YFP-control cells [Figure 15]. These cells also demonstrated a rounded cell shape in culture in addition to the elongated cells pictured in Figure 15 [See also Figure 14, 18]. Mutants T34A and the double mutant E2A had a MT cytoskeletal structure that more closely resembled that of YFP control [Figure 15]. MTs of both were easily seen and appeared highly expanded. Rev appeared to localize to nucleoli of both [Figure 15].

B23

B23 is known to bind to Rev and is found in the nucleoli of the YFP Control cell line [Figure 16] (Dundr et al. 1997). In cells over-expressing WT Rev, B23 localizes in the cytoplasm as well as showing abundant nucleolar localization during interphase [Figure 16]. All mutants demonstrated primarily nucleolar localization of B23 in interphase [Figure 16]. Mutants A37D, R50A, and E2A demonstrated more cytoplasmic localization of B23, in addition to its nucleolar localization, than other cell lines.

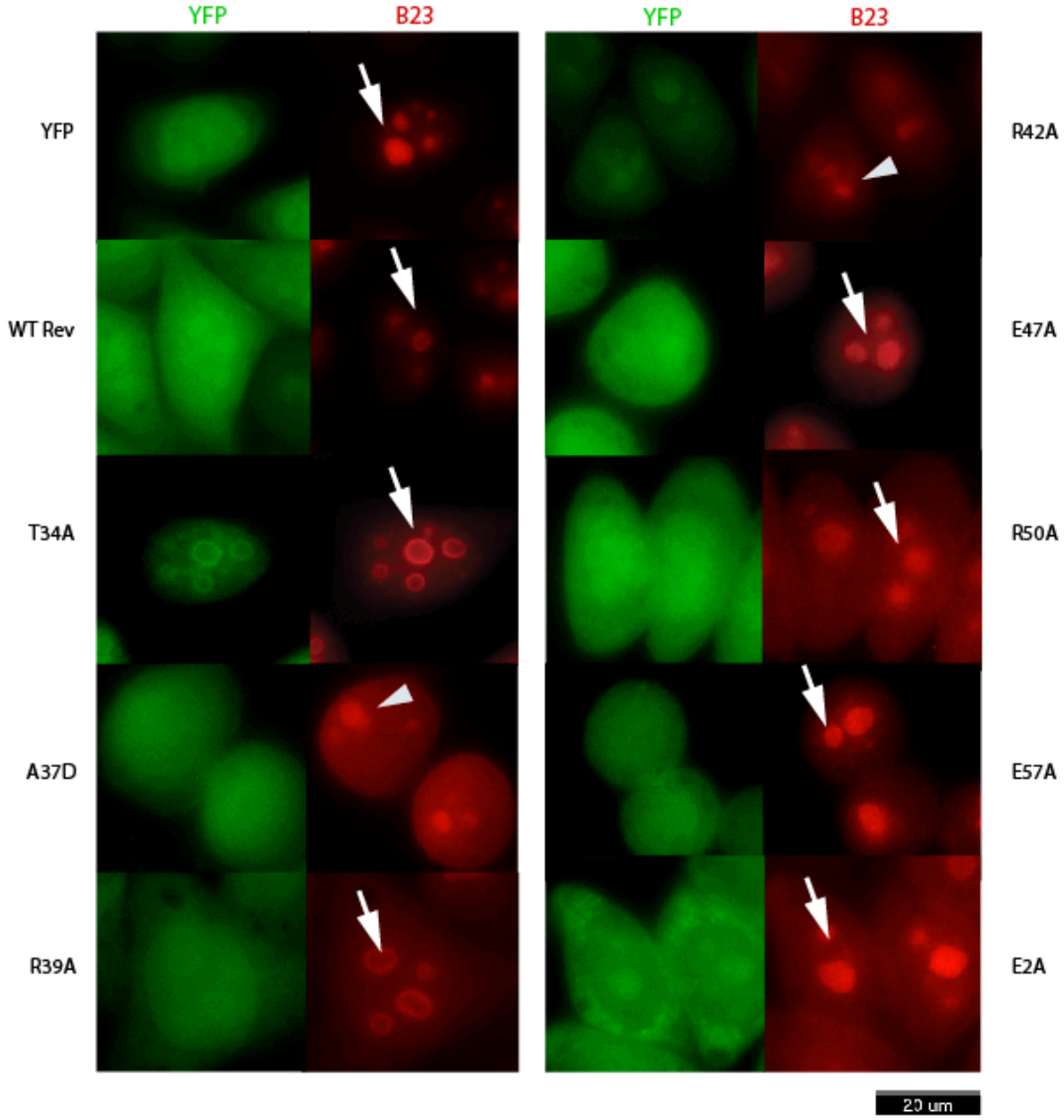


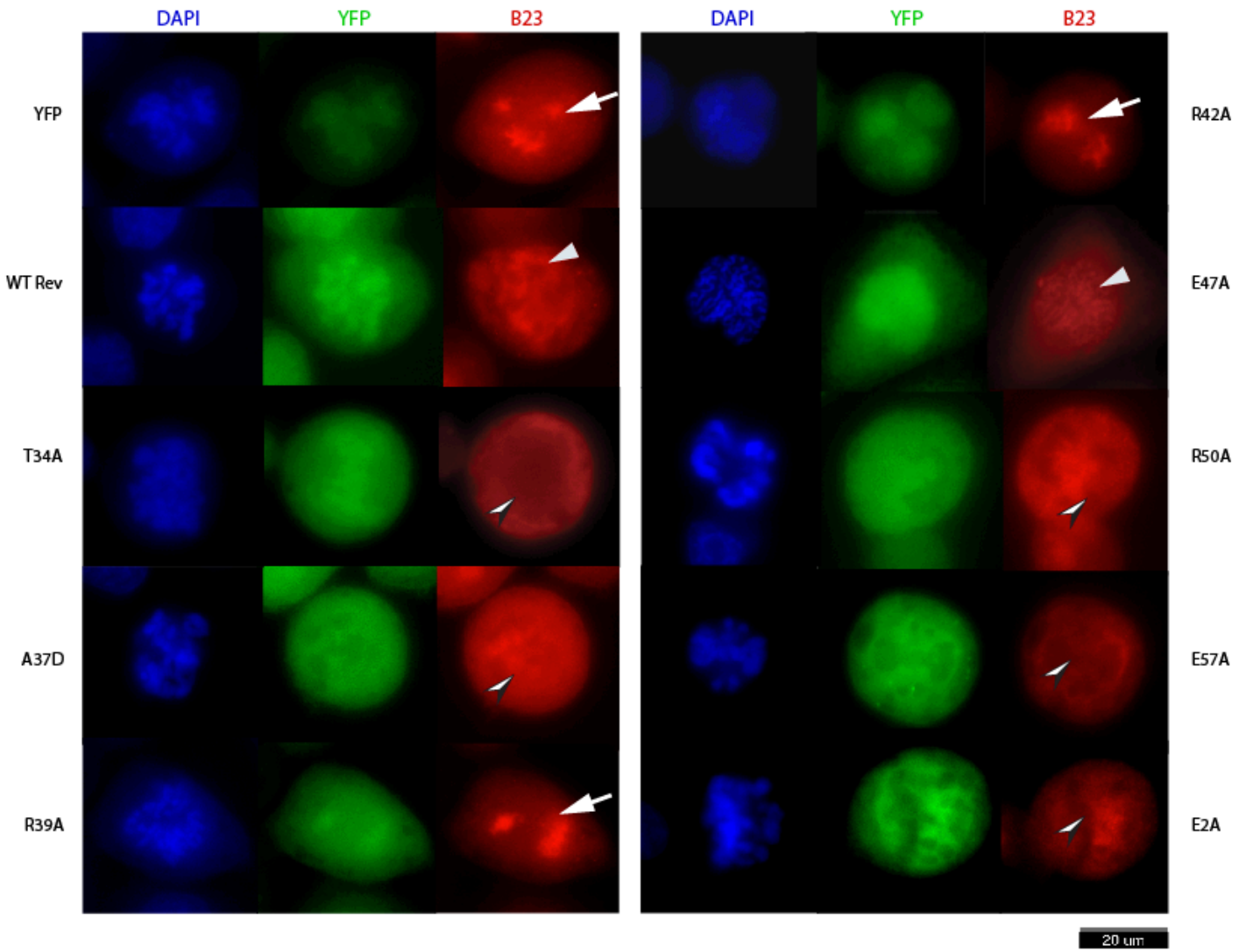
Figure 16: B23 localization of the YFP control, WT Rev, and mutant cell lines fell into two patterns during interphase.

The YFP-expressing cells demonstrated nucleolar localization in interphase cells, as expected (see vertical white arrows). All of the mutants and WT Rev cell lines demonstrated this phenotype in interphase cells to some degree. White arrowheads in A37D and R42A indicate oddities in B23/nucleolar morphology.

During prophase, B23 in the YFP control concentrates in foci of the cytoplasm of cells around the condensing chromosomes [Figure 17]. These foci could be prenucleolar bodies (PNBs). WT Rev expressing mitotic cells appeared to have B23 localizing to chromosomes during prophase [Figure 17]. In most mutant cells, B23 localization followed two localization patterns between interphase and prophase cells. Mutants T34A, A37D, R50A, E57A, and the double mutant E2A exhibit perichromosomal localization of B23 in cells during prophase [Figure 17]. In some instances, Rev and B23 localize to small clusters in the cytoplasm of prophase cells [Figure 17]. Mutant E47A Rev does not localize to nucleoli as whereas B23 remains nucleolar during interphase. In prophase, B23 localizes to chromosomes where E47A Rev is also found [Figure 17].

Figure 17: B23 localization differed during Prophase between the mutants and control cell lines.

The YFP expressing cells during prophase demonstrated B23 localizing to foci in the cytoplasm (see white arrow). The WT Rev control, on the other hand, showed B23 enriched around chromosomes during prophase (see white arrowhead). Mutants R39A and R42A most closely resembled the YFP control, and mutant E47A had B23 localizing to chromosomes like the WT Rev control. Mutants T34A, A37D, R50A, E57A, and E2A, however, followed neither of these patterns. What seemed to be occurring was B23 localized perichromosomally or predominantly in the cytoplasm, but not concentrating in PNBs as seen in the YFP control (see black and white arrowheads).


DAPI
YFP
B23
DAPI
YFP
B23
YFP
WT Rev
T34A
A37D
R39A
R42A
E47A
R50A
E57A
E2A
20 um

Importin β

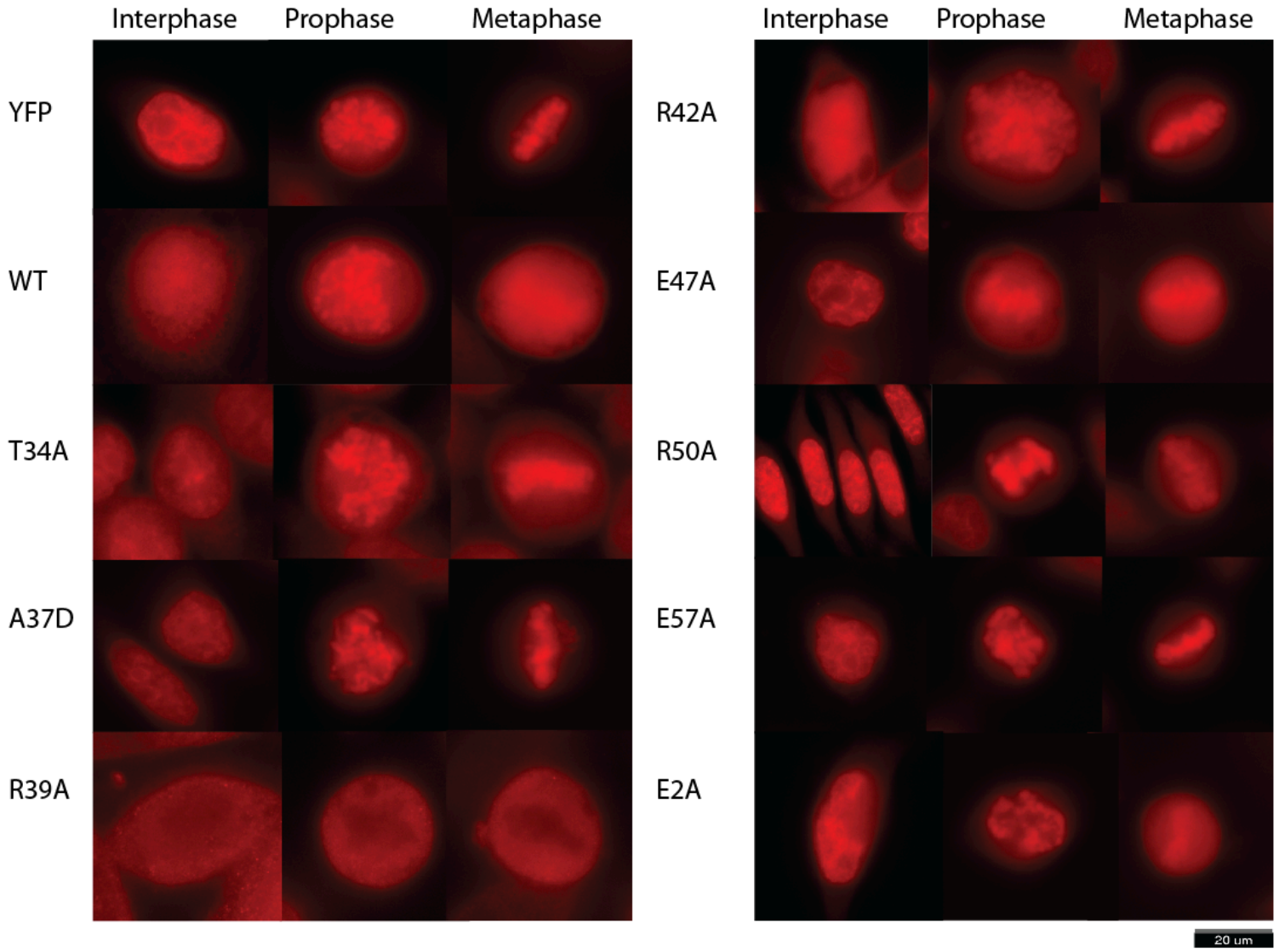
As importin β is a binding partner of Rev via its NLS and because importin β plays an important role in spindle nucleation, this study sought to determine whether any Rev mutations interfered with importin β localization. Certainly, if the NLS were mutated, then Rev should accumulate in the cytoplasm. Theoretically, this means there would be higher Rev concentrations in the cytoplasm to interact with other cytoplasmic proteins such as tubulin.

Importin β in the YFP-control cells was enriched in the nuclei of cells [Figure 18]. Interphase cells showed both nuclear and cytoplasmic localization of importin β in WT Rev over expressing cells [Figure 18]. Mutants, for the most part, demonstrated a phenotype similar to that of the YFP Control cell line: cells in interphase demonstrated predominantly nuclear localization although some mutants did have cytoplasmic localization of importin β [Figure 18].

Importin β in the YFP control localized around chromosomes during mitosis as expected. The WT Rev control also had importin β localizing around chromosomes during prophase, but this was not consistent during metaphase, where it appeared to be primarily cytoplasmic. The mutants that followed this phenotypic pattern were T34A, A37D, E47A, R50A, E57A, and E2A. These mutant forms of Rev all had enrichment of importin β in the nucleus and chromosomes [Figure 18]. However, not all mutants followed this pattern. Mutant R39A and mutant R42A both demonstrated differences in localization patterns of their respective Rev mutant and importin β. R39A followed a completely different localization pattern from what was seen previously. Neither mutant R39A Rev nor importin β localized to chromosomes during mitosis and appeared to be primarily found in the cytoplasm [Figure 18]. Mutant R42A demonstrated a different pattern altogether in which during interphase, R42A had similar localization patterns to the WT

Figure 18: For the most part, mutant Rev proteins restored YFP phenotype in Importin β localization.

In interphase cells, Importin β primarily had a nuclear localization in YFP expressing cells. In prophase and metaphase cells, Importin β localized primarily around chromosomes (see white arrows). However, WT Rev only localized chromosomally during prophase and seemed spread throughout the cytoplasm during interphase and metaphase (see white arrowhead). Mutants T34A, A37D, E47A, R50A, E57A and E2A all demonstrated localization patterns similar to that of the YFP expressing cells. The remaining two mutants, however, showed variations on this theme. Mutant R42A did have some similarities with the YFP control cells. R42A, like the YFP, also had predominantly chromosomal localization during prophase and metaphase. But, during interphase, Importin β localized throughout the cell in a similar manner to the WT Rev cells. Mutant R39A demonstrated a different phenotype entirely. Importin β was found exclusively in the cytoplasm, excluded from the nucleus during interphase and chromosomes during prophase and metaphase (see black and white arrowheads).


Interphase
Prophase
Metaphase
YFP
WT
T34A
A37D
R39A
Interphase
Prophase
Metaphase
R42A
E47A
R50A
E57A
E2A
20 um

control, but during prophase and metaphase, it more closely resembled the YFP control [Figure 18].

Mad2

Mad2 is a spindle checkpoint protein first identified in yeast (Li and Murray 1991). Some cells exhibit an unusual distribution of Rev before and after the mitotic checkpoint. To identify if Rev is perturbing the spindle checkpoint, I performed an immunofluorescence experiment to see if there were any visible changes in the different mutant Rev cell lines. One would think the most distinct differences between the cell lines would occur between prometaphase and metaphase. These are highlighted in Figure 19. In the YFP-Control cell line, Mad2 appeared mostly cytoplasmic [Figure 19]. The WT Rev control cells, in both mitotic and non-mitotic cells, also had equal distribution of Mad2 throughout the nucleus and cytoplasm, except during prometaphase where Mad2 appeared to localize to chromosomes [Figure 19].

All mutants except for R42A, E47A, and E57A appeared to have Mad2 localizing in areas that resembled the YFP control line [Figure 20]. R42A and E57A appeared to follow a localization pattern that was similar to the WT Rev control with some slight variations [Figure 20]. Mutant E47A had Mad2 localizing in the cytoplasm during prometaphase, but unlike the others, it did localize chromosomally. Mad2 should be localizing to kinetochores; however, this was not seen in this study.

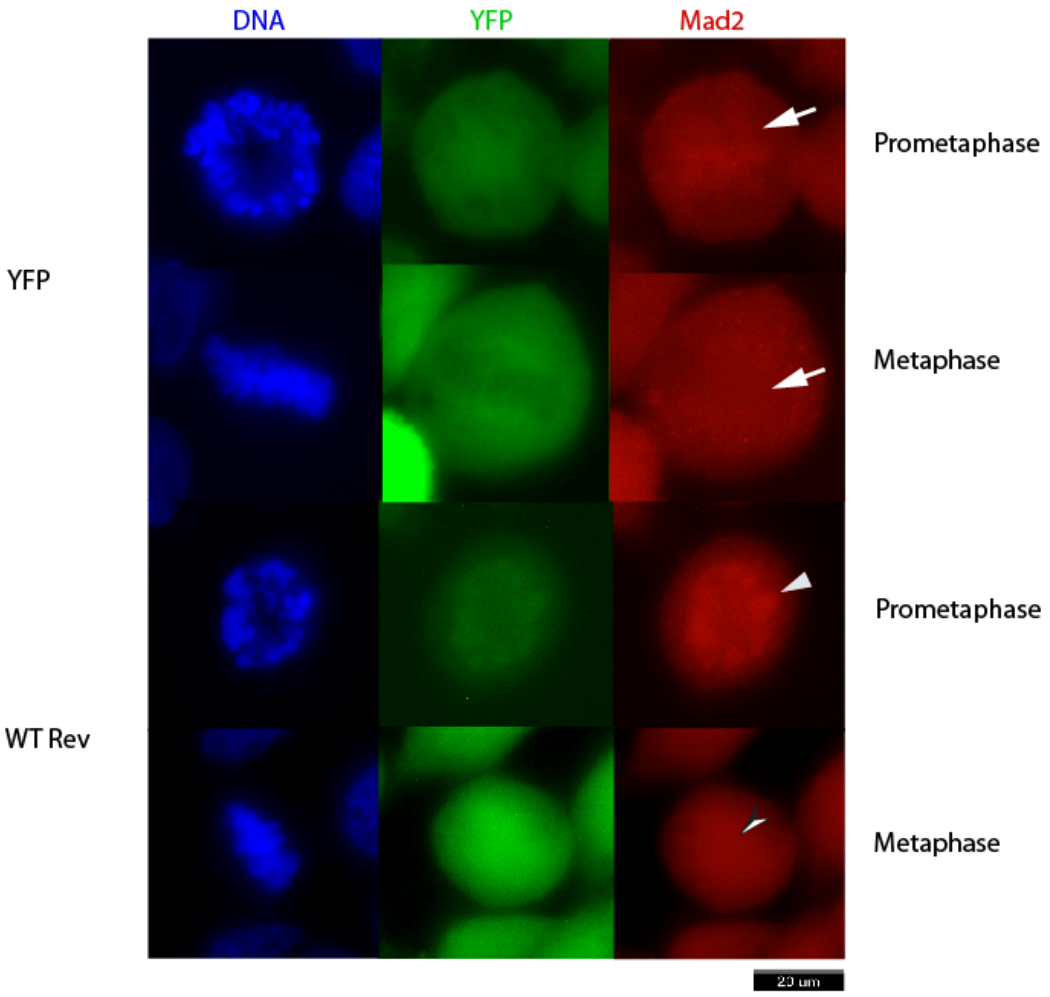
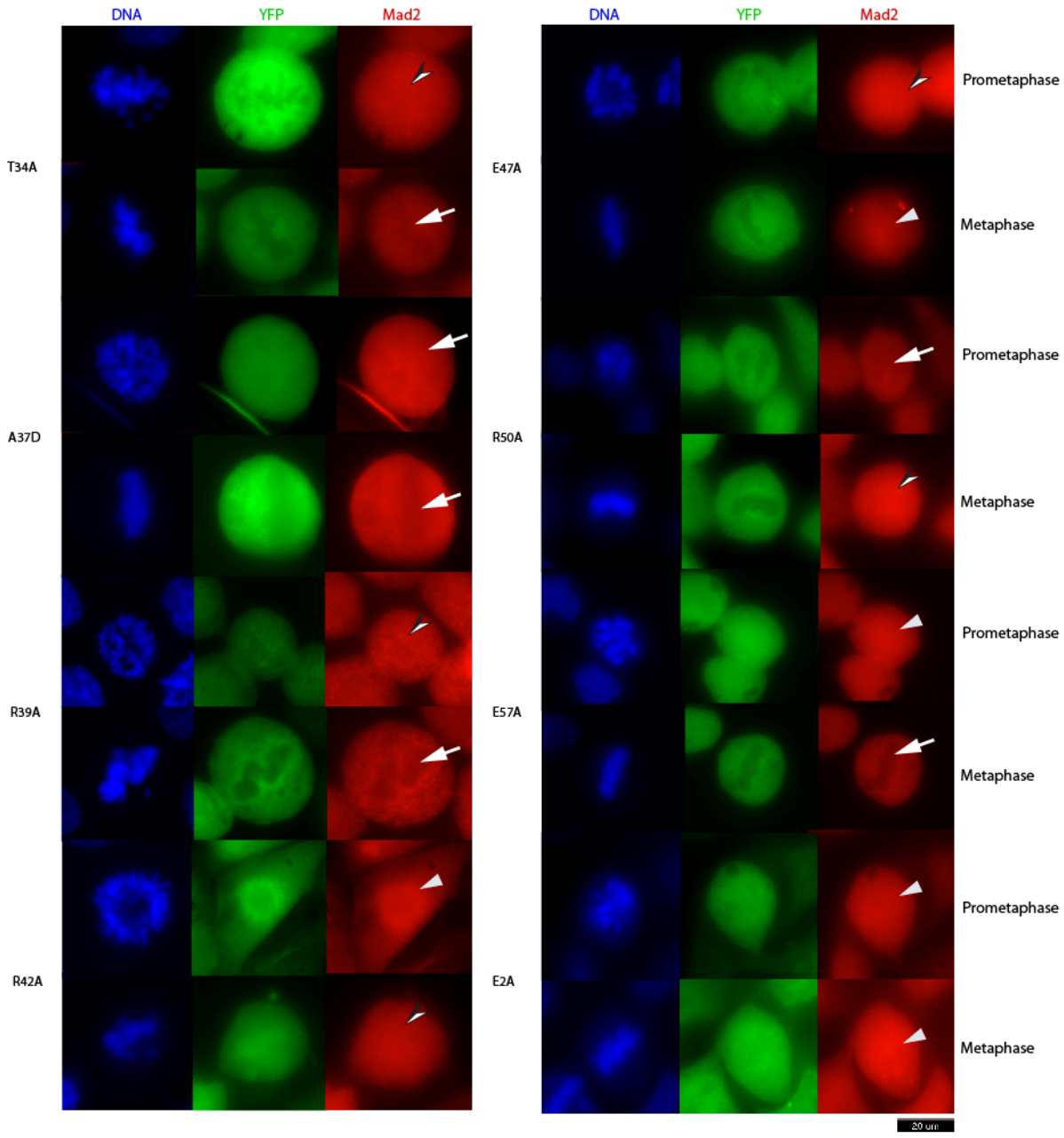


Figure 19: Mad2 in control cell lines showed differing localizations during prometaphase and metaphase.

Mad2 in YFP expressing cells demonstrated primarily cytoplasmic localization in prometaphase and metaphase. It appeared to localize perichromosomally as demonstrated by the white arrows. Mad2 in the WT control cells had a different localization pattern. Although in metaphase it localized primarily in the cytoplasm (as shown by the black and white arrow), during prometaphase, Mad2 appeared to be enriched around chromosomes (see white arrowhead).

Figure 20: Mad2 in mutant cell lines demonstrated a variety of localization patterns.

Only two mutants appeared to have similar localization patterns as that of the control cell lines. White arrows denote perichromosomal/cytoplasmic localization of Mad2, white arrowheads denote enrichment around chromosomes, and black and white arrowheads denote equal distribution of Mad2 between chromosomes and cytoplasm. Mutant A37D most closely resembled the YFP control cell line, having perichromosomal localization of Mad2 in prometaphase and metaphase. Mutant R42A appeared to have Mad2 localizing to chromosomes during prometaphase, but appearing more perichromosomal during metaphase. This was similar to the pattern seen in the WT Rev control cell line. Other mutants, however, demonstrated different patterns altogether. Mutants T34A and R39A both had equal distribution of Mad2 throughout the cell in prometaphase (black and white arrowheads) and perichromosomal localization of Mad2 during metaphase (white arrows). Mutant E47A had Mad2 localizing throughout the cell during prometaphase and around chromosomes during metaphase. R50A appeared to have Mad2 avoiding chromosomes during prometaphase and then appears equally distributed throughout the cell later in metaphase. Mutant E57A appeared at first to localize to chromosomes during prometaphase, but then appeared to localize perichromosomally during metaphase. The double mutant E2A, in both prometaphase and metaphase, appeared to have Mad2 equally distributed throughout the cell.

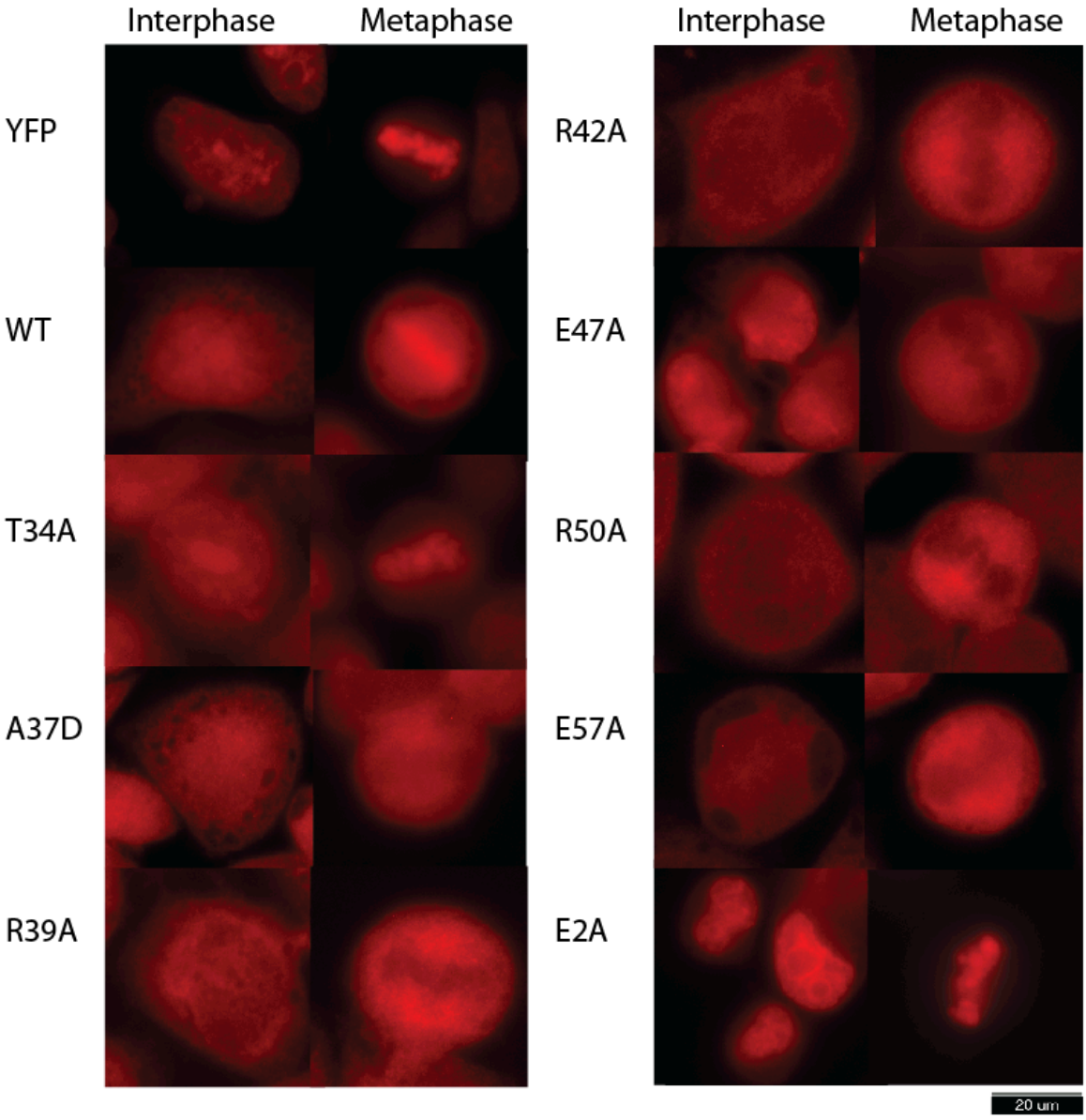

DNA
YFP
Mad2
DNA
YFP
Mad2
T34A
E47A
Prometaphase
Metaphase
A37D
R50A
Prometaphase
Metaphase
R39A
E57A
Prometaphase
Metaphase
R42A
E2A
Prometaphase
Metaphase
20 um

Ran

YFP Control cells showed an enrichment of Ran in the nucleus. Ran did not appear to have cytoplasmic localization in these cells although there must some because Ran shuttles into and out of the nucleus. The WT control, however, saw an equal distribution of Ran in the nucleus and the cytoplasm in interphase cells and, in mitotic cells, Ran only appeared to slightly localize around chromosomes [Figure 21]. WT Rev and Ran both localized to similar regions of the cell in interphase and in metaphase [image not shown]. YFP and Rev seem to have comparable phenotypes, although more Ran was seen in the cytoplasm of WT Rev expressing cells. During mitosis, there was chromosomal Ran localization seen in WT Rev and mutants T34A and E2A. The striking effect is the localization of Ran in these mutants, where in the rest of the mutants, there appears to be an increased concentration of Ran in the cytoplasm and failure to localize to chromosomes [Figure 21].

Figure 21: Control and mutant cell lines demonstrated a variety of localization patterns for Ran.

The YFP expressing cells all appeared to localize Ran to the nucleus of interphase cells. During metaphase, Ran localized around or near chromosomes as expected (see white arrow). WT Rev expressing cells appeared to have both Ran localizing throughout the cell to both the nucleus and cytoplasm in interphase (see white arrowhead). It also appeared to localize to chromosomes as well as cytoplasmic areas during metaphase (see also white arrowhead). The mutants, however, varied greatly from both of these controls. The double mutant E2A was the only mutant to express a phenotype that resembled the YFP control cell line: Ran was highly localized to the nucleus during interphase and to chromosomes during metaphase. Mutant T34A demonstrated the other extreme in which it closely resembled the WT Rev control line: Ran appeared to localize to the cytoplasm and nucleus during interphase, and it also localized to the cytoplasm, in addition to chromosomes, during metaphase. Mutants A37D and E57A fell in between these two extremes in which equal staining of Ran could be seen in interphase. But, during metaphase, Ran appeared to localize perichromosomally (see black and white arrowheads). Mutant E47A somewhat resembled the WT control in which Ran appeared to localize to nuclei, but during metaphase, Ran seemed to avoid localizing to chromosomes (see also black and white arrowheads). Mutants R39A, R42A, and R50A differed from these patterns. These mutants all localized primarily to the cytoplasm, avoiding the nucleus during interphase and chromosomes during metaphase completely (see black and white arrowheads in E57A).


Interphase
Metaphase
Interphase
Metaphase
YFP
WT
T34A
A37D
R39A
R42A
E47A
R50A
E57A
E2A
20 um

Discussion

These experiments attempted to identify amino acids that mediate the defects in cell growth when Rev is over-expressed. By doing so, it should be possible to formulate a model to explain how these defects occur. Although the mutations used in this study targeted residues shared with Kin13s, it should be possible to distinguish between other models in which Ran/importin β or B23 function were affected.

Does Rev mediate inhibition of cell growth by depolymerizing MTs by a mechanism shared with Kin13?

If this hypothesis is true, then every mutant examined should to relieve the inhibition of growth as well as moderate the defects on the MT cytoskeleton. Figure 10 confirms the former expectation insofar as each mutation shortens either the doubling time or the length of time in a stage of the cell cycle. This conclusion is tempered by the lack of statistical significance, which almost certainly is due to small sample sizes. This observation is bolstered by the data in Table 2 showing that all mutations except T34A and A37D restored mitotic indices to YFP control levels. T34A increased indices well beyond control levels although it is clear that it ameliorated the Rev phenotype. A37D appears to be an intermediate effect. It is interesting that both mutations restored the proportion of cells before the metaphase checkpoint (Table 2).

Two mutations, T34A and R50A, were found to correct for the defects seen in cells overexpressing WT Rev, indicating that these are important for WT Rev function. Immunofluorescence imaging demonstrated mutants T34A, A37D, R39A, R50A, and E2A most closely resembled the YFP control in MT cytoskeletal structure [Figure 15]. As these residues are also important for MT binding, and possibly depolymerization, it could be suggested that these defects could be attributable to Rev-MT interactions.

Amino acids E47 and E57 separately do not appear to be very important for interactions with tubulin. E57 is an important residue in Kin13s arguing that Rev and Kin13s might not be as alike as previously thought. Mutant R42A appears to worsen the effects seen by WT Rev in cells for reasons as yet unknown. In vitro studies from our lab showed that R42A does not interact with tubulin; however, it does appear to interact with tubulin in the in vivo studies. In which case, R42A may interact specifically with MTs. This data suggest that the α-helical region where these residues are important for MT and tubulin binding, but not necessarily for depolymerization. Therefore this simple hypothesis is not sufficient to fully explain Rev-MT interactions. Rather than focusing on MT depolymerization, Rev-MT binding studies should be conducted to see if Rev sequestration of tubulin could be responsible for the cellular and mitotic defects seen in this study. As these amino acids are important for MT binding, it could be that Rev is slowing MT polymerization and accelerating depolymerization by removing the free tubulin available in the cytoplasm. This observation is supported by the fact that the M4 and M6 Rev mutants do not depolymerize MTs *in vitro* also demonstrate mitotic defects in culture (Sharma, unpublished).

Another reason why Rev-MT interactions do not completely account for the defects seen in cells is due to what was seen in interactions between Rev mutant R42A and MTs. Mutant R42A in *in vitro* studies from our lab demonstrate that it is not able to bind to MTs to form RTTs (Miller unpublished). However, R42A was still able to cause severe mitotic defects as well as forming multinucleated and polyploid cells [Figure 11]. Based on these results, the Kin13 hypothesis does not completely account for the effects seen. However, as seen in previous studies, Rev's interactions with free tubulin heterodimer could be a cause for the phenotypes exhibited in the tubulin immunostaining experiments and in the growth rates.

Based on growth rates, this hypothesis is consistent with Rev causing these defects via its interactions with tubulin and MTs. All amino acid mutations were able to partially restore normal growth rates. However, R39A is an exception because while it restored growth, it had a more potent effect, which suggests that there is another factor that was not examined. This simple hypothesis therefore is not correct and will need to account for these aberrant cell lines. We would expect the arginine residues, R39, R42, and R50, to be important, as these are the residues predicted to bind to tubulin. These residues, plus a few others, are important for these interactions. A37D and T34A are not expected to be important, but both were found to restore somewhat the normal phenotype.

Does Rev mediate inhibition of cell growth by affecting the Ran-importin β axis of regulation?

Rev also interacts with nuclear import and export proteins such as importin β and Ran. This second hypothesis suggests that since Ran is also an important factor in MT nucleation at chromosomes, and because Rev can interact either directly or indirectly with tubulin, importin β, or Ran, Rev could cause the cellular effects seen in my study through these protein-protein interactions.

R42 is an important residue for nuclear localization and importin β binding (Truant and Cullen 1999). R42A is located in the NLS. This interaction between Rev and importin β is also an indirect means by which Rev can affect MTs *in vivo*. Importin β is implicated in Ran-dependent MT assembly (Wiese et al. 2001). The association of the guanine exchange factor (GEF) RCC1to chromatin sets up a gradient of RanGTP and RanGDP. This gradient is used to help direct mitotic spindle formation (Kalab and Heald 2008). In transport, Ran is responsible for also creating a gradient important for directional movement of proteins in and out of the nucleus. Importin β binding of RanGTP in the nucleus allows for importin β's cargo to be released after

nuclear entry (Wiese et al. 2001). Importin β and Ran diffuses throughout the cell in interphase. It is during mitosis where importin β and Ran both play a role in MT spindle assembly. They are both found perichromosomally and at spindle poles (Harel and Forbes 2004). During interphase and metaphase in this study, importin β and Ran are both found either localizing to nuclei or chromosomes with very little or a slight localization in the cytoplasm in YFP control cells [Figure 18, 21].

Residues R39 and R42 lie in the NLS/Importin β binding domain. In YFP Importin β and Ran have nuclear/chromosomal localization and in WT Rev cells they have an increased cytoplasmic localization. Mutant R39A neither restores YFP control cell phenotypes nor showed a WT Rev control cell line localization of importin β or Ran. Instead, R39A reversed the phenotype seen in the YFP control by having importin β and Ran localizing predominantly to the cytoplasm in interphase and the early stages of mitosis [Figure 18, 21]. Mutant R42A demonstrated a variety of effects on importin β and Ran localization. Although R42 is a residue important for importin β binding, its mutation did not restore the YFP phenotype in interphase. However, it was found to have a corrective effect when the cell entered metaphase [Figure 18]. Mutant R42A does appear to have the same effect on Ran as R39A. This mutation also reversed the effect seen in YFP control cells where Ran was mostly found in the cytoplasm. Mutations of R39 and R42 are expected to give similar results if they are working through the same mechanism. However, this does not seem to be the case as we see differing results in both mutations.

Another complication in this hypothesis is that amino acid mutations outside of this region should not show an effect. This was not the case. Mutants A37D, E47A, E57A, and the E47A/E57A double mutant restored YFP characteristics in cells in this study [Figure 18, 20]. Interactions with importin β do not appear to be consistent with the hypothesis, but Rev's

interactions with Ran might provide a reasonable explanation for why these effects are occurring since this hypothesis can explain Rev-MT interactions without Rev specifically interacting with MTs.

Although this hypothesis is also not correct, it can be modified and further tested. As Ran is an important protein for MT polymerization, Rev could be affecting MT dynamics by affecting the Ran-GTP and Ran-GDP gradients that induce MT nucleation and polymerization at chromosomes. *In vitro* assays by Watts et al. (2000) do demonstrate that spindle and aster formation is inhibited when WT Rev is present in egg extracts. These experiments could be adapted to study Rev-Ran binding, which could further elucidate the interaction between Rev and this protein and could better explain how Rev is causing the cytoskeletal and mitotic defects seen in these experiments.

Does Rev mediate inhibition of cell growth by affecting B23 metabolism?

The final hypothesis involves Rev's interactions with the nucleolar protein B23. If Rev is affecting B23, we would either see an increase in multipolar spindles and/or a general lengthening of the cell cycle. When the cell enters prophase, B23 in WT Rev cells localize to chromosomes instead of to foci within the cytoplasm [Figure 17]. Mutants T34A, A37D, R50A, E57A, and E2A do not correct for the defect caused by WT Rev. Instead, these mutants primarily localize to the cytoplasm, completely avoiding chromosomes [Figure 17]. These mutant Rev proteins appear to colocalize with B23 indicating that the Rev-B23 interaction is not disrupted by these mutations. However, in mutant T34A overexpressing cells, B23-Rev binding appears to be hindered as T34A mutant Rev localizes to chromosomes, but B23 is found only in the rest of the cell [Figure 17]. This indicates that T34 is an important amino acid for binding B23.

B23 also functions in centrosome duplication during the cell cycle (Lindstrom 2011). As the centrosome is the primary MT organizing center for the cell and is a regulator of cell cycle

progression, B23 provides another way in which Rev can affect MT dynamics indirectly. If Rev is moving B23 to other areas of the cell during mitosis, then Rev-B23 interactions could be one way in which Rev is disrupting MTs during the cell cycle. Rev binds the nucleolar protein B23 somewhere within the NLS/RNA binding region as well. Therefore, all the amino acids except E57A should be important for Rev-B23 interactions. B23 is important in ribosome maturation and centrosome duplication both of which can play a part in mitosis. If ribosome manufacture were being impaired, we would expect to see a lengthening of the time the cell spends in mitosis, which was what was seen in the cell growth experiments. Also if centrosome duplication were affected, there would also be an effect in mitosis as well.

Mutants R39A and R42A were the only two amino acid substitutions that were found to restore YFP control characteristics. A37D, E47A, R50A, E57A, and E47A/E57A did not cause an effect indicating they are not important for this interaction although they were predicted to be so. Mutant T34A had a different effect and seems to be important for B23 binding and localization. Therefore, this hypothesis is consistent with the arginine residues being important for affecting B23-Rev effects. However, it is difficult to distinguish if Rev's interactions with B23 are causing the cellular and mitotic defects seen in this study. This hypothesis, while correct, still leaves many questions to be answered. These experiments do not differentiate whether centrosome duplication or ribosome production is being affected by Rev. Centrosome imaging studies using immunolabeled centrosome proteins such as CENP-E with these Rev mutants could elucidate this B23 effect. Ribosome quantification could also be performed to see if ribosome production is affected when Rev is introduced into the cell. Rate of protein synthesis could also be measured in these cells to see if Rev-B23 interactions cause a decrease of ribosome function to induce the mitotic defects seen.

Which amino acids are important for cell growth?

Although it would be reasonable to discuss the amino acids mutated in this study according to biochemical characteristics, throughout this study the results seen did not often fall into such neat categories. Overall, amino acids T34 and R50 are important amino acids in Rev function. Mutants T34A and R50A had the most neutralizing effects observed in all the mutants according to cell growth data [Figure 10]. Both mutations also affected mitotic indices in a statistically significant manner (Table 2). In fact, the mitotic index of the R50A cell line was not found to be significantly different from the YFP control ($p > 0.3$). The R50A mutant also restored control cell importin β/Ran localization patterns [Figure 18, 21]. T34A's mitotic index, however, was found to be almost significantly lower than the YFP control ($p \leq 0.06$). The consequence of these results indicates that these two amino acids are important for Rev's ability to cause mitotic defects in cells. Mutations in T34 and R50 restored cell doubling times seen in the YFP control (Appendix 3). Both mutations were found to be statistically different in mitotic index from the WT control but not from the YFP control, indicating they more closely resembled the YFP control (Table 2). Figure 10 also shows that both mutations were able to restore YFP control times spent during phases of the cell cycle. Interestingly, these are the first and last amino acids bordering the RNA binding area/NLS, respectively [Figure 2]. The corresponding Kin13 residue of R50, R522, is important for MT binding/depolymerization (Ems-McClung et al. 2007).

Cellular abnormalities present in cells that overexpress Rev and Rev mutants

During my experiments, certain cell lines exhibited unusual phenotypes that may or may not be attributable to Rev-MT interactions. One such phenotype was the presence of multinucleated cells. Abnormal chromosome number is a main characteristic of tumor cells (Cahill et al. 1998). A possible cause for abnormal chromosome content is centrosome

duplication (Borel et al. 2002). Duplication of centrosomes is often a consequence of failure to undergo mitotic cleavage, and this duplication could allow for proliferation of centrosomes, causing cells to go through multipolar divisions (Borel et al 2002, Krzywicka-Racka and Sluder 2011). Having extra centrosomes also causes chromosomal instability, which can increase the appearance of unequal chromosome distribution on multipolar spindles (Brinkley 2001, Krzywicka-Racka and Sluder 2011).

Krzywicka-Racka and Sluder (2011) used HCT116 cells lacking p53, a tumor suppressor protein, and cultured the cells that repeatedly underwent cleavage failure. These cells resemble those seen in mutant R39A, R42A, and the double mutant E2A cell lines. What is important to note is the cell lines used in my study were made in HeLa cells. HeLa cells treated with cytochalasin D, however, were not able to survive past the third mitosis cycle when chromosome number got to be too large (Krzywicka-Racka and Sluder 2011). In cells transfected with WT Rev and certain Rev mutants, similarly sized and multinucleated cells were also formed in which the cell was of much larger size and upwards of ten nuclei could be seen [Figure 11,12]. These cells closely resembled the HCT116 cells lacking p53 treated with cytochalasin D (Krzywicka-Racka and Sluder 2011).

Multinucleation can occur naturally in HeLa, with two or three present in a single cell. These cells normally do not survive past the third mitosis cycle (Krzywicka-Racka and Sluder 2011). However, in my experiments, these cells are able to remain and grow in culture with five to ten nuclei present in the cell. These nuclei were usually smaller than normal nuclei and also had nuclei of different sizes present in one cell [Figure 11,12]. Cell lines that produced multinucleated cells were WT Rev, A37D, R39A, R42A, and E2A. In terms of severity, WT Rev had the mildest phenotype with cells having four or five extra nuclei [Figure 12]. A37D and R42A were the cell lines that had the most severely multinucleated cells having cells that had

upwards of ten nuclei present in cells. R39A and E2A fell in between WT Rev and A37D and R42A in terms of severity of multinucleation [Figure 11,12].

Limitations of this Study

From one point of view, the data obtained here are consistent with the growth defects with every residue playing an important role in affecting MT binding and depolymerization. Drawing meaningful conclusions from the results presented is difficult for several reasons. Firstly, 'cell growth' is an ill-defined metric. In this study, growth is measured by calculating doubling times, flow cytometry, and mitotic indices. Whereas each metric clearly measures growth, they do not measure identical growth parameters. Moreover, 'growth' can be estimated by casual observation when there are obvious, abnormal cell morphologies, i.e., rounded versus elongated cells, the presence of stressed phenotypes and/or by the appearance of many cytoplasmic vesicles.

Secondly, Rev expression may impact cell growth simultaneously by different and potentially overlapping mechanisms. It is difficult to separate each proposed mechanism from the other. Moreover, each amino acid targeted by mutation may not be equally important in its role for eliciting the phenotype and there may be redundancy of function.

Thirdly, the conclusions are confounded by unexpected observations. Previous results have shown that Rev over-expression results in slower doubling times, altered flow cytometry profiles, reduced mitotic indices, and potential defects before the metaphase checkpoint. Therefore, it is reasonable to hypothesize that point mutations will lessen the growth defect such that cells more closely resemble the controls. While this was often the case, there were several instances where the phenotypes of mutant cell lines were unpredictable, e.g., changes in cell shape and highly multinucleated cells.

Given the selection methods of this study, the defects seen and abnormal phenotypes could be the result of isolating and perpetuating these irregular cells. Previous flow cytometry studies by our lab have shown that between transiently and stably transfected cell lines there was no statistically significant differences, suggesting that this is not the case (N. Smith, unpublished). The presence of multinucleated cells was also seen in multiple mutants, and these cells are not distinguishable from other cells until after several doubling periods have passed.

The cellular concentrations of Rev are expected to be higher in these experiments than are relevant to HIV expression because a CMV promoter controls Rev-YFP expression. Up to a 60-fold higher level of expression can be seen in these Rev expressing cells over HIV-infected cells (Wilkinson and Akrigg 1992). It is therefore unclear how directly relevant these experiments are to conditions seen in HIV infected cells. Watts et al. (2000) showed that Rev concentrations ≈1 mg/mL are sufficient to abolish aster formation in a cell-like environment. Tenfold lower concentrations are unable did not induce large-scale aster defects. However, MT dynamics were not measured in these studies and, by analogy with drugs like taxol and nocodazole that are highly effective anti-mitotics at concentrations 1000-fold lower than that required to visible inhibit aster formation, it is plausible to presume Rev exhibits comparable effects on spindle dynamics at lower concentrations. Thus it seems reasonable to extrapolate from data these experiments to physiological conditions.

Future Directions

Even though there are correlations that could be made for how these various Rev mutants are affecting cell cycle progression in living cells, there is still much speculation as to how these processes are occurring and whether or not these proteins do indeed interact with WT Rev and the associated Rev mutants. Further deconvolution microscopy could also be done to give a clearer picture of what is happening within the cell and to provide more accurate evidence for

colocalization. To address the effect of Rev and Rev mutants on the spindle checkpoint, live cell imaging through a time course experiment could be used to further pinpoint which phase of mitosis is being perturbed as well as the mechanism by which these defects are occurring.

Also, several amino acids were found to be of more importance than others. Mutants T34A, R42A, R50A, and the E47A/E57A double mutant were found to consistently be important residues across all three studies and should be further studied. T34 and R50 are important residues for affecting growth rates in cells overexpressing WT Rev, but the mechanism by which these amino acids allow Rev to disrupt cell cycle progression is unknown. R42A and the E47A/E57A (E2A) double mutant cause more severe effects in cells than WT Rev. The location of R42 in Rev must greatly affect Rev function and is important in its interactions with other cellular entities. The E2A double mutant eliminates the two acidic residues within this section of Rev thus changing the biochemistry of the protein. These mutations have the potential to drastically affect Rev's electrostatic interactions in the cell and should be further studied.

Appendices

***Appendix 1*:** Concentrations of primary and secondary antibodies used in immunofluorescence experiments.

Experiment		**1^O Ab (conc)**	**2^O Ab (conc)**	**Manufacturers**
Western Blotting	Rev	Sheep α Rev (1:500)	HRP conjugated α Sheep (1:2000)	US Biologicals (1^O) Jackson (2^O)
	Actin	Mouse α β-actin (1:1000)	HRP conjugated α Mouse (1:100,000)	Sigma (1^O) Jackson (2^O)
	YFP	Rabbit α YFP/Living colors (1:100)	HRP conjugated α Rabbit antibody (1:100,000)	Clonetech (1^O) Jackson (2^O)
Immunofluorescence	Alpha Tubulin	Alpha tubulin antibody raised in rabbit (1:500)	Rhodamine-conjugated α rabbit antibody (1:500)	Sigma (1^O) Jackson (2^O)
	B23	B23 antibody raised in mouse (1:500)	Rhodamine-conjugated α mouse antibody (1:500)	Sigma (1^O) Jackson (2^O)
	Importin β	Importin β antibody raised in mouse (1:500)	Rhodamine-conjugated α mouse antibody (1:500)	Santa Cruz Biologicals (1^O) Jackson (2^O)
	Mad2	Mad2 antibody raised in rabbit (1:1000)	Rhodamine-conjugated α rabbit antibody (1:500)	Bethyl Labs Inc. (1^O) Jackson (2^O)
	Ran	Ran antibody raised in mouse (1:5000)	Rhodamine-conjugated α mouse antibody(1:500)	BD Transduction (1^O) Jackson (2^O)

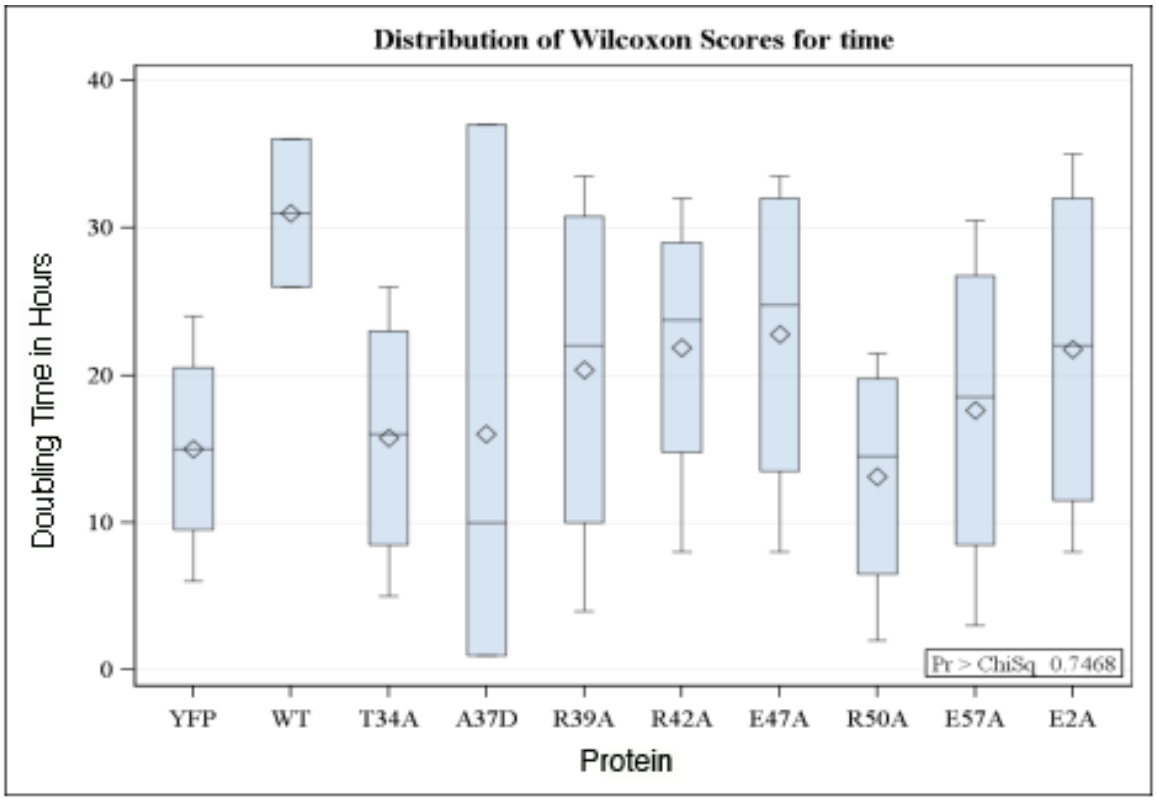
***Appendix 2*:** Raw percentages of cells of each stable cell line in G1, S, G2, and polyploid/aggregate stages. Cells were also scored for percentages of Sub-G1 cells found (not shown).

	YFPC	**WT**	**T34A**	**A37D**	**R39A**	**R42A**	**E47A**	**R50A**	**E57A**	**E2A**
G1	74.8	66.0	65.6	58.5	71.8	70.0	71.3	75.2	72.7	72.8
S	9.6	17.5	15.6	22.7	9.4	11.2	13.9	11.8	14.0	10.5
G2	13.2	15.8	15.7	13.4	15.1	18.6	13.9	11.8	10.9	15.1
Polyploid	0.20	0.85	0.75	0.33	0.89	0.35	0.34	0.31	0.62	1.1

***Appendix 3*:** Doubling times of HeLa cells stably expressing YFP, WT Rev, and mutant Rev proteins.

Raw data is shown in the table. Although there was not found to be a statistically significant difference (as determined by Chi Squared analyses) between the control cell lines and the mutant cell lines, the graph below shows a trend can be discerned in which the average doubling times of the mutants (depicted in open diamonds) was shortened compared to the WT Rev control line. A few mutants did appear to have doubling times that were very close to the YFP expressing control cell line. The horizontal line indicates the median doubling time. The error bars represent standard deviation lines.

YFP	WT	T34A	A37D	R39A	R42A	E47A	R50A	E57A	E2A
39 hrs	**61 hrs**	**37 hrs**	**51 hrs**	**47 hrs**	**50 hrs**	**47 hrs**	**41 hrs**	**44 hrs**	**52 hrs**



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