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Rev Interacts with Tubulin Heterodimers to Cause Cell Cycle Defects

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Poornima Kotha Lakshmi Narayan ENTITLED Rev Interacts With Tubulin Heterodiners To Cause Cell Cycle Defects. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Rev is a regulatory protein that plays an important role in the replication of HIV virus by post-transcriptionally promoting expression of viral proteins late in infection. Rev expression also slows cell growth, leads to an accumulation of cells in G2/M specifically before the spindle checkpoint, and can produce changes in ploidy. Because Rev is capable of depolymerizing microtubules (MTs) *in vitro,* possibly by a mechanism shared with Kinesin-13 proteins, themselves potent cellular MT depolymerases, I tested the hypothesis that these cellular defects were due to an interaction between Rev and tubulin.

To this end, Rev and select Rev mutants defective in RNA binding and nuclear import (M6), nuclear export (M10), and Rev multimerization (M4) were expressed in HeLa cells. Rev's ability to interact with tubulin was monitored by reciprocal co-immunoprecipitation experiments using antibodies specific for tubulin and the Rev transgene. Results from these experiments are consistent with this hypothesis as Rev and tubulin can be detected in the same immunoprecipitates.

To extend these results, deconvolution microscopy was used to colocalize Rev and spindle microtubules. Whereas Rev, M4, M6, and M10 fused to green or yellow fluorescent protein are largely dispersed throughout the cytoplasm of mitotic cells, the use of colocalization software indicates there is a shallow gradient of Rev accumulation proximal to the spindle. Some M6 appears to colocalize at or near spindle poles although

this is also seen in control cells. However, while these data suggest there is a potential for substantial colocalization between Rev and tubulin, visual inspection shows there is little compelling colocalization with spindle MTs. However, because immunostaining readily detects tubulin polymerized into MTs and not soluble tubulin heterodimers, the results of the colocalization and co-immunoprecipitation assays are both consistent with the hypothesis that Rev and Rev mutants are interacting with the heterodimer and not the polymerized tubulin. Intriguingly, significant amounts of wild-type Rev, M4 and M10 accumulate perichromosomally where a large fraction of spindle MTs nucleates early in mitosis. Thus Rev is spatially positioned within the cell to affect spindle assembly during early mitosis. Indeed, the previously discovered cell cycle defects of wild-type Rev, M4, M6, and M10 are all consistent with this hypothesis. Taken together, these results suggest that cells have the ability to correct spindle defects that occur during prometaphase. In conclusion, these results suggest that Rev and Rev mutants interact with tubulin heterodimers and might interfere with cell cycle progression.

Since Rev expressing cells accumulate in G2/M phase, the mitotic defects in cells expressing Rev and Rev mutants were examined. Previous research has suggested that expression of Rev and Rev mutants alters progression through mitosis with cells accumulating before the spindle assembly checkpoint. These results suggest that Rev expression may interfere with chromosomal congression and therefore alter tension across the spindle and between kinetochores. To investigate this, the distances between spindle poles and interkinetchore distances were measured in metaphase cells. No

significant differences were found between cells expressing Rev or Rev mutants and control cells.

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Introduction:

Acquired immune deficiency syndrome (AIDS) is caused by human immune deficiency virus, a retrovirus (52). After the viral entry into the host cell, its RNA genome is reverse transcribed, creating a DNA provirus that then integrates into the host chromosome (46). This provirus DNA is transcribed by the host cell polymerases to form a 9 kb long primary transcript which is alternatively spliced, forming fully spliced 2 kb mRNAs and partially spliced 4Kb mRNAs. Only 2 kb transcripts accumulate during early stages of the infection and are exported into the cytoplasm where they are translated into regulatory proteins Tat, Nef and Rev. Under-spliced 9 and 4 kb mRNA that encode the structural proteins such as GAG and Pol are not exported into the cytoplasm and translated until late in infection due to function of Rev (46).

Rev is a 13 KD protein with 116 amino acids that shuttles between the nucleus and the cytoplasm. In the nucleus Rev multimerizes through two multimerization domains onto the Rev responsive element (RRE) (5. 31, 33, 44, 46 and 68) an RNA motif with complex stem and loop structure present in the 3' intron of 9 and 4 kb viral mRNAs (4, 6, 46). In the absence of Rev function, partially spliced and unspliced 4 and 9 Kb RNA are degraded in the nucleus. However, in the presence of Rev, these RNAs are transported across the nuclear envelope via the CRM-1 export pathway to the cytoplasm where they are translated (31). It is currently hypothesized that nonproductive or latent infections result when Rev expression is low and the cell is unable to express late viral proteins (15).

Importance of studying Rev

Study of Rev is important because Rev is a potential anti-viral HIV target (11). Since Rev is a regulatory protein that facilitates the expression of viral structural protein, inhibition of Rev function should inhibit HIV infection. In fact, certain Rev mutations such as M10, a mutation that inactivates the nuclear export sequence, exert *trans*-dominant inhibition of HIV infection by forming inactive mixed multimers with the wild-type protein. It is believed that one mutant Rev molecule is sufficient to effectively inactivate a multimer containing number of wild-type Rev molecules (11, 31).

Functional domains of Rev:

Rev protein can be divided into discrete *N*- and *C*-terminal domains. The *N*-terminal domain is rich in arginine residues and contains both a nuclear localization sequence that promotes nuclear import and an overlapping RNA binding domain that binds to the RRE (6, 9, 22, 28, 31, 33, 44, 45, 46, 67 and 68). The arginine-rich region is flanked on both sides by multimerization domains (5, 31, 33, 44, 46 and 68). Rev multimerization on the RRE is essential for RRE export. At least three or four Rev monomers are required for mutimerization and proper Rev function. Multimerization deficient mutants exhibit defective RNA export and do not induce conformational changes in the RRE that normally occur (46). The *C*-terminus contains a nuclear export signal (16, 38, 46 and 63) which when disrupted produces a protein that localizes to the nucleus, binds and multimerizes to the RRE but is not exported into the cytoplasm (10, 23, 46, 53 and 56). This region is also called the activation domain because it activates the expression of late viral proteins (16, 46).

The presence of separate nuclear import and export sequences allows Rev to shuttle in and out of the nucleus. The import sequence promotes the binding of importin β, a protein import receptor, to Rev (54). This Rev-receptor complex is then efficiently transported into the nucleus through nuclear pore complex by the nucleotide-regulated activity of the G-protein Ran (46). In the nucleus, the arginine-rich region of Rev binds and multimerizes on the RRE (17, 18, and 46). The nuclear export signal then stimulates the binding of Ran-GTP and the export receptor CRM1 (also called Exportin1) to the Rev-RRE (46). CRM1 belongs to the importin β family of transport factors (17). The CRM1/Rev-RRE/Ran GTP complex is transported into the cytoplasm where nucleotide bound to Ran is hydrolyzed and the complex dissociates, liberating underspliced viral RNA.

Biochemical Properties of Rev:

In vitro, purified Rev protein polymerizes to form two distinct structures in a concentration dependant manner. In the presence of RNA it forms poorly ordered filaments that are about 8 nm in diameter. Filament length is proportional to the length of the RNA (61). In the absence of RNA, Rev forms ordered filaments with an outer diameter of 15nm (20, 61 and 65) that have a tendency to aggregate, a property that has hindered attempts to solve its three dimensional structure (61). Nonetheless, the *N*-terminal half of the protein is believed to form a helix-loop-helix structure based on analyses of wild-type and mutant proteins using circular dichroism, NMR, Raman spectroscopy and computer modeling (61 and 62).

Rev, being positively charged (pI 9.2), not only binds to the RNA but also to other polyanions like polyguanylate, polydeoxyguanylate and polyglutamate, possibly through its arginine-rich motif (62). Rev filaments are effectively depolymerized by these polyanions suggesting that their addition may limit aggregation. When Rev is mixed with taxol stabilized microtubules (MTs), themselves composed of acidic proteins α and β tubulin (pI 4.8-5.2), bilayered rings called Rev tubulin toroidal complexes (RTTs) form (62). RTTs resemble rings formed when MTs are disassembled by cooling (36 and 62) or exposed to drugs like the dolastatin-10 (2 and 62). RTT formation is not due merely to simple electrostatic interactions as toroids form at different pH. Even at pH 8 where the charges of Rev are neutralized, RTT formation is not affected. RTTs also form when tubulin's *C*-terminal polyglutamate tails are removed with subtilisin showing that Rev-tubulin interactions are more complex than simple electrostatic interactions (62). Watts et al. (62) showed that Rev depolymerizes MTs formed in *Xenopus* egg extracts, further suggesting that Rev-MT interactions can occur in cellular environments. They observed that Rev inhibited 94% of aster formation and the few asters that formed were abnormally small. MT asters in these assays were induced by addition of chromatin. The addition of chromatin creates a locally high concentration of RAN-GTP due to the presence of RCC1, a chromatin RAN nucleotide exchange factor. RAN-GTP interacts with importin β thus releasing the NLS containing proteins like TPX2 and NuMA that are essential for the chromatin mediated MT nucleation (3, 19). These observations collectively suggest that Rev might have a distinct and specific mechanism by which it can bind and depolymerize MTs in cells.

Sequence similarity of Rev to Kinesin 13 proteins:

A mechanism that explains Rev-MT interaction is suggested by a limited sequence similarity between Rev and the motor domain Kinesin 13 family of kinesins (formerly called Kin I and Kin M) distinguished by the unique "internal" or "middle" location of the motor domain (Figure 1). In contrast, the motor domains of most kinesins are present either at the *N*- or *C*-terminus (62). Specifically Rev shares sequence similarity with loop11-α helix 4-loop12 (L11-α-4-L12) of MCAK (mitotic centromere associated kinesin, formerly called XKCM1) and XKIF2 (Figure 1) (42 and 51). MCAK is a potent MT depolymerizing agent that plays an important role in the MT dynamics during mitosis. Depletion of MCAK in *Xenopus* egg extracts in the presence of chromatin results in abnormally large MT asters with centrally located chromatin (12 and 57). These results are reversed upon the re-addition of MCAK, indicating that MCAK is important for the spindle assembly and maintenance. (12 and 57).

MCAK binds the ends of the MT, where it induces conformational changes in tubulin subunits, stimulating depolymerization in an ATP independent manner (12). ATP hydrolysis triggers release of the tubulin from the tubulin dimer/MCAK complex (12). The minimal region required for depolymerizing activity of MCAK is the motor domain and the adjacent 64 amino acids called the neck (35). Rev, like MCAK depolymerizes microtubules form both ends (62). As seen in Figure 1 many amino acids that are shared between Kin13 kinesins and Rev are not present in the motor kinesins indicating that these amino acids might be critical for MT binding and depolymerization. In Rev, the shared hydrophilic residues are surface exposed on the c-terminal α-helix residues (e.g.,

R39, R42, R44, E47, R50 and E5). Similarly these shared residues of Kin 13 are also exposed on the surface of α4 helix. In kinesins, these residues are important for MT binding (42 and 51) Studies of the protozoan homologue of MCAK pKinI (Plasmodium falciparum KinI) revealed that aminoacids KEC (268-270) are important for depolymerization activity. Similarly mutation of glutamic acid in KEC in MCAK inhibits the depolymerization activity (14). E57 in Rev is homologous to the glutamic acid in the KEC motif of the Kin13 kinesins suggesting its importance in the depolymerization of MT. A second glutamic acid E47 may be similarly important for MT depolymerization. R39 and R42 involved in RRE binding are conserved among the Kin 13 are speculated to be involved in MT binding. The depolymerization activity of Rev could be attributed to the region of Rev that is similar to MCAK (62). Should this be true, then the analysis of Rev-MT interaction will provide an insight into the mechanism of Kin-13 proteins.

Two distinct populations of MCAK are present in the cell. This includes the soluble cytoplasmic MCAK that is essential for maintaining the MT dynamics during interphase and the centromeric MCAK that is essential for spindle assembly and maintenance during mitosis. Depletion of centromeric MCAK results in the chromosomal misalignment along the metaphase plate which shows its importance in proper alignment of chromosomes (58). Tension across the sister kinetochore also plays an important role in the alignment of chromosomes along the metaphase plate. Less tension across the sister kinetochore is an indication for improper attachment to the opposite spindle poles (69). Experimental studies by Kline-Smith et al. (27) have shown that depletion of MCAK results in 17-36% decrease in the interkinetochore stretch. The reason for this

decrease might be the influence of MCAK on MT dynamics (27). Also it was shown that chromosomes display reduced centromere stretch under conditions of reduced MT dynamics (60). With this in mind, it is possible that Rev that has sequence similarity with MCAK might have an effect on the interkinetochore distance. It will be useful to test this hypothesis as it will provide an insight on Rev's effect on MT dynamics during mitosis.

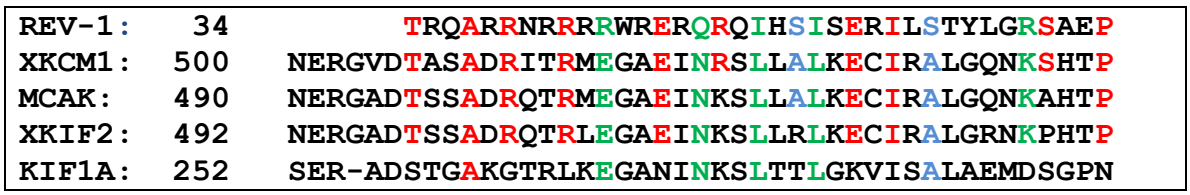


Figure 1 Similarity between Rev and Kinesin 13 proteins Sequence

Identical amino acids are highlighted in red, very similar amino acids are highlighted in green and similar amino acids are highlighted in blue (adapted from Watts et al., 2000 [62] and Ogawa et al., 2000 [42]). XKCM1 is the *xenopus* homolog of MCAK, XKIF2A, the Xenopus paralog of MCAK and a member of Kin 13 family. KIF1A is a motile kinesin.

Significance of studying Rev-MT interaction:

The observation that Rev can bind and depolymerize MT *in vitro* suggests that over-expression of Rev may affect MT dynamics during HIV infection. The addition of Rev to *Xenopus* extracts inhibits aster formation, showing that Rev affects MT dynamics in cell-like environment (62). These effects on aster formation, coupled with Rev's ability to form RTTs, suggest that Rev may bind tubulin *in vivo* and affect MT formation. It is also possible that Rev might interfere with MT dynamics by sequestering tubulin heterodimers by a mechanism similar to Stathmin/OP18 (62). Stathmin is a phosphoprotein that results in MT depolymerization by either sequestering the tubulin heterodimers or by triggering the MT catastrophe at the MT ends (25). If Rev interacts and depolymerizes MT *in vivo*, then Rev derived peptide might be used as a potential anti-mitotic drug.

Previous data from our lab have shown that Rev can be co-immunoprecipitated using tubulin specific antibodies from HeLa cell extracts transfected with Rev expression plasmid, suggesting that there is physical interaction between Rev and tubulin (39) However this interaction was not seen when Rev specific antibody was used. This observation is substantiated by a limited colocalization of Rev and MT detected by immunofluorescence staining (29) However, the latter results were not conclusive given the broad intracellular distribution of Rev and the limited resolution of epifuorescence microscopy. Higher resolution microscopy should provide more useful information on the potential colocalization of the two proteins.

Flow cytometric analysis of HeLa cells stably and transiently expressing wild-type Rev (wt Rev) have shown accumulation of cells in G2/M phase of cell cycle (Smith, N., personal communication). This is consistent with the results of Miyazaki et al. (40) who observed Rev over-expression in COS-7 cells results in its accumulation in G2/M and prophase stages of the cell cycle (40). Because Rev is known to interact with the nucleolar protein B23 and Rev expression also deforms nucleoli, Miyazaki et al. (40) suggested that these defects might be due to Rev inhibiting B23 function. B23 is a major nucleolar phosphoprotein and is important in the maturation of ribosomal proteins (47). Consequently, Rev expression may create cell cycle defects by indirectly interfering with ribosome metabolism. Altrnatively the accumulation of Rev expressing cells in G2/M phase might be attributed to Rev's potential to bind and depolymerize MTs.

More recently, B23 was shown to be involved in centrosome duplication (43, 50). It localizes to the unduplicated centrosome early in G1 until it is phosphorylated by cyclin E dependent CDK2 kinase when it dissociates from the centrosome. Dissociation appears to trigger the centrosome duplication (43, 50) as blocking of B23 phophorylation inhibits centrosome duplication, producing monopolar spindles. Loss of B23 is also partially cytotoxic as cells are observed to undergo apoptosis which might be due to defective mitosis (43). Therefore some of the cell cycle defects observed following Rev over-expression may be due to Rev inhibiting B23 function in centrosome duplication.

In total, there are many mechanisms by which Rev over-expression can create cell cycle defects. They are summarized below and in Table 1.

1. Rev may directly bind MTs and alter their dynamics. The fact that Rev depolymerizes MTs *in vitro* suggests that Rev may similarly depolymerize MTs *in vivo* (62). Since Rev also binds tubulin heterodimers (62), it is possible that over-expression of Rev affects MT dynamics by sequestering tubulin by a manner similar to stathmin (59).
2. The observation that Rev shares sequence similarity with MCAK suggests that Rev has a potential to bind the same site on tubulin as MCAK. Consequently, Rev may synergize or inhibit the activity of the cell's most potent MT depolymerase. If true, Rev expression may either affect MT dynamics by displacing MCAK or binding to the same regions of MTs as MCAK, thereby enhancing MT depolymerization.
3. Rev may affect cell progression by interacting with B23, which might result in reduced rates of translation or altered centrosome duplication. If latter is true, then B23's ability to target centrosomes may be affected and/or the interaction between Rev and B23 might result in the formation of monopolar spindles.
4. Since cells over-expressing Rev do not progress well through mitosis, it is possible that Rev is interacting with cellular proteins important for chromatin-mediated nucleation of spindle MTs. Two such proteins include importin β and Ran. Rev interacts directly with importin β and indirectly with Ran during its translocation through the nuclear membrane (54). Importin β binds NuMa and Tpx2 that are critical in the formation of normal spindle during mitosis (55). NuMa and Tpx2 are released during prophase due to a sharp Ran-GTP gradient

originating from condensing chromosomes (8). It is therefore possible that Rev may be inducing mitotic defects through its interaction with Importin β causing defects early in spindle formation.

To test some of these hypotheses, I propose to search for physical interaction between Rev and its effector proteins by immunoprecipitation. I will also use immunofluorescence to detect whether Rev expression affects normal cellular localization of the effector protein and determine whether there is any colocalization between Rev and its binding partners.

Lastly I will repeat these experiments in cells expressing well characterized Rev mutants that are predicted to interfere with Rev's ability to bind its effector proteins. The Rev mutants employed in this study are M4, M6 and M10 first described by Malim and Cullen (33). The M4 mutation (YSN→DDL, amino acids 23, 25 and 26) inhibits Rev's ability to multimerize and form high molecular weight complexes on the RRE. M4 localizes more in the cytoplasm than the wild-type protein but it is able to localize to the nucleolus. Examination of the interaction between M4 and tubulin and/or MT in vitro shows that M4 can bind tubulin heterodimers and MT but can neither depolymerize stabilized MTs nor form RTTs (Sharma, A. personal communication.) However, M4 expression still causes cell cycle defects (Smith, N., personal communication).

The M6 mutation (^{41}RRRR→DL) inhibits nuclear import by abrogating Rev's interaction with importin β. Consequently, M6 is predominately localized to the cytoplasm although there is significant nuclear accumulation owing to Rev's ability to diffuse through nuclear pore complexes. Moreover, this mutation reduces Rev's affinity

for B23 (38, 40). *In vitro* analysis has shown that M6 reduces Rev's ability to bind purified tubulin heterodimers and polymerized MTs (Sharma, A. personal communication). However it still affects the ability of the cell to progress through mitosis (Smith,N., personal communication).

The M10 mutation (^{78}LE→DL) inhibits nuclear export of Rev by reducing Rev's ability to bind the export factor Crm1 (exportin 1) (46), a member of the importin β of transport factors. The M10 mutant accumulates almost exclusively to the nucleolus. Presently there is no data available that assess M10's ability to bind tubulin and depolymerize MTs although it is predicted not to affect either based on the observation that the amino acids 1-59 lacking the NES are sufficient to interact with tubulin (62).

Possible effects of Rev in over-expressed cells are shown in Table 1 and possible interaction between Rev and its mutants with tubulin and other cellular mitotic proteins are summarized in Table 2.

Table 1 Possible effects of Rev in over-expressed HeLa cells

Nature of interaction of Rev with the cellular protein	**Role of the cellular protein**	**Possible effects of Rev over-expression**	**Expected observation *in Vivo***
Physical interaction with tubulin	MT is required for the formation of spindles during mitosis , cellular trafficking	Abnormal spindle formation. Activates check point proteins	Abnormal spindle formation. Activates check point proteins like BubR1
Physical interaction with B23	B23 is required for the maturation of the ribosomal proteins, centrososme duplication	Abnormal centrosome duplication, slow growth	Cells with monopolar spindles. Abnormal chromosome congression
Physical interaction with Importin β	Importin β is required for the normal spindle formation and nuclear import	Abnormal spindle growth, activates check point protein and apoptosis	Poor spindle formation and spindle growth
Compete with MCAK for binding site on the tubulin and depolymerize MT	MCAK is required for normal spindle dynamics, chromosome movement	Abnormal spindle growth. Alteration in MT dynamics	Abnormal spindle growth
Synergize with MCAK to depolymerize MT	MCAK is required for normal spindle dynamics, chromosome movement	Abnormal spindle growth. Altered spindle dynamics and chromosomal congression	Abnormal spindle growth

Table 2 Possible interaction between Rev and its nutants with Tubulin and other cellular mitotic proteins

protein	wt-Rev	M4	M6	M10
Tubulin	+++	+++	+	+++
B23	+++	++	-	+++

+++ Strongly interacting, ++ Moderately interacting, + Weakly interacting, - not interacting,

Specific Aims

I aim to confirm cellular interactions between wild-type and mutant Rev proteins and effector proteins and correlate these interactions with changes in cell cycle progression, MT behavior and subcellular localization of effector proteins. To this end, I propose to

1) Assess Rev-MT interactions in HeLa cells by looking at the frequency of physical interactions between Rev and tubulin using co-immunoprecipitation, and deconvolution microscopy. It is essential to show that Rev and these proteins interact *in vivo* because it might provide a clue for a potential role of Rev in alteration of microtubule dynamics in HIV infected cells. Since Rev and MT interact *in vitro* causing depolymerization of microtubules, it is logical to see if these interactions have any significance *in vivo*. Rev M4, M6 and M10 that are deficient in multimerization, nuclear import and nuclear export respectively, were used to identify the different domains that are involved. This will give an insight as to which domain has the most impact on the MT binding and depolymerization.

2) To document the impact of Rev over-expression on centrososme duplication. Deconvolution microscopy was used to observe any alteration in the localization of the

cellular proteins that might result in the presence of Rev. For this purpose normal HeLa cells were compared with those that were over-expressed with Rev/mutants. Colocalization assay also provided information on various proteins interacting with Rev. Rev mutant M4, M6 and M10 were used to determine the various domains involved in the interaction.

3) To quantify the effects of wild-type and mutant Rev expression on interkinetochore tension and spindle formation.

Materials and Methods:

Cloning. The coding sequence of wild-type Rev and the mutants were obtained from pRevG9 where Rev coding sequences were subcloned from pXRGG (30) in frame with GFP encoded with pEGFP N1. The coding region was fused to the N terminus of YFP encoded by the pEYFP-N1. The Rev's coding sequence was excised from pRevG9 using unique restriction enzyme Kpn1 and Nhe 1, gel purified and ligated into pEYFP-N1 vector previously linearized with Kpn1 and Nhe1. The ligation mixture was transformed into DH5α cells and plated on LB agar containing 10 μg/ml concentration of kanamycin. Kanamycin-resistant colonies were grown in 10 ml LB-kan overnight cultures and plasmid DNA was isolated and sequenced (Davis Sequencing, Davis, CA). Rev-YFP and its mutants were expressed in HeLa cells and the localization pattern was observed to check if the fluorescent tag did not alter the gene expression.

Over-expression of Rev in HeLa. HeLa cells were plated on coverslips, 24 hours prior to transfection, in 100 mm dish containing DMEM media with high glucose, 10% fetal bovine serum, 100μg/ml penicillin, 100μg/ml streptomycin at 37□C in a humidified atmosphere with 5% CO_2. Transfection was done using 100 μl of the polyfect transfection reagent (Qiagen), 6 μg of pERev-YFP/ YFP M4, M6 and M10. After 24 hrs of incubation the cells were immunostained as described below.

Immunofluoresce staining. HeLa cells were plated on coverslips in 100 mm dish containing DMEM media with high glucose and 10% fetal bovine serum 24 hours prior to transfection. Transfection was performed as above. Fourty-eight hours post

transfection the cells were rinsed briefly in 1X PBS (38 mM sodium phosphate monobasic, 162mM sodium phosphate dibasic and 150mM sodium chloride, pH- 7.2) and fixed for 15 min in freshly prepared 4% paraformaldehyde dissolved in 1X PBS. Cells were washed thrice for 5 min each in 0.5% NGS (normal goat serum) in 1X PBS and then permeabilized with 0.2% Triton X-100 in 1X PBS. Primary antibodies were added for 1 hour at room temperature. The cells were then washed in 0.5% NGS in 1X PBS three times 5 min each, followed by the addition of secondary anti-species-specific antibodies for 1 hour at room temperature. The primary and the secondary antibodies used and the concentrations at which antibodies were used are listed in Table 3 and Table 4. Cells were then washed with 0.5% NGS in 1 X PBS three times 5 min each. The cells were then stained with DAPI and mounted using PPD media.

Light Microscopy. The subcellular localization of Rev and microtubule was examined using DeltaVision RT Microscope (Applied Precision) using Olympus IX70 inverted microscope, with the assistance of Dr. Paula Bubulya. SoftWoRx Explorer Image Viewer software was used to collect digital images. Images were acquired by collecting a stack of 0.5 μm increments in the Z-axis in all the required filters. Such a stack consists 7.5 μm-18 μm (15-36 sections) total depth. Stacked images were then compressed for display. The images were saved as TIFF images. Individual Z-images were also examined to confirm results obtained from projected images. The extent of colocalization between Rev and MT was analyzed using Velocity software.

Co-Immunoprecipitation. HeLa cells were seeded at 1 x 10^7 in a 100 mm dish. Forty-eight hours after transfection cells were washed with 5ml of 1X PBS and scraped

off the plate using a rubber policeman and 1 ml 1X PBS. The cells were collected in 15 ml conical and spun at 1500 rpm for 5 min. The cell pellet was transferred into a 1.5 ml tube and spun at 2000 rpm for 2 min. Excess PBS was removed and 1 ml lysis buffer (300mM NaCl, 100mM tris pH-8, 0.2mM EDTA, 0.1%NP40, 10% glycerol, protease inhibitor cocktail) was added. Samples were placed in a rotator at 4°C for 45 mins. The lysate was then spun at 2000 rpm for 10 min. The supernatant (whole cell extract) was collected and aliquoted in 3 tubes and snap frozen and stored at -80°C until use. 300 µl aliquots were diluted 3 fold with dilution buffer (100mM tris pH-8, 0.2mM EDTA, 0.1%NP40, 10% glycerol, protease inhibitor cocktail) to dilute the concentration of the salt to 100 mM. Protein G coated agarose beads were added to the extract and incubated at 4°C for 30 min. The beads were removed by centrifugation and discarded. The Appropriate primary antibody was added to the supernatant at specified dilution (Tables 3) and incubated at 4ºC with rocking for 1 hr. The supernatant was recovered, 50 µl of G plus agarose beads was added, vortexed and incubated at 4ºC with rocking for 2 hrs. This was followed by centrifugation at 16,000 g in a microfuge for 15 sec. Both bound and unbound fractions were saved for further examination. The bound fraction was washed twice with TE buffer (1M tris HCl , pH 7.4, 10mM EDTA). 50µl of 2x gel sample buffer (124mM Tris HCL pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% SDS, 0.05% bromophenol blue) was added to 50µl each of bound and unbound fraction. Each sample was boiled for 5 min, the beads were spun down by centrifugation at 14,000 g for 10 min and the supernatants were resolved by 12% SDS-PAGE at 35mA per gel for 1 hr. Proteins were transferred to nitrocellulose membranes for 2 hrs at 100

V, 100 mA in 1 x transfer buffer (25mM tris HCL, 190 mM glycine and 20% methanol). Blots incubated with blocking buffer (5% donkey serum, 0.1% NaN_3 in 1X TBS-T) for 1 hr and then incubated in blocking solution with diluted primary antibody in blocking solution for 1 hr. After washing the membrane in TBS-T (TBS, 0.1% Tween, pH 7.8) 3 times for 10 min each, the blots were incubated in solution containing secondary antibody conjugated to horseradish peroxidase in blocking solution for 1 hr followed by 3 washes 10 min each with TBS-T. Blots were then developed by incubating in Pierce, super signal west pico chemiluminescent substrate/ enhancer for 5 min and then imaged with LAS 4000 Fuji chemiluminescence. If necessary the blots were stripped in stripping buffer and re probed for another protein and re developed as described above.

Table 3 Concentration of the primary antibodies used in immunofluorescence and immunoprecipitation experiments

Experiment description	Primary antibody used/ (Manufacturer)	Working antibody concentration
Co-immunoprecipiation	• Rev antibody raised in sheep (US Biological) • Rev antibody raised in rabbit • α- tubulin antibody raised in mouse(Sigma Aldrich)	1:500 1:500 1:500
Colocalization	• α tubulin antibody raised in mouse	1: 1000
Western blot analysis	• α tubulin antibody raised in rabbit (NeoMarkers) • Rev antibody raised in sheep • Rev antibody raised in rabbit	1: 2000 1: 2000 1: 1000

Table 4 Concentration of the secondary antibodies used in immunofluorescence and western blot analyses.

Name of the experiment	Type of Seondary antibody used (Jackson Laboratories)	Concentration of the antibody
Co-immunoprecipitation	• None used	
Colocalization	• Texas Red conjugated Donkey anti mouse antibody • Texas red conjugated donkey anti rabbit antibody • Texas red conjugated donkey anti goat antibody • Cy 5 conjugated donkey anti mouse antibody	1:500 1:500 1:500 1:500
Western blot analysis	• HRP conjugated donkey anti mouse antibody • HRP conjugated goat anti mouse antibody • HRP conjugated donkey anti goat antibody	1:100,000 1:100,000 1:100,000

Results:

Specific Aim 1:

Rev Interacts with tubulin:

I used co-immunoprecipitation (co-Ip) assays to study the interaction between Rev and MTs *in vivo*. This assay demonstrates the interaction between two proteins when one protein is isolated using a precipitating antibody specific for another protein. The presence of the antigen and its co-purifing partners can be detected by western blot analysis. To test the hypothesis that Rev is binding to tubulin in cells, whole HeLa cell extracts (WCEs) transfected with Rev-YFP were prepared. WCRs were then immunoprecipitated using DM1a antibody that is specific for α-tubulin. Precipitates were then resolved by SDS-PAGE. Control WCEs prepared from untransfected cells and HeLa cells transfected with YFP were also used as negative controls. As shown in Fig 2a, tubulin precipitation is inefficient. Significant amounts of the tubulin are present in the unbound fraction. When these blots were reprobed using Rev-specific antibody detectable amounts of Rev were co-immunoprecipitated with the tubulin (Figure 2a right panel). This is consistent with the hypothesis that Rev is interacting with Rev in live cells.

To test whether the multimerization, the RRE binding and activation domains of Rev are involved in the interaction with tubulin, I repeated the co-Ip in cells transiently transfected with M4-YFP, M6-YFP or M10-YFP DNA. WCEs were prepared and tubulin was immunoprecipitated with DM1a, resolved by SDS-PAGE, and subjected to immunodetection As expected, tubulin was immunoprecipitated in each extract (Figure

2b-2d). When the blots were re-probed with Rev-specific antibody, detectable amounts of each mutant Rev were present in each experiment sample. This is consistent with the hypothesis that Rev is physically interacting with tubulin despite the presence of M4, M6 and M10 mutations. Thus mutation in any one of these domains does not block tubulin binding.

To confirm these results, I performed the reciprocal co-Ip using Rev-specific antibody. Rev was readily immunoprecipited from WCEs (Figure 3). When these blots were re-probed with DM1a, tubulin was detected in Rev-expressing cells (Figure 3). However, faint bands were also detected in both HeLa and YFP controls suggesting that the Rev antibody cross react with tubulin. Similar results are obtained when co-precipitating from M4, M6 or M10 expressing cells (Figure 3b-3d). Although the amount of bound tubulin present in Rev expressing samples was greater than that of the controls suggesting interaction between Rev and tubulin, the background signal seen in the controls suggest that the Rev antibody cross reacts with the tubulin. To clarify these results, co-Ips were repeated using a GFP antibody that should precipitate the Rev-YFP fusion protein. The results shown in Figure 4 suggest that very little tubulin is present in the GFP precipitates. Trace amounts of tubulin are present in precipitates from cell WCEs expressing Rev that seem not to be present in the non-expressing controls. However, these signal strengths are so close to background that a definitive conclusion is not possible

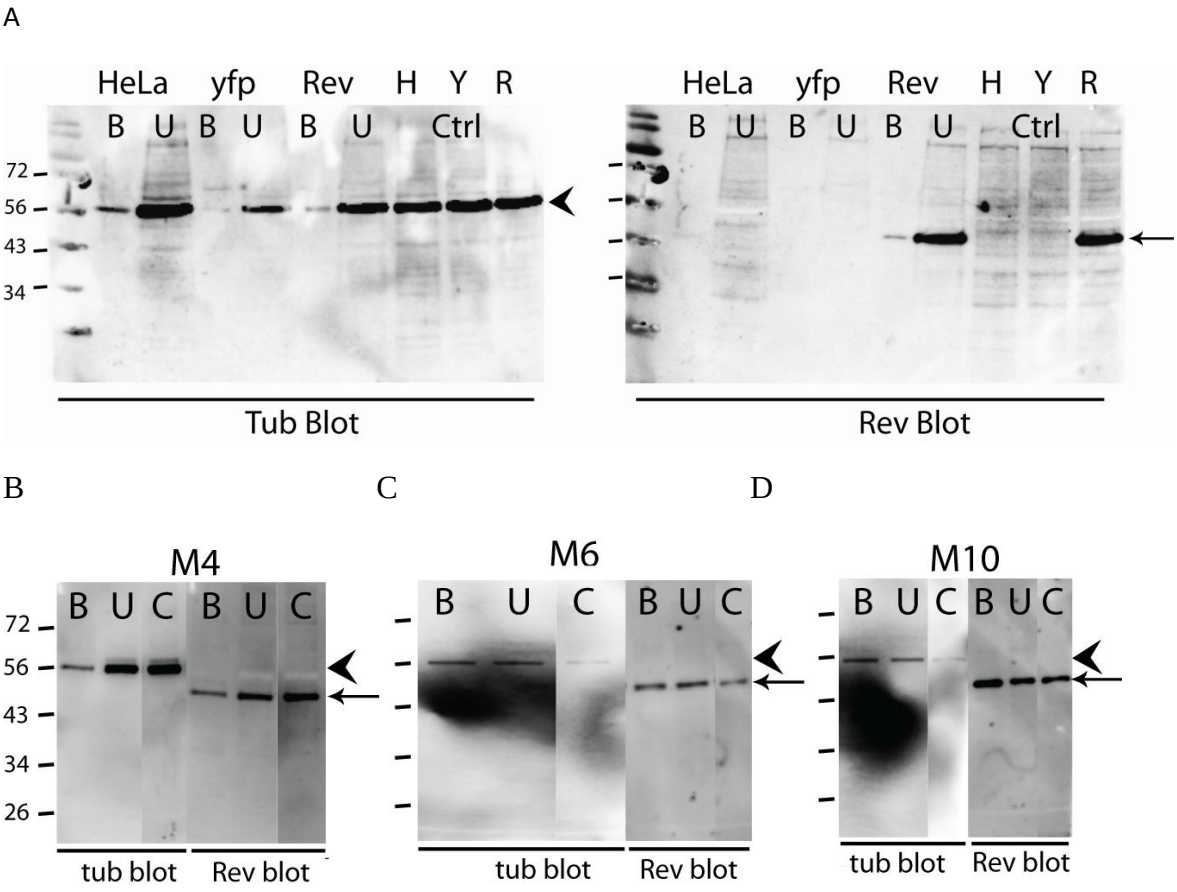


Figure 2 Rev is present in tubulin precipitates

A. Proteins were immunoprecipitated using antibodies specific for α tubulin as discussed in the "Materials and Methods. Immune complexes bound (B) and not bound (U) to the antibody were resolved by SDS PAGE. WCEs prepared from untransfected HeLa cells (H), and transfected with YFP (Y) or Rev-Yfp (R) were also electrophoretically resolved and serve as positive controls for immunobloting. Proteins were transferred to nitrocellulose and probed with DM1a (Tub blot). The location of tubulin is shown by an arrowhead. The blot is stripped and re-probed with Rev-specific antibody (rev Blot). The location of Rev is shown by the arrow. Molecular weight standards renging from 72 kDa to 34 kDa are shown in the left.

B, C and D. Proteins were immunoprecipitated using tubulin specific antibodies from WCEs prepared from HeLa cells transfected with the mutants M4, M6 and M 10 respectively. WCEs not subjected to immunoprecipiation were resolved by SDS-PAGE and provide positive controls for blotting (C). The convention for labeling these figures is the same as 2A.

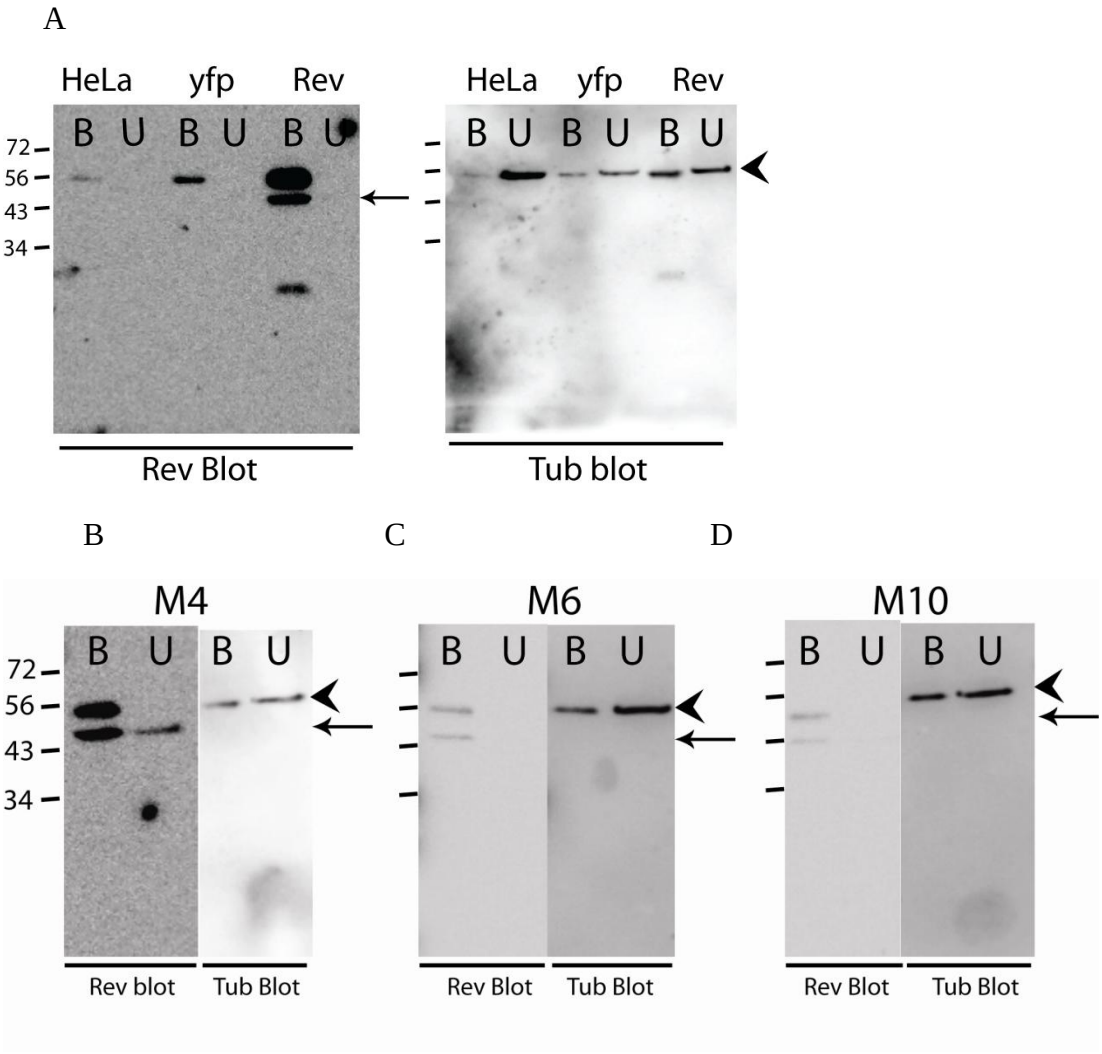


Figure 3 Tubulin may be present in the Rev immunoprecipitates

A. Proteins were immunoprecipitaed using Rev specific anitbodis and detected using antibodies specific for Rev (Rev blot) or tubulin (Tub blot) as described in the "Materials and Methods." The figure is labeled according to the convention described in Figure 2.

B, C and D. Mutant Rev was immunoprecipiated using Rev specific antibody from HeLa cells transfected with the mutants, M4, M6 and M10.

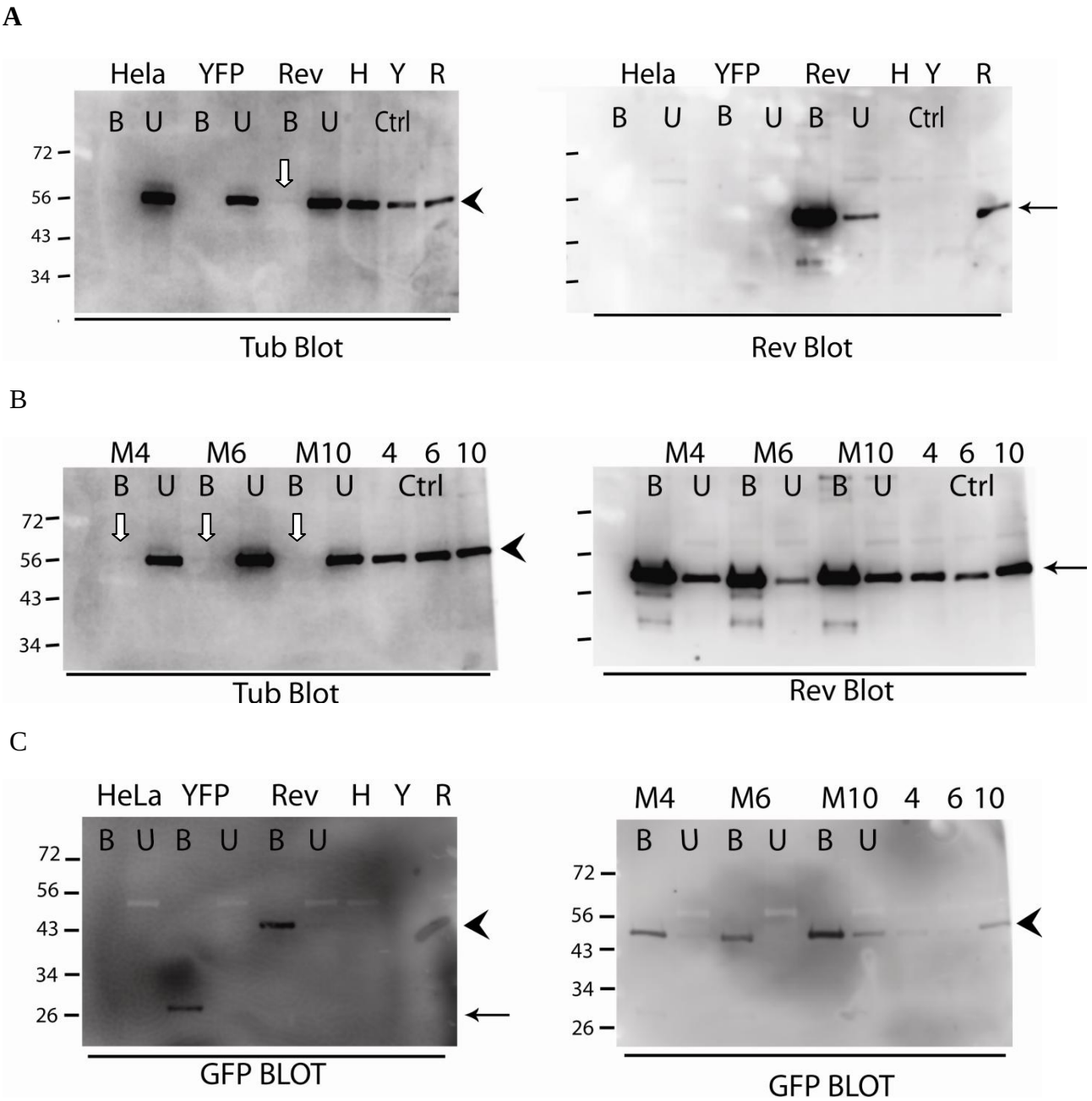


Figure 4 Trace amounts of tubulin present in GFP immunoprecipitates

A. Proteins were immunoprecipitated using GFP specific antibodies and detected using antibodies specific for Rev (Rev Blot) or tubulin (Tub Blot) as described in the "Materials and methods." Trace amount of Tubulin (white arrow) was detected in the Rev YFP precipitates. The figure is labeled using convention described in Figure 2.

B and C Mutant Rev was immunoprecipiated using GFP specific antibody from HeLa cells transfected with the mutants, M4 YFP, M6 YFP and M10 YFP. Trace amounts of Tubulin was detected (white arrow) in the bound fractions of the precipitates.

Specific Aim 2:

Colocalization between Rev and tubulin

During interphase Rev primarily localizes to the nucleus with substantial amounts localizing in the nucleoli (e.g., 13). In contrast, tubulin exclusively localizes to the cytoplasm, suggesting that there is limited opportunity for both the proteins to interact in interphase. Conversely, the nuclear envelope breaks down during mitosis allowing the nucleoplasm and cytoplasm to mix. To detect colocalization between Rev and MTs, HeLa cells stably expressing fluorescently tagged Rev were immunostained for tubulin and examined using deconvolution microscopy.

Figure 5A and 5B are representative images of HeLa cells stably expressing Rev GFP. Rev is widely distributed throughout the cytoplasm of mitotic cells through their distinct perichromosomal accumulation (arrow). This has been observed previously (13) and may be driven by the Ran-GTP gradient that originates with chromatin (19). Given the localized distribution of MTs to the spindle, there is a substantial amount of Rev close to the spindle although visual inspection suggests there is no compelling colocalization of Rev and spindle MT. To determine the extent that Rev and MT may colocalize in an unbiased manner, Z-series stack of images were analyzed using fluorescent softWoRx colocalization software. Figure 5C shows a representative correlation between the intensities of tubulin and Rev on a pixel by pixel basis. The Pearson coefficient of correlation is therefore a measure of potential colocalization. The region highlighted in red indicates those pixels with highest probability of colocalization. As is evident from

the corresponding image (Figure 5D), these pixels are closely adjacent to the spindle. The average Pearson coefficient of correlation of three images is 0.712.

Because potential toxic effects may be attenuated in cell lines stably expressing Rev HeLa cells transiently transfected with Rev were also examined. Similar results were seen when HeLa cells were transiently transfected with Rev-YFP, n=8. Figure 6A is a representative image showing perichromosomal accumulation (arrow). The perichromosomal accumulation is relatively low in this cell due to the low expression of Rev. As before visual inspection shows no compelling colocalization between Rev and tubulin. As with stable cell lines, the extent of Rev-MT colocalization was determined using SoftWoRx colocalization software and Rev was accumulated with spindle. The average Pearson coefficients of correlation of three images were 0.748 which was comparable with that seen in stable cell lines. These results collectively suggest that there is a potential for Rev to colocalize with the tubulin.

Likewise Rev mutants were also examined to see if there is an interaction between the mutants and tubulin, n= 4. Figure 7-9 are representative images showing HeLa cells transiently transfected with Rev M4, M6 and M10 respectively. In general the co-localization results for mutants were similar to wt-Rev. Pearson coefficients of correlation suggest the possibility of colocalization, but there is no compelling colocalization that can be detected. On average the Pearson coefficients of correlation for M4, M6 and M10 are 0.614, 0.503 and 0.610 respectively In both Rev M4 and M10 expressing cells the perichromosomal accumulation is not greatly affected by the mutations. On the other hand, though M6 has a diffused dispersal. There is limited

perichromosomal accumulation and distinct enrichment near the spindle pole (Figure 8A arrow). This phenomenon was observed in almost 75% of the cells expressing Rev M6 suggesting that the NLS region might be important for perichromosomal accumulation. Occasionally, finger-like projections appeared to be decorate the spindle MTs (Figure 8B arrow).

Figure 10 and 11 depict representative images of control untransfected and YFP transfected HeLa cells respectively. Figure 11B shows that similar to Rev M6 YFP has a diffused localization with considerable amounts seen at close proximity to spindle pole (arrow) and is also seen by Hutchins et al., 2009 (26). Colocalization analysis suggested that there is potential for YFP to colocalize at the spindle (Figure 11C). The average Pearson coefficient of correlation is 0.594. Comparison of wt-Rev/mutants with YFP suggested that the potential colocalization seen with the wt-Rev/mutants might not be true localization.

Rev expressing cells may have mitotic defects

Since a statistically larger fraction of Rev expressing cells accumulate in G2/M than control cells, cells expressing wt-Rev and mutant Rev were examined for detection of any obvious mitotic defects (Smith, N., personal communication). HeLa cells stably expressing Rev-GFP were first examined. However, it was difficult to identify misaligned chromosome for static images and moreover, stable cell lines attenuate the possible toxic effects of Rev. For the same reason, HeLa cells transiently transfected with wt-Rev or mutant Rev were stained with antibody specific for kinetochore which that will help identification of misaligned chromosomes

Figure 12 is representative image showing stable Rev expressing HeLa cell. Each panel is a single section of Z-series of images that displays a misaligned chromosome (arrow). Similar results were observed with transiently transfected HeLa cells. Figure 13 is representative image of HeLa cell transiently transfected with Rev YFP. The image displays misalignment of chromosome that is detected by the kinetochore staining (arrow). Figure 14-16 are representative images of HeLa cells transiently transfected with M4, M6 and M10 respectively. Chromosomal misalignment (arrow) is seen in cells expressing either M4 or M6 mutant. Representative images showing control untransfected and YFP transfected cells in Figure 17 and 18, respectively however show there are misaligned chromatids. Given the small sample size of this study n= 5, it is difficult to determine whether these defects are due to Rev expression.

Specific Aim 3

Rev effects on centrosome duplication:

To observe whether Rev/mutants have any effect on the centrosome duplication by interfering with centrosomal proteins, like B23, HeLa cells transfected with Rev and Rev mutants were examined. Antibody specific for γ-tubulin that allows the visualization of centrosomes was used to immunostain HeLa cells. Figure 19 is representative image of HeLa cell transfected with Rev YFP showing the presence of 3 centrosomes (arrow). Figure 20-22 show representative images of HeLa cells transfected with M4, M6 and M10 respectively. These cells do not show any alteration in the number of centrosomes present in the mitotic phase. However, control HeLa cells transfected with YFP plasmid (Figure 24) show the presence of three centrosomes (arrow) suggesting that the defect

seen with wt-Rev could be an artifact. A larger sample might provide conclusive results. Figure 23 is control untransfected HeLa that displays normal centrosomes.

Rev effects on spindle tension:

To test whether the Rev and Rev mutant expressing cells show any difference in tension across the spindle, interkinetochore distance as well as the spindle pole distance was measured and was compared to the untransfected control HeLa cells, n~10. Likewise to detect any difference in the spindle tension in mutant expressing cells the measured interkinetochore and spindle pole distance were compared to the wt-Rev expressing cells. Statistical analysis were used detect significant difference. The results of the measurements are summarized in the Table 5. A general ANOVA test suggests that there is a significant difference in the group. However, a post hoc testing using Tukey's HSD suggested that there is no statistically significant difference except the interkinetochore distance of M10 when compared to HeLa had a P value close to 0.05. A larger sample size will provide conclusive results.

Table 5 Spindle tension measurement

Interkinetochore distance measurement:

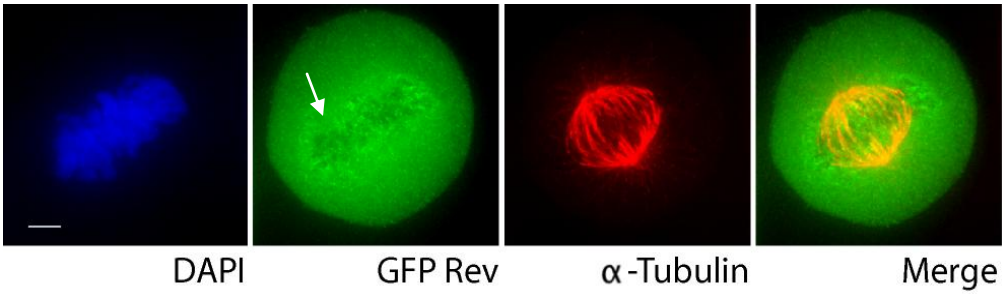
Measurement	HeLa	YFP Rev	M4	M6	M10	YFP
Average distance (μm) (St. dev.) n~10	0.987 (0.135)	0.906 (0.198)	0.984 (0.188)	0.885 (0.139)	0.745 (0.279)	0.926 (0.241)
Tukey's HSD Vs HeLa	NS	P>0.05	P>0.05	P>0.05	P~0.05	P>0.05
Tukey' HSD Vs Rev	NS	NS	P>0.05	P>0.05	P>0.05	P>0.05

Spindle Pole distance:

Measurement	HeLa	YFP Rev	M4	M6	M10	YFP
Average distance (μm) (Std. dev.) n~10	9.379 (3.288)	11.23 (2.850)	7.65 (1.488)	7.47 (1.358)	7.94 (1.233)	8.00 (1.130)
Tukey's HSD Vs HeLa	NS	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
Tukey's HSD Vs Rev	NS	NS	P>0.05	P>0.05	P>0.05	P>0.05

NS= Not significant

A



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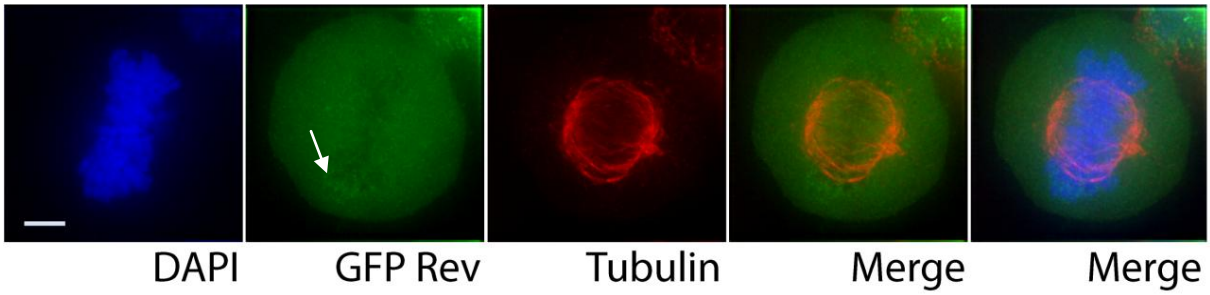

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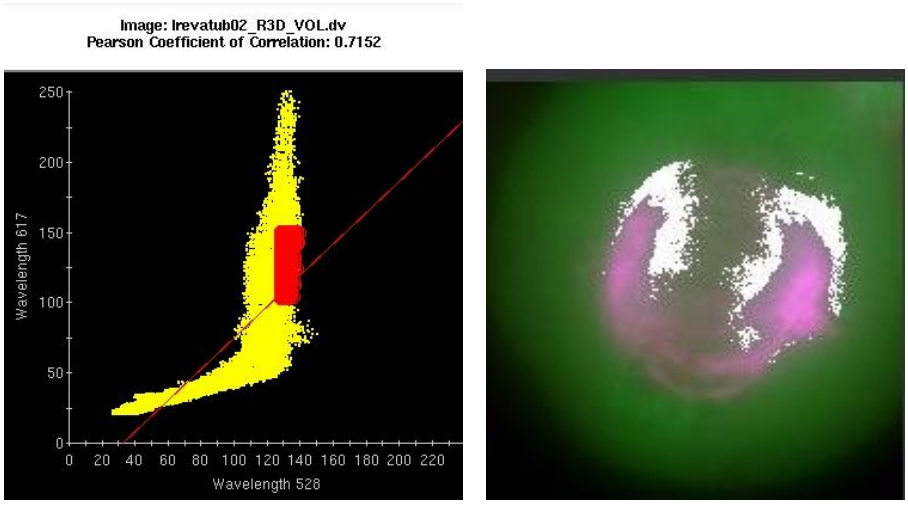
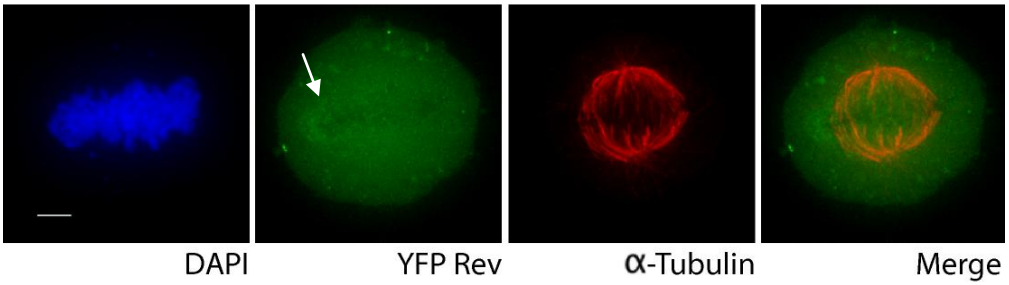
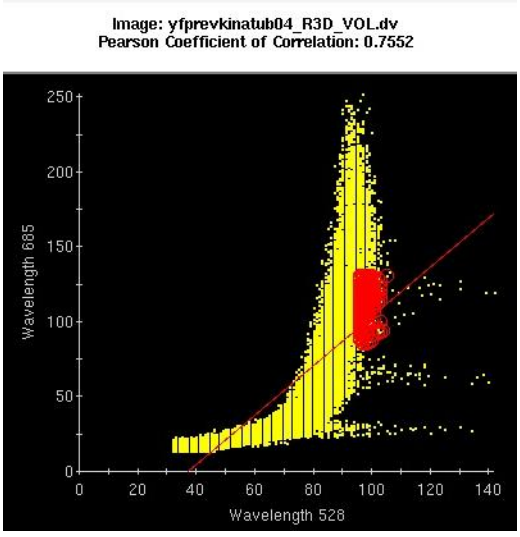


Figure 5 Colocalization between stably expressed Rev and tubulin in a HeLa cell
A and B HeLa cells stably expressing Rev-GFP were immunostained DAPI and tubulin specific antibody. Each of the panels (Left to right) displays DAPI, GFP Rev, tubulin and merge channel that shows the Rev-MT colocalization interaction. Perichromosomal accumulation is shown by arrow. C. Representative graph that was produced as a result of analysis with SoftWoRx colocalization measurement software. The region highlighted in red shows highest region of colocalization.

A



B C



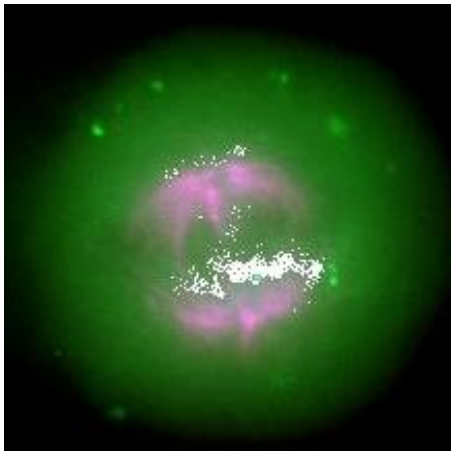
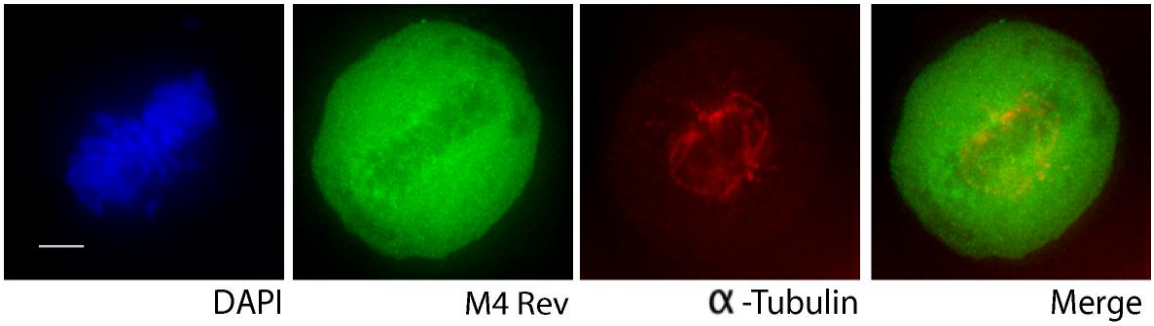


Figure 6 Colocalization between transiently transfected with Rev and tubulin in HeLa cells

Representative image of HeLa cell transiently transfected with Rev-YFP. The image is labeled according to the convention in Figure 5

A



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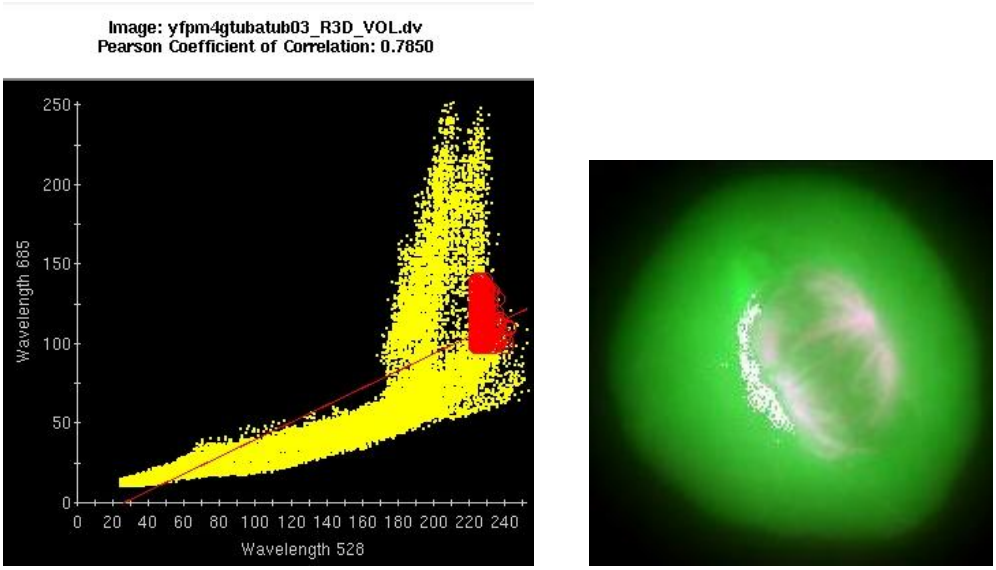
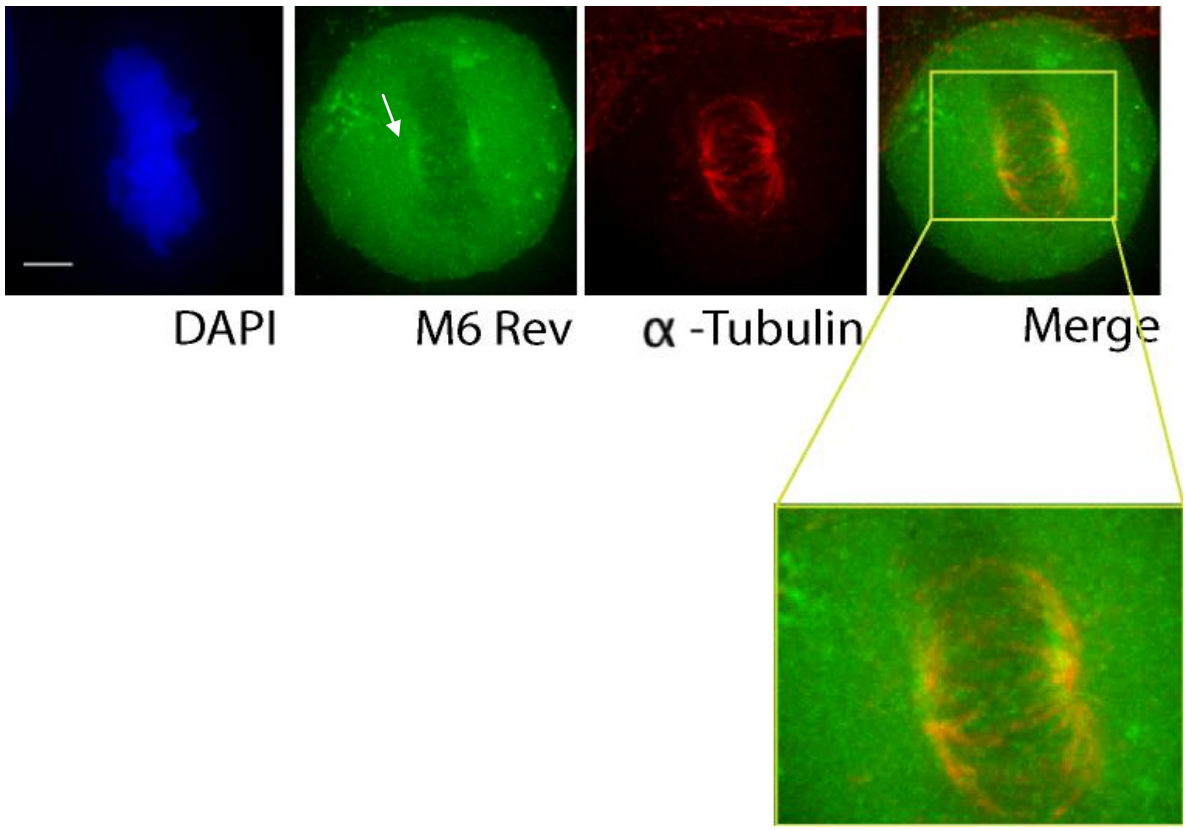


Figure 7 Colocalization between transiently transfected with M4 Rev and tubulin in a HeLa cell

Representative image of HeLa cell transiently transfected with YFP tagged M4 Rev. The image is labeled according to the convention in Figure 5

A



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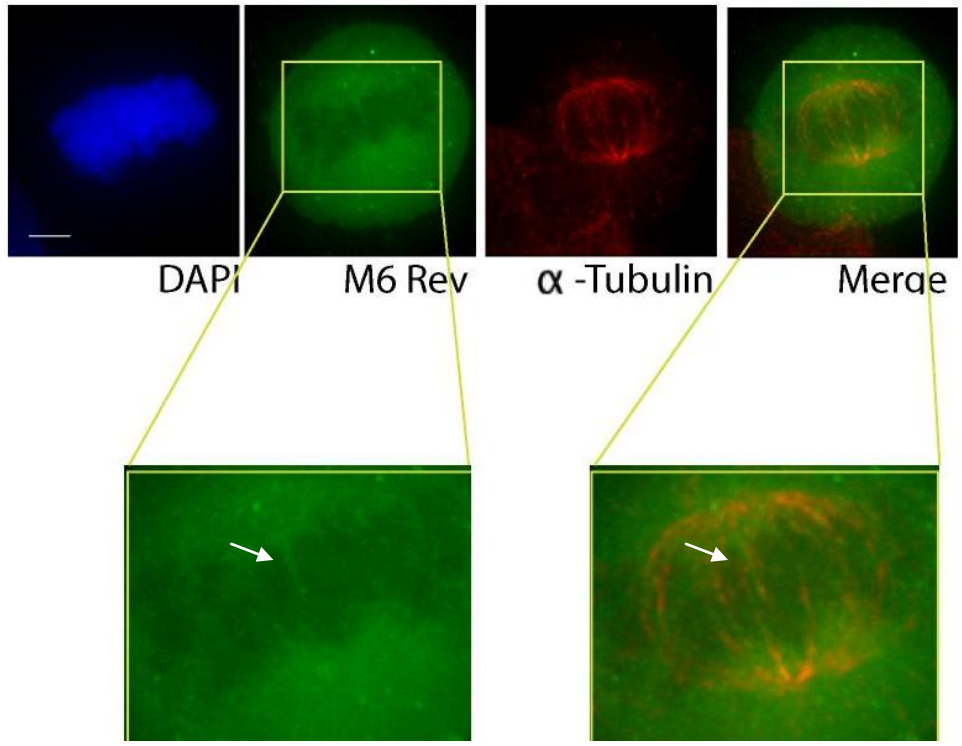

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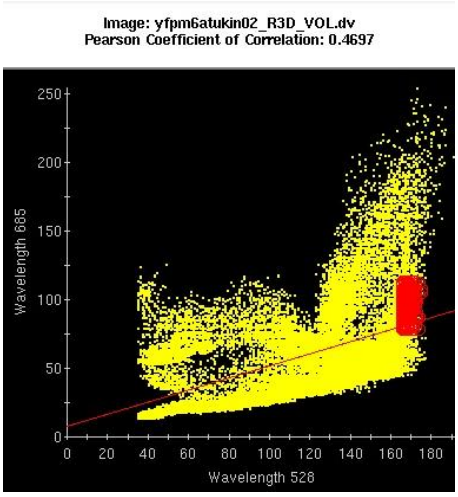


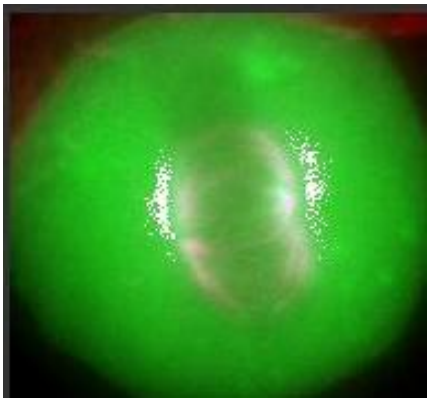
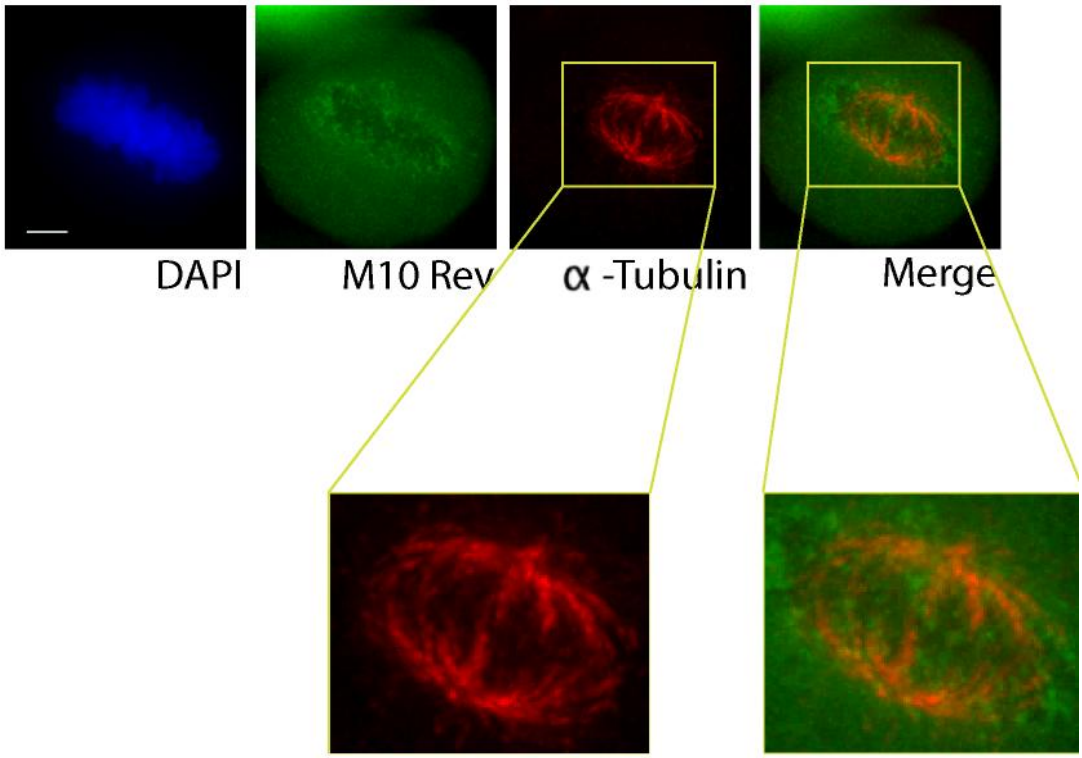


Figure 8 Colocalization between transiently transfected with M6 Rev and tubulin in a HeLa cell

Representative image of HeLa cell transiently transfected with YFP tagged M6 Rev . The enarged panel in A and B shows the Rev-MT colocalization. The image is labeled according to the convention in figure 5

A



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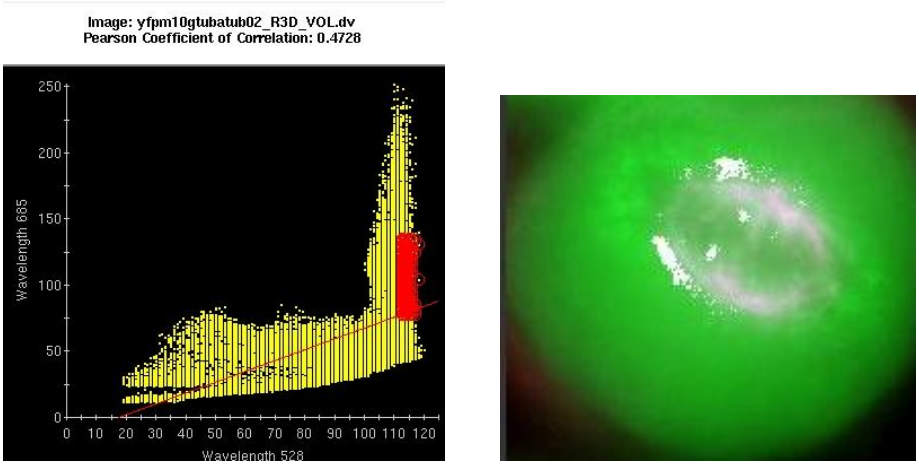


Figure 9 Colocalization between transiently transfected with M10 Rev and tubulin in a HeLa cell

Representative image of HeLa cell transiently transfected with YFP tagged M10 Rev The image is labeled according to the convention in Figure 5

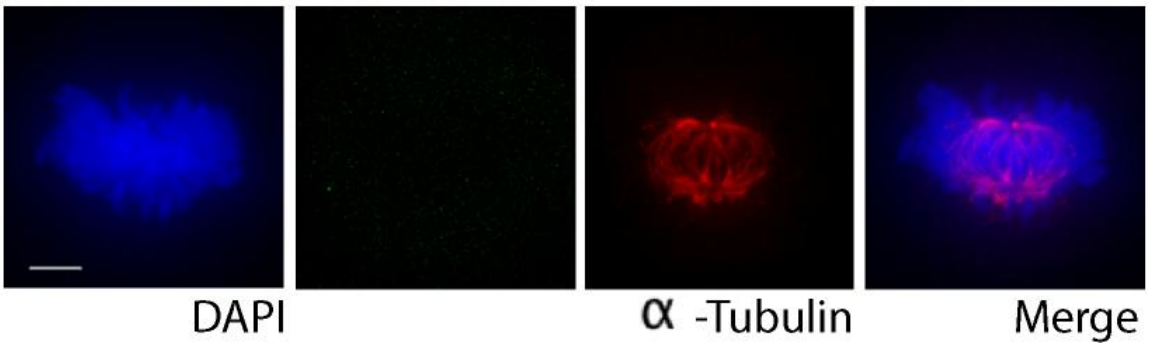
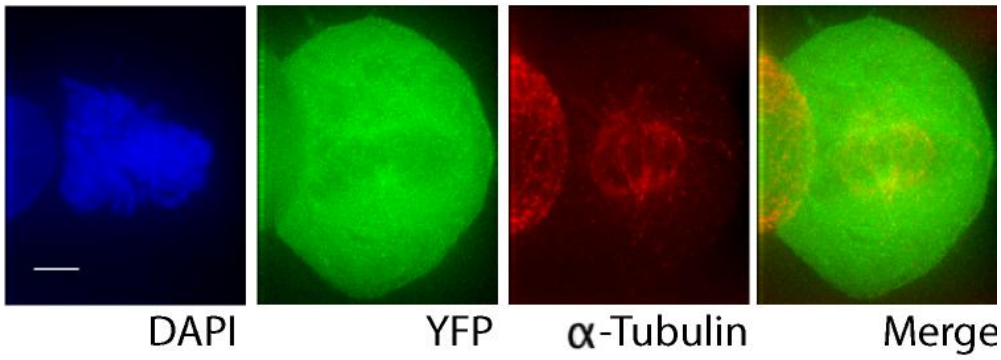


Figure 10 Untransfected control HeLa cell
Representative image of untransfected HeLa cell stained for DAPI and tubulin.

A



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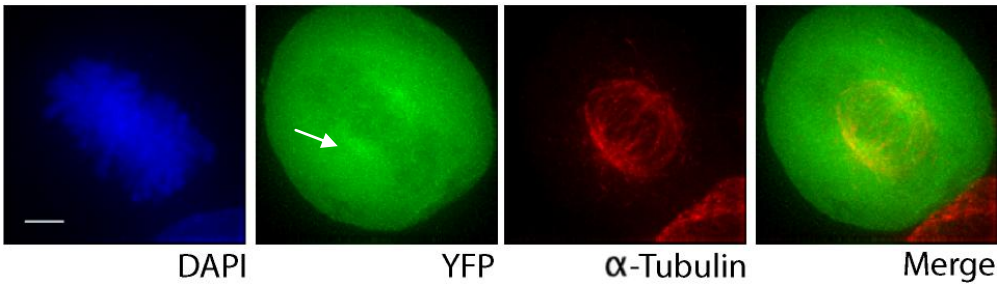

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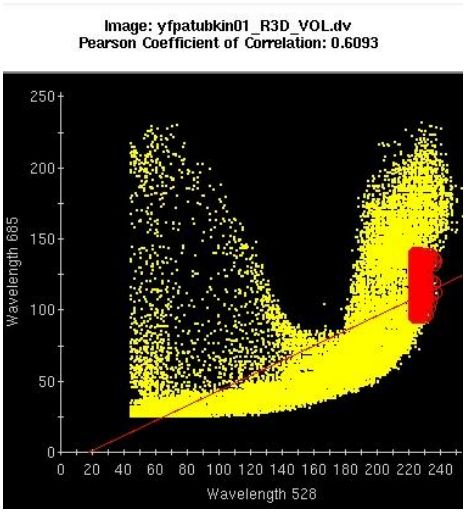


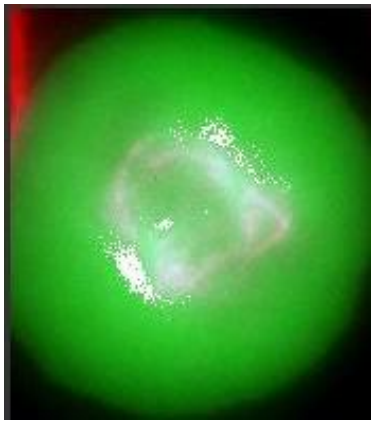


Figure 11 Colocalization between transiently transfected YFP control and tubulin in a HeLa cell

Representative image of HeLa cell transiently transfected with YFP. The image is labeled according to the convention in Figure 5

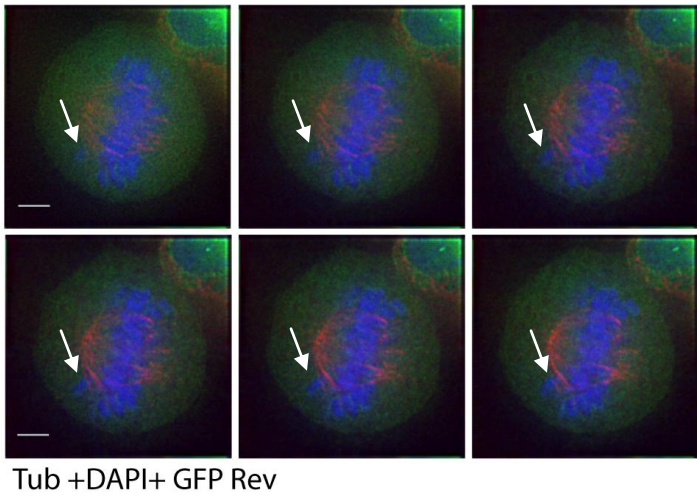


Figure 12 Visualization of mitotic defects in HeLa cells stably expressing GFP Rev
Representative image showing HeLa cells stably transfected with Rev GFP. Each panel shows a single section of z-series of the cell. A potential lagging chromosome is shown by arrow.

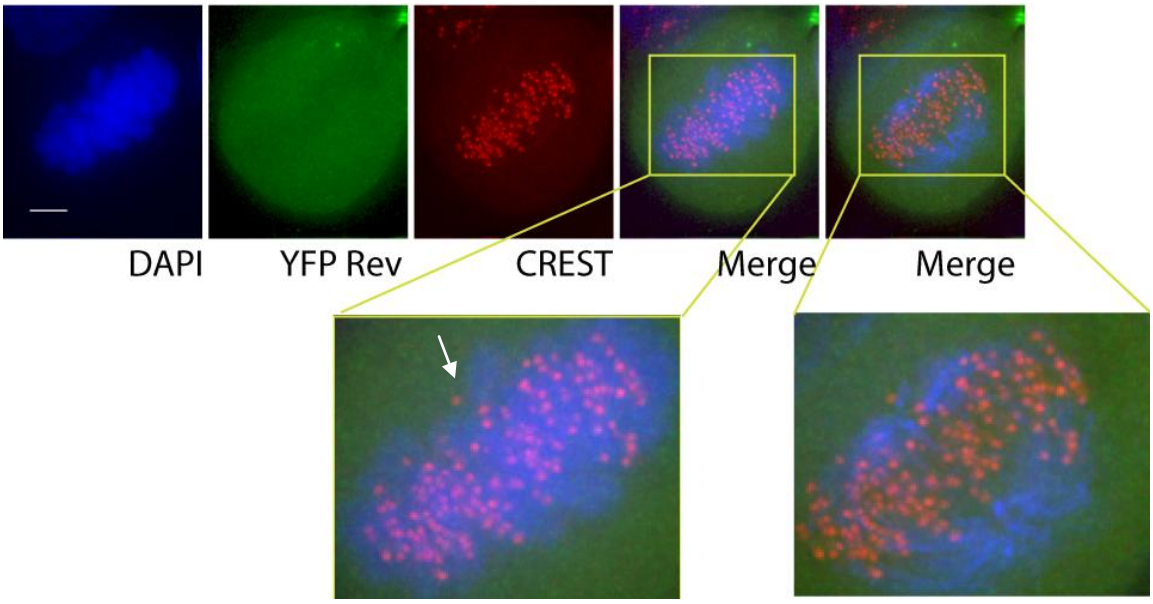


Figure 13 Visualization of mitotic defects in HeLa cells transiently transfected with Rev YFP

Repreentative image of HeLa cell transiently trnasfected with Rev YFP. The cell is stained for DAPI , CREST (specific for kinetochore), and tubulin. The lagging chromosome is shown by an arrow in the enlarged panel

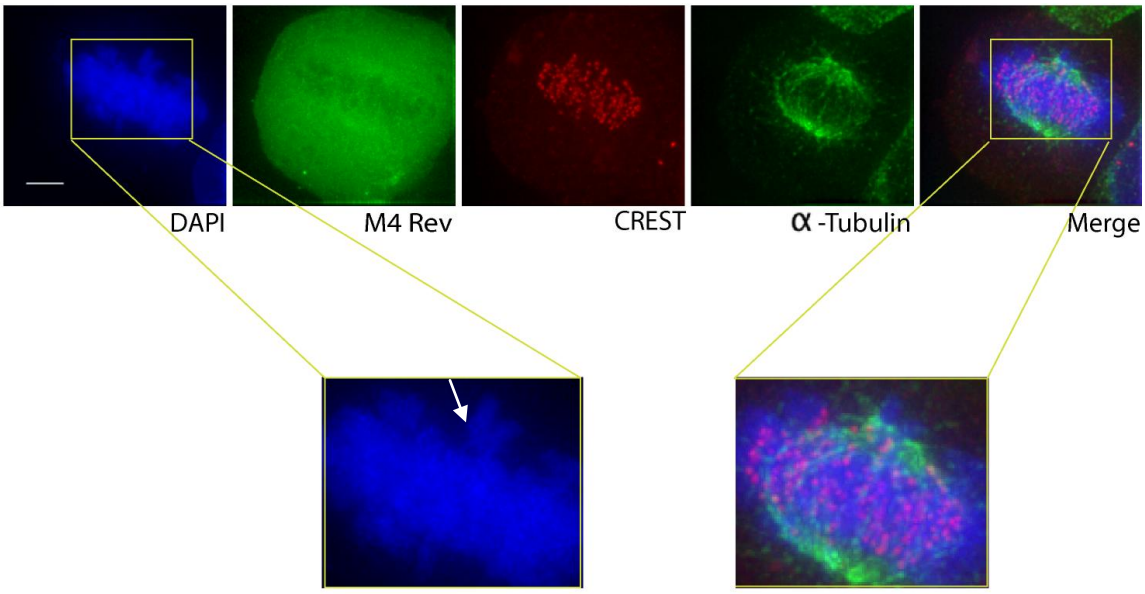


Figure 14 Visualization of mitotic defects in HeLa cells transiently transfected with M4 Rev

Repreentative image of HeLa cell transiently trnasfected with YFP tagged Rev M4. The cell is stained for DAPI , CREST (specific for kinetochore), and tubulin. The lagging chromosome is shown by an arrow in the enlarged panel

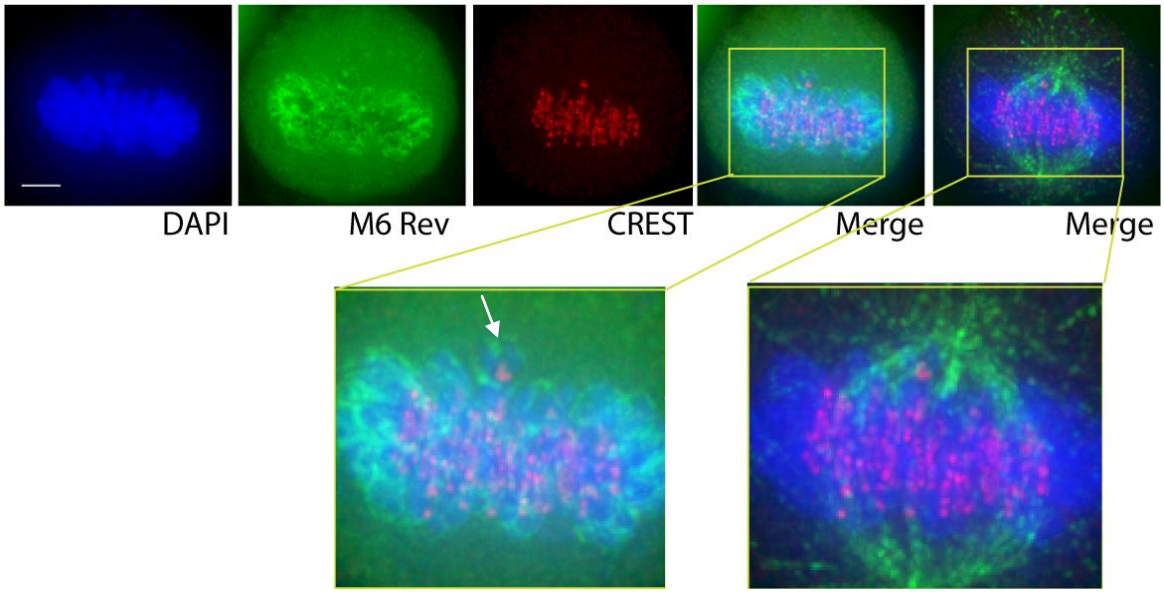


Figure 15 Visualization of mitotic defects in HeLa cells transiently transfected with M6 Rev

Repreentative image of HeLa cell transiently trnasfected with YFP tagged Rev M6. The cell is stained for DAPI , CREST (specific for kinetochore), and tubulin. The lagging chromosome is shown by an arrow in the enlarged panel

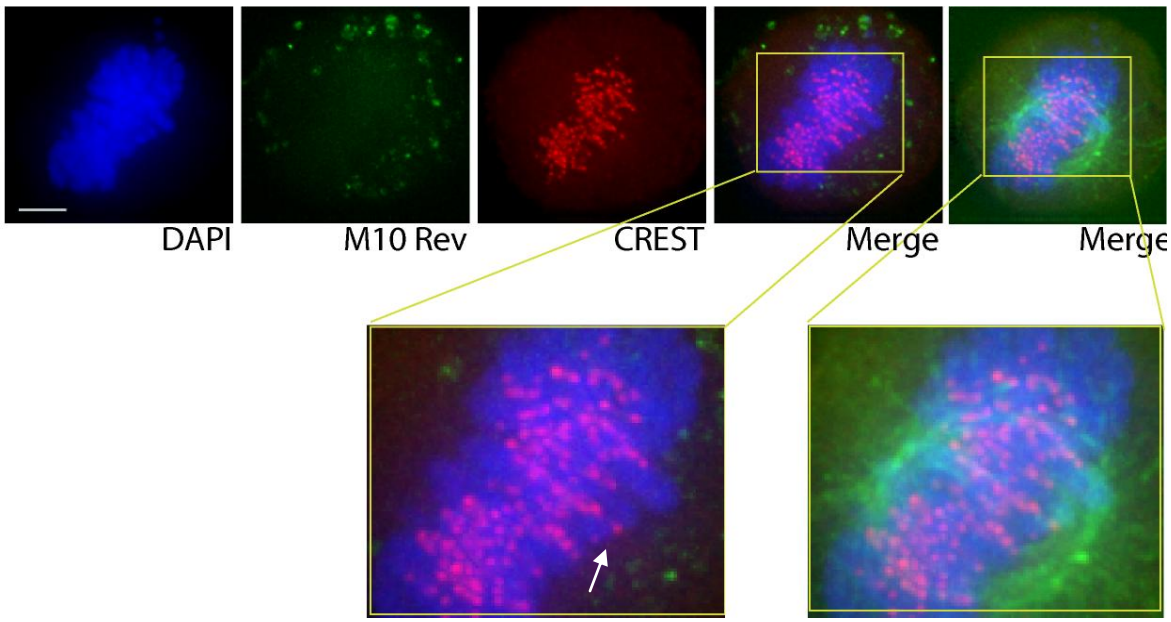


Figure 16 Visualization of mitotic defects in HeLa cells transiently transfected with M10 Rev

Repreentative image of HeLa cell transiently trnasfected with YFP tagged Rev M10. The cell is stained for DAPI , CREST (specific for kinetochore), and tubulin. The lagging chromosome is shown by an arrow in the enlarged panel

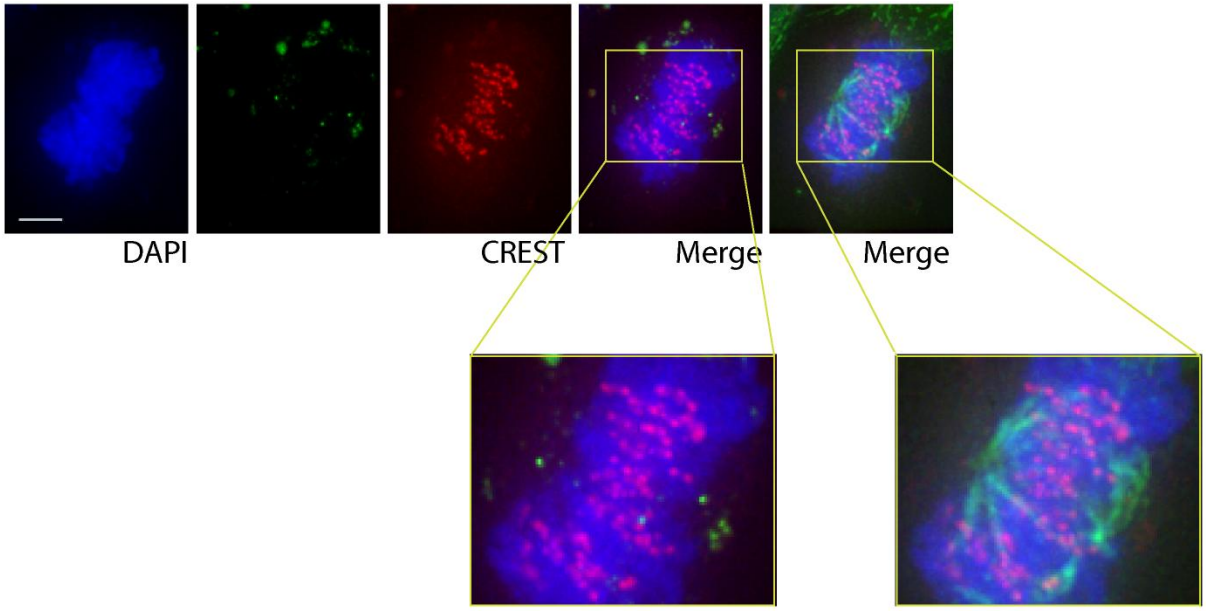
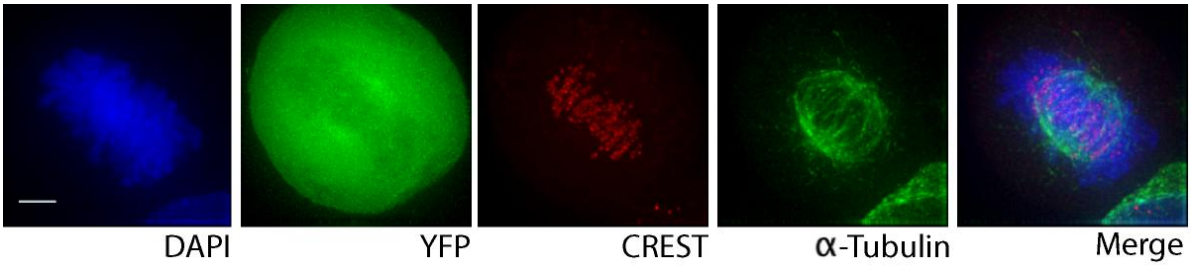


Figure 17 Visualization of mitotic defects in untransfected control HeLa cell. Representative image of control untransfected HeLa cell. The cell is stained for DAPI , CREST (specific for kinetochore), and tubulin.

A



B

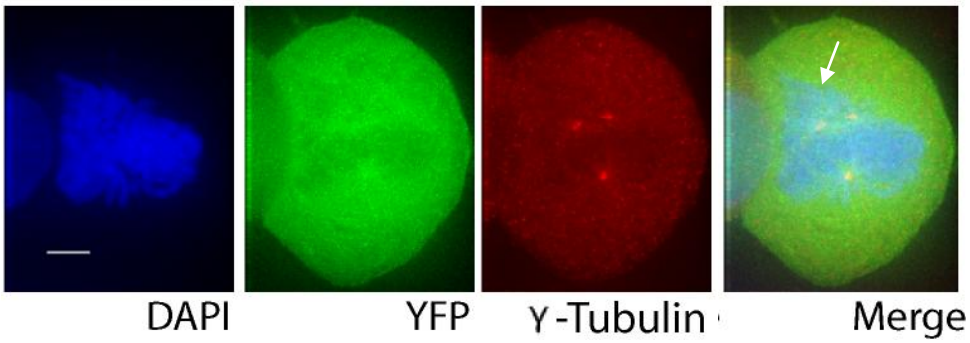


Figure 18 Visualization of mitotic defects in HeLa cells transfected with YFP control.

Representative images of HeLa cell transiently trnasfected with YFP. A. The cell is stained for DAPI , CREST (specific for kinetochore), and tubulin. B. The cell is stained for DAPI and γ tubulin (specific for centrosomes). The lagging chromosome is shown by an arrow

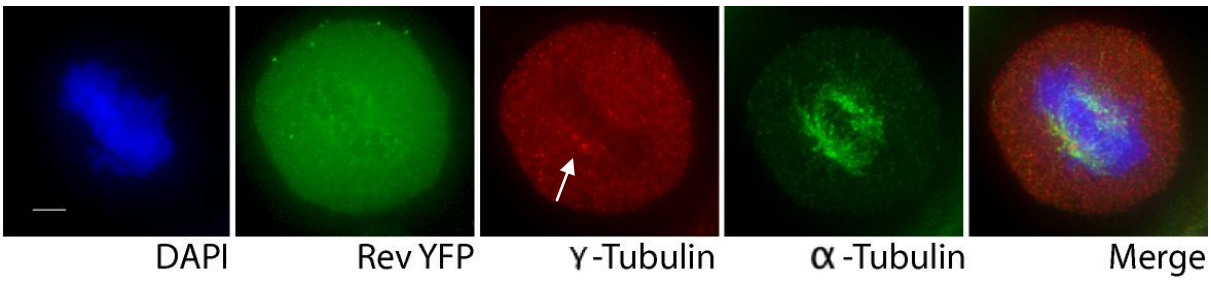


Figure 19 Visualization of centrosomes in a HeLa cell transfected with YFP Rev. Representative image of HeLa cell transiently trnasfected with Rev-YFP. The cell is stained for DAPI, γ tubulin (specific for centrosomes) and α- tubulin. More than 3 centrosomes in a cell is shown by arrow.

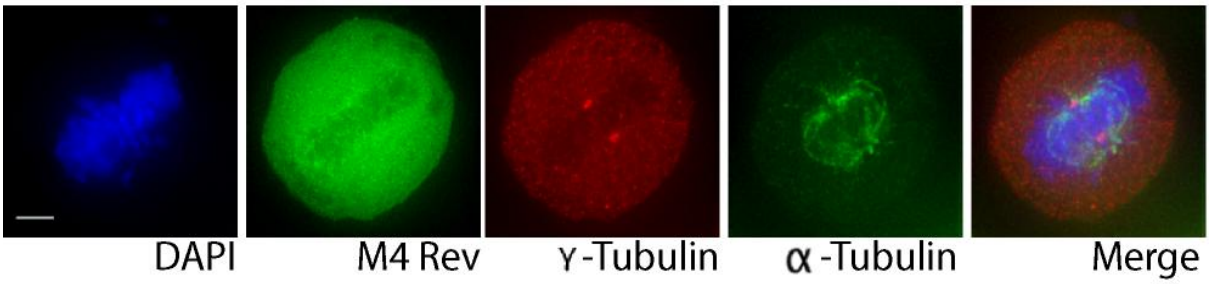


Figure 20 Visualization of centrosomes in a HeLa cell transfected with M4 Rev
Representative image of HeLa cell transiently trnasfected with YFP tagged Rev-M4. The cell is stained for DAPI, γ tubulin (specific for centrosomes) and α- tubulin.

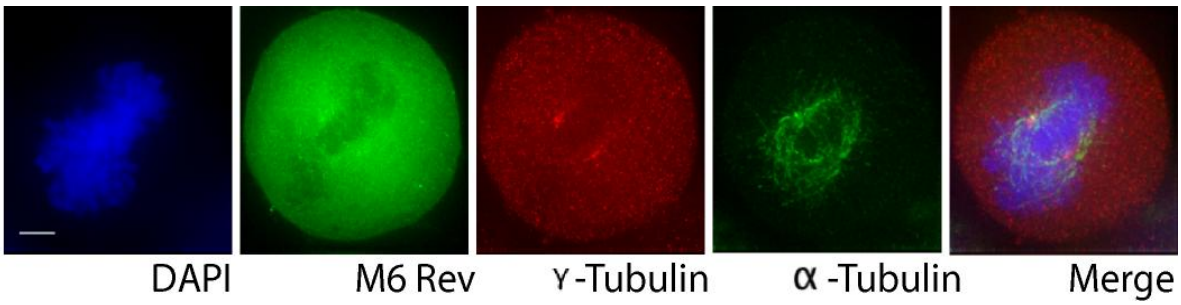


Figure 21 Visualization of centrosomes in a HeLa cell transfected with M6 Rev. Representative image of HeLa cell transiently trnasfected with YFP tagged Rev-M6. The cell is stained for DAPI, γ tubulin (specific for centrosomes) and α- tubulin.

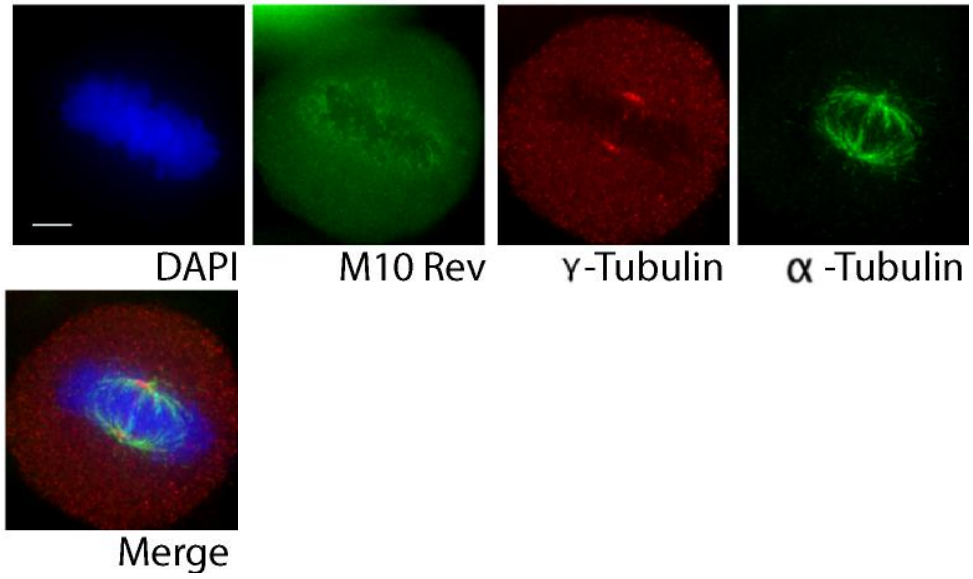


Figure 22 Visualization of centrosomes in a HeLa cell transfected with M10 Rev
Representative image of HeLa cell transiently trnasfected with YFP tagged.Rev-M10. The cell is stained for DAPI, γ tubulin (specific for centrosomes) and α- tubulin.

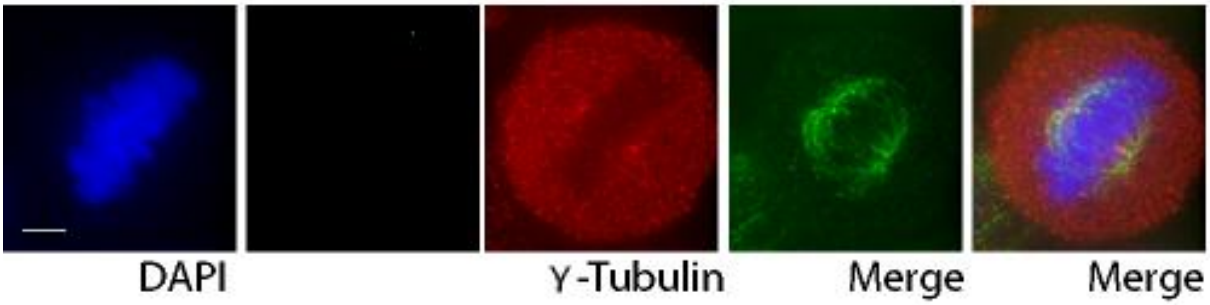


Figure 23 Visualization of centrosomes in untransfected contol HeLa cell.
Representative image of control untransfected HeLa cell . The cell is stained for DAPI, γ tubulin (specific for centrosomes) and α- tubulin.

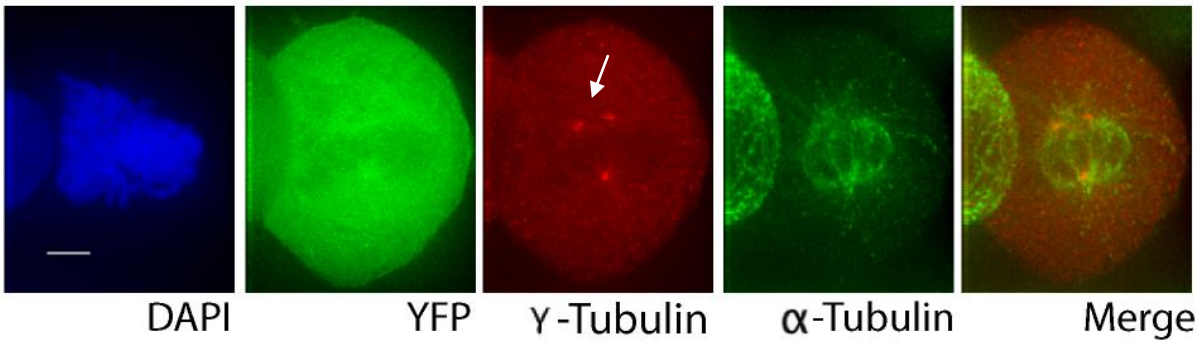


Figure 24 Visualization of centrosomes in a HeLa cell transfected withYFP control. Representative image of HeLa cell transfected with YFP . The cell is stained for DAPI, γ tubulin (specific for centrosomes) and α- tubulin. More than 3 centrosomes in a cell is shown by arrow.

Discussion

Previous studies have shown that Rev over-expression slows cell growth apparently by interfering with events occurring in G2/M phase (40, Smith, N., personal communication). Rev inhibits spindle formation *in vitro* (62), cells accumulate prior to the spindle assembly check point (Smith, N., personal communication) and chromosomal abnormalities ensue (40). These defects may result from Rev's ability to alter spindle dynamics by depolymerizing microtubules, interfering with microtubule polymerization by sequestering tubulin heterodimers, antagonizing or synergizing MCAK function, or interfering with centrosome duplication. To differentiate among these possibilities, the ability of wt-Rev and select Rev mutants (M4, M6 and M10) were compared to negative controls for their ability to bind and co-localize with tubulin.

Rev's ability to depolymerize MT

If Rev interacts and depolymerizes spindle microtubules, one would expect that antibodies specific for tubulin would precipitate Rev. Since M4, M6 and M10 similarly affect cell cycle progression, tubulin precipitation is predicted to pull down these mutant proteins as well. Figure 2 confirms this prediction. Reciprocal precipitations using Rev and GFP-specific antibodies that confirm these findings are less compelling but nonetheless consistent with these observations (Figures 3 and 4).

There are also other observations however contradict the hypothesis that Rev promotes depolymerization of cellular MTs. First, and perhaps most compelling, deconvolution microscopy reveals that there is little overt colocalization of Rev or Rev mutants with spindle microtubules (Figures 5-10). These results do not completely reject

this hypothesis because there is the potential for colocalization as indicated by Pearson correlation coefficients. Moreover, Rev's affinity for MTs may be difficult to detect if MT depolymerization occurs quickly after Rev's binding. Indeed, *in vitro* depolymerization at stoichiometric concentrations of Rev and tubulin is instantaneous (62, Sharma, A., Bedi, S., Robbins, K., personal communication). It is however unlikely that Rev concentrations in transfected cells approach cellular levels of tubulin.

Finally, wt-Rev, M4, M6 and M10 each alter cell cycle progression (*Smith, personal communication*) yet *in vitro* studies show that only wt-Rev retains its ability to depolymerize GMP-CPP stabilized MTs (Sharma, A., Personal communication). M4 appears capable of binding MTs, but is unable to depolymerize them. M6, on the other hand, appears to have a reduced affinity for MT yet, like wt-Rev and M4, it retains the ability to interact with tubulin heterodimers. Unfortunately, there are no data available concerning the *in vitro* interactions between MT, tubulin and M10. These findings coupled with the data presented here suggest that the cell cycle defects observed by Smith, N. and Miyazaki et al. (40) are not likely to be due Rev mediated MT depolymerization. Instead these findings are consistent with the hypothesis that Rev is sequestering tubulin heterodimers. Immunoprecipitation studies show that there is a physical interaction between wt-Rev and Rev mutants with tubulin heterodimers. By sequestering tubulin, Rev has the ability to affect the polymerization of cellular microtubules by a mechanism analogous to stathmin (25, 59) The lack of compelling colocalization with MT is consistent with Rev binding to tubulin heterodimers because tubulin heterodimers are not readily visible in epifluorescence micrographs. Thus the

results presented here are consistent with the hypothesis that Rev is affecting MT dynamics by sequestering tubulin heterodimers.

Rev's ability to affect chromatin mediated nucleation

wt-Rev, M4 and M10 show perichromosomal accumulation during mitosis. From previous studies Rev is known to interact with nuclear transport receptors importin β and CRM1 (18, 46, 54). It is also well established that the spindle is populated by MTs nucleated by the centrosomes, as well as by a steep Ran-GTP gradient created by chromatin-bound RCC1. Thus, we hypothesized that perichromosomal accumulation of Rev interferes with the chromatin-mediated nucleation by interacting with importin β and thereby leading to the accumulation of cells in the G2/M phase. Upon interaction with importin α and β Ran-GTP release NuMa and TPX2 that are critical for chromatin mediated nucleation. In such a model wt-Rev is expected to have the greatest inhibitory activity on cell cycle progression. On the other hand M6, which has a reduced affinity for importin β, should not affect the cell cycle progression greatly. Similarly wt-Rev should interfere with chromatin-mediated nucleation through its interaction with CRM1. CRM1, an importin β homologue, is also regulated by chromatin stimulated Ran-GTP gradient and is known to promote the activities of certain spindle activator proteins. Thus wt-Rev should have a greater effect in reducing the MT nucleation than M10. Indeed over expression of M6 and M10 increases the proportion of cells in G2/M phase over the non expressing controls but not to the extent seen by over-expression of wt-Rev. Moreover in this study it is shown that considerable amounts of M6 accumulated at the spindle pole with reduced perichromosomal accumulation. However, M10 did not have much effect

on the perichromosomal accumulation. Fluorescence resonance energy transfer between fluorescently labeled Rev and tubulin should provide conclusive evidence

Mitotic defects seen as a result of Rev over expression

If wt-Rev interferes with chromatin-mediated nucleation, then Rev expression should slow spindle assembly, affect chromosomal congression and possibly alter tension across the spindle and kinetochore. Although these effects are best measured using time-lapse videography, it is possible to estimate defects by looking for misaligned chromosomes. Likewise measuring the spindle pole distance and interkinetochore distance will provide an estimate on the amount of tension across MTs.

wt-Rev and the mutants showed some degree of chromosomal misalignment (Figures 12, 13, 15 and 16); however, the results were comparable to control untransfected and YFP transfected HeLa cells. HeLa cells are cancerous cells that have large number of chromosomes than that of normal cells and have many mitotic defects themselves. Hence, these cells are not appropriate for these studies.

The results obtained from measuring the spindle tension suggest that M10 reduces spindle tension when compared to untransfected and YFP-transfected controls in a manner that was nearly statistically significant ($p \approx 0.05$, Table 5). Furthermore, the data in Table 5, although not statistically significant, also suggests that M6 and possibly even the wild-type protein may reduce interkinetochore tension. Finally, data in Table 5 suggest that expression of wild-type Rev and its mutants may alter tension across the spindle as there is a trend towards altered inter-centrosomal distances. Clearly, larger sample sizes

are required in order to draw conclusions with confidence. Based on published data, sample sizes need to be increased by 5-fold (27).

However, if these results become statistically different, there are several consequences. The observation that M10 reduces interkinetochore tension seems to implicate the NES and its receptor CRM1. Indeed, CRM1 plays a role in kinetochore functions including recruitment of Ran GAP activity (Crm1 is a mitotic effector of Ran-GTP in somatic cells (1)). Inhibition of CRM1 activity by leptomycin B is known to increase kinetochore tension (1). In this case, one would predict wt-Rev should similarly increase tension and that the M10 mutant, inhibited in CRM1 binding should relieve tension. The data in Table 5 show that this is not the case and in fact, is opposite to predictions. Thus, while it is formally possible that M10 mutation is altering CRM1 function in a manner different from the wild-type, this mechanism is not clear.

If the trend that M6 reduces interkinetochore tension is correct despite the lack of statistical support, then the NLS and its receptor are implicated. It is well-established that importin β sequesters spindle assembly factors (55). It is difficult to predict the effects of Rev overexpression. Simplistically, over-expression of Rev and its NLS should stimulate premature spindle formation. However, there is no evidence to support this contention. Therefore, if Rev binds importin β and does not stimulate release of spindle assembly factors, it is possible that Rev can inhibit spindle assembly by blocking the activity of Ran-GTP. If this mechanism is correct, wild-type Rev should to reduce spindle tension by inhibiting MT nucleation. This inhibition should be reversed by the M6 mutation. As with M10, these predictions are not confirmed by the data in Table 5.

If these results are not statistically different from each other, then they are consistent with the hypothesis that Rev is affecting MT dynamics by sequestering tubulin heterodimers. If is this hypothesis is correct, one would predict that tension across the spindle may or may not be affected. However, based on the binding affinities measure *in vitro (A.Sharma, personal communication),* the effects should be the same for the wild-type and M4, M6, and M10 mutants.

The results presented do not provide direct evidence on the ability of Rev to affect chromosomal congression or spindle tension across the MTs. However, if Rev expression is altering MT nucleation, it is expected that these effects will be noticeable during prophase when the spindle assembles and during prometaphase when chromosomes congress. Ultimately, it is more appropriate to study the effects of Rev mutants on the cell cycle by examining primary cell lines. One such cell line is the PtK2 cell line that has very few chromosomes, remains flattened during mitosis and is commonly used for mitotic studies. Alternatively, a time lapse experiment will provide an insight into the cell cycle progression through mitosis. Likewise spindle tension can be measured on primary cell line using a larger sample size to obtain conclusive results.

Effect of Rev on centrosome duplication

Cells in interphase have a single centrosome that divides into two while entering the mitosis. B23 is a centrosomal protein whose phosphorylation triggers centrosome duplication. It is well established that Rev interacts with B23. To determine whether wt-Rev/Mutants have an effect on centrosome duplication by interfering with B23 function, centrosmes were immunostained and examined to check for any alteration in their

number. The results suggested that although there are instances where three centrosomes are seen in Rev-YFP expressing HeLa cells, it cannot be attributed to Rev's effect as such a phenotype is not seen all the time. Moreover, control untransfected and YFP transfected cells show similar result. Thus, similar study carried out in primary cell line could provide evidence on any alteration in centrosome number. Studies using mutants M4, M6 and M10 have shown similar results; hence it is difficult to determine the effects of Rev on centrosome duplication.

Summary of conclusions

The co-immunoprecipitation results suggest that Rev interacts with tubulin. Co-localization results suggest that Rev does not co-localize with the MTs. Thus, the results collectively indicate that Rev might be interacting with tubulin heterodimers which is why the interaction is seen in co-ips and is consistent with no co-localization seen between Rev and MTs.

The perichromosomal accumulation was seen with wt-Rev, Rev M4, and Rev M10 mutant However, over-expression of M6 which did not show perichromosomal accumulation because of the mutation in the nuclear localization signal. These results are consistent with the hypothesis that Rev might be interacting with importin β on the surface of the chromosome. If true, then chromatin mediated nucleation of MTs will be greatly affected as importin β is essential for the release of spindle assembly factors.

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