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Investigation of Interactions between Rev and Microtubules:
Purification of Wild-type and Mutant Rev Protein and
Optimization of Microtubule Depolymerization Assays

By

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2007
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SCHOOL OF GRADUATE STUDIES

June 6, 2007

Date

     I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY <u>Kelly Robbins Miller</u> ENTITLED <u>Investigation of Interactions between Rev and Microtubules: Purification of Wild-type and Mutant Rev and Optimization of Microtubule Depolymerization Assays</u> BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF <u>Master of Science</u>.

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ABSTRACT

Robbins Miller, Kelly. M.S., Department of Biological Sciences, Wright State
University, 2007. Investigation of Interactions between Rev and Microtubules:
Purification of Wild-type and Mutant Rev Protein and Optimization of Microtubule
Depolymerization Assays.

      As a logical pharmaceutical target for antiviral drugs, HIV-1 Rev is a regulatory

protein essential for viral infection (Hope, 1999). The development of antiviral drugs

that target Rev has been hindered by the lack of high-resolution structural information

due to the protein's tendency to aggregate in solution. While searching for solution

conditions rendering Rev amenable to crystallographic analyses, Watts *et al*., (2000)

discovered a novel *in vitro* interaction between Rev and microtubules (MTs) whereby

addition of equimolar Rev and tubulin forms bilayered rings called Rev-tubulin toroidal

complexes (RTTs). RTTs are similar to those seen when MTs are mixed with certain

anti-MT drugs and KinI kinesins (Watts *et al*., 2000). Coupled with the sequence

homology that exists between KinI kinesins and Rev, I hypothesize that Rev and KinI's

are depolymerizing MTs by a shared mechanism. This mechanism may include the

binding of the Rev/KinI to the end of the MT inducing a curved confirmation in the MT

thereby destabilizing it to promote depolymerization.

      I propose to test this hypothesis through measuring Rev-MT interactions by

adapting biochemical and microscopy-based assays used to measure MT

depolymerization by KinI proteins. These assays require microgram amounts of highly

purified wild-type and mutant Rev proteins as well as purified tubulin from which MTs

can be polymerized *in vitro*. To this end, I have purified wild type Rev with no visible

contaminants on coomasie stained gels. Rev mutants R42A and E57A can also be

purified with limited visible contaminants. However, the appropriate controls can be

generated from non-expressing cells to address this issue. Rev mutants A37D and R39A

can also be partially purified.

Using purified Rev proteins, I then applied sedimentation assays to measure Rev-

stimulated MT depolymerization. There was a statistically significant time dependence

for wild type Rev to depolymerize MTs although there was no evidence for concentration

dependence. Visual assays demonstrate no significant difference in the length of MTs

treated with Rev although Rev decreased the number of MTs on the coverslip over time.

This could contribute to the finding that MT depolymerization by Rev is time dependent.

These results demonstrate that it is possible to measure Rev-MT interactions *in

vitro* although it is clear that these assays are deficient in certain ways, including the

ability of MTs to depolymerize on their own and RTTs potentially pelleting during the

depolymerization assay. However, it is likely that cycling tubulin and/or using taxol to

further stabilize the MTs can remedy the deficiency of MT depolymerization on their

own. EM could also be used to determine if RTTs are pelleting during the

depolymerization assay.

After using mutant Rev proteins in the depolymerization and visual assays, it is

the long-term goal that mechanistic information about Rev will lend valuable evidence to

the study of KinI kinesins.   Generating structural Rev information may also be helpful in

the drug design of anti-mitotic peptides.

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Introduction

Rev is an essential 116 amino acid regulatory protein that is expressed early during infection and is essential for human immunodeficiency virus (HIV) replication (Hope, 1999). Without Rev, there is no production of Pol, Env, and Gag, proteins encoded by underspliced RNAs that are necessary for virion production. Overexpression of Rev has been indicated to cause general structural deformity within cells, and accumulation of cells that have failed cytokinesis, suggesting slowing of cell cycle progression (Miyazaki *et al*., 1995). Multiple studies have identified the mechanism by which Rev functions (Malim *et al*. 1998, reviewed by Hope, 1999 and Pollard *et al*., 1998). Immediately after its translation, Rev is imported into the nucleus where it binds a stem-loop structure unique to an intron of viral RNAs called RRE (Rev Response Element). Multiple Rev monomers multimerize on the RRE which subsequently promotes nuclear export of these RNA's to the cytoplasm allowing translation of Pol, Env, and Gag proteins.

Rev's Functional Domains

Mutational analysis initiated by Malim *et al*. (1989) (reviewed by Hope, 1989 and Pollard and Malim, 1998) has identified four functional domains of Rev responsible for nuclear import, RRE binding, multimerization, and nuclear export. They systematically mutated every charged residue and potential phosphorylation site and overexpressed each in COS cells. They identified a nuclear localization signal (NLS) defined by mutants M5 and M6 where Rev localizes more in the cytoplasm than the nucleus (Figure 1). Both mutations are placed in an arginine-rich region of Rev, which interacts with importin β, a nuclear transport factor shown to mediate Rev import in digitonin permeabilized cells

(Truant and Cullen, 1999). Both M5 and M6 mutants are also deficient in RRE binding. RRE binding is mediated by both interaction with the phosphate backbone and specific nucleotides (reviewed by Hope, 1999 and Pollard *et al*., 1998; Daly *et al*, 1990; Heaphy *et al*., 1991). The competition of the RRE and importin β for this arginine-rich region is thought to prevent re-import of Rev before it has released RRE-containing transcripts in the cytoplasm (Hope, 1999).

There are two multimerization domains in Rev defined by M4 and M7 mutants (Figure 1) (Malim *et al*., 1989). Rev multimerization is typically monitored using gel retardation assays that measure the formation of high-order protein complexes formed in labeled RRE. Measurement of binding affinities for monomer, dimer, trimer for the RRE indicate that both Rev M4 and M7 form multimeric complexes more poorly than wild-type Rev. A comparison of monomer and multimer binding affinities for M4 suggest that this mutant is truly deficient in multimerization. In contrast, Rev M7 can readily form multimers although it is deficient in binding the initial monomer (Brice *et al*., 1999). The deletions of the two amino acids in Rev M7 may be more responsible for protein folding that multimerization.

There is a nuclear export sequence (NES) present in wild-type Rev that promotes export of viral mRNA out of the nucleus into the cytoplasm. Mutational analysis has confirmed that mutant M10 residues are part of the NES of Rev (Malim *et al*., 1989, Figure 1), which binds the nuclear export factor Crm1 (Truant and Cullen, 1999). Unlike previous mutations that are recessive, the M10 mutant is a trans-dominant inhibitor of wild-type Rev function (Malim *et al*., 1989). M10 expression reduces the levels of late,

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structural gene expression following infection of HIV virus. Moreover, M10 expression does not support provirus production in Rev deficient cells (Malim *et al*., 1989).

Rev is required for virion production and the transdominance exhibited by M10 demonstrates that Rev is a potential protein target for HIV-1 drug therapy. Clearly, structural data about Rev would facilitate drug design; however, there exists only limited high-resolution structural information about Rev due to its high tendency to aggregate in solution. Circular dichroism (CD) spectra have indicated that approximately half of the Rev sequence has a helical secondary structure (Wingfield *et al*. 1991; Daly *et al*., 1990). CD spectra on Rev mutants with deletions from amino acids 68-112 or 92-112 suggest that these helical structures are on the N-terminal part of the protein (Auer *et al*., 1994). Blanco *et al*. (2001) have used solid state NMR to confirm this helix-loop-helix model in full length Rev protein. They propose a helix-loop-helix model where residues 8-26 form an N-terminal α-helix that interacts with a second helix formed by amino acids 34-59. Thomas et al. (1998) suggest that hydrophobic residues present in each helix tether the two together.

In vitro, Rev persists as monomers, dimers, multimers, non-physiological filaments, and non-functional aggregates in a concentration dependent manner (Wingfield *et al*., 1991; Blanco *et al*., 2001). Rev is dimeric at 0.1 mg/ml, and forms increasing multimers at concentrations reaching 1 mg/ml (Wingfield *et al*., 1991). Rev can, however, be kept in its monomeric form when denatured with 6M urea or stored in high salt (>750 mM) (Wingfield *et al*., 1991; Karn *et al*., (1995). In more physiological conditions, Rev forms filaments at increasing temperatures above freezing and concentration (1-2 mg/ml), and aggregates follow when stored in sodium phosphate

buffer (Wingfield *et al*., 1991). However, solvation conditions that prevent filament aggregation have been determined (Watts *et al*., 1998). After overexpression and purification of Rev (Wingfield *et al*., 1991), Rev can be unfolded in 6 M urea and refolded by sequentially dialyzed against first, ammonium sulfate, and second, sodium citrate as described in Materials and Methods (Watts *et al*., 1998). Rev filaments are stored at 4ºC in the third dialysis buffer. Although these filaments are probably not the physiologically relevant form of Rev, they retain their functionality.

While searching for solution conditions required to provide more structural information about Rev, Watts et al., (2000) discovered a novel interaction between Rev and microtubules (MTs). Since previous experiments indicated that Rev exhibits high affinity binding with RNA, poly G, poly dG, and polyglutamate and that these polyanions can depolymerize Rev filaments *in vitro* (Heaphy *et al*., 1991; Wingfield *et al*., 1991; Watts *et al*., 1998), Watts *et al*. reasoned that tubulin may prevent nonspecific aggregation of Rev allowing X-ray crystallography. Alpha and beta tubulin are acidic proteins and their 3D structures have been solved with X-ray crystallography (Nogales, *et al*., 1998) to reveal highly acidic C-termini that may interact with Rev's basic residues. They speculated that Rev might bind the acidic C-termini of tubulin while the bulk of tubulin may block higher order aggregation.

Upon the addition of equimolar amounts of Rev-filaments to tubulin monomers, electron microscopy reveals immediate formation of Rev tubulin toroidal complexes (RTTs) or rings (Watts *et al*. 2000). RTTs have a mass of approximately 3-4 MD and 28, 30, and 32 fold symmetry (Watts *et al*., 2000). When MTs are added in molar excess, the MT ends appear curled and RTTs are seen forming. The depolymerization of MTs by

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Rev and appearance of RTTs occurs by a method that is typical of MTs in cells and those treated with MT destabilizing drugs like the anti-cancer drug Dolastatin (Watts *et al*., 2000). Interestingly, Maytansine, another anti-cancer drug whose binding site is the same as Dolastatin, blocks the formation of the RTTs, indicating that Rev may bind at or near the vinca binding site of β-tubulin (Watts *et al*., 2000). Taxol and colchicine, MT drugs that bind to different sites on tubulin, do not block ring formation suggesting that Rev binds tubulin specifically. RTTs also form in the presence of Rev and tubulin heterodimer suggesting that RTTs form in the presence of polymerized and depolymerized tubulin (Watts *et al*., 2000).

The specificity of Rev for MTs is further suggested by the findings that RTT formation occurs in the presence of 200 mM NaCl and at 4°C and 25°C (Watts *et al*., 2000). At 500 mM NaCl both Rev filaments and MTs are depolymerized. Rev/MT interactions appear to be more complex than simple electrostatic interactions because RTT formation is resistant to changes in pH (6-8). Furthermore, ringed structures are not produced when MTs are mixed with other basic proteins including histones, lysozyme, polylysines, and polyarginines (Watts *et al*., 2000). Ring formation is also noticed with GDP-tubulin and MTs mixed with KinI kinesins and certain MT destabilizing drugs like Dolastatin-10 (Watts *et al*., 2000).

A molecular explanation of Rev/MT interactions is suggested by a statistically significant sequence similarity between Rev and XKCM1, a microtubule destabilizing protein found in *Xenopus* eggs (Watts *et al*., 2000) (Figure 2). XKCM1 is a member of the KinI class (recently named Kinesin 13 (Lawrence *et al*., 2004)) within the Kinesin super family of proteins. Most kinesins are microtubule motor proteins that translocate

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along MT surfaces. Unlike conventional kinesins, KinI kinesins bind to MTs and promote MT catastrophes, events that reverse MT polymerization and promote rapid depolymerization. XKCM1's KinI homologs include MCAK (hamster mitotic centromere associated kinesin), Kif2c (mouse), and pKinI (*P. falciparum*). The Rev/XKCM1 sequence similarity is conserved in these homologs (Figure 2). Furthermore, pKinI has been shown to bind and destabilize MTs producing rings (Moores *et al*., 2002).

In cells, XKCM1 and its homologs are crucial participants in spindle disassembly and MT dynamics. (Kline-Smith & C. E. Walczak, 2002). Inhibiting XKCM1 in prophase PtK2 cells by microinjecting inhibitory anti-XKCM1 antibody causes a decrease in the catastrophe frequencies of MTs, resulting in long MTs that disrupt spindle assembly and mitotic delay or arrest (Kline-Smith & C.E. Walczak, 2002). The mitotic spindle has dynamic properties, and a recent study indicated that some of these dynamic properties could be reconstituted by mixing tubulin with the XKCM1 and the stabilizing microtubule associated protein XMAP215 (Becker *et al*., 2002). This experiment implies that there is a balance of destabilizing and stabilizing factors that regulate spindle dynamics with XKCM1 being at least one component. Rev may also be a catastrophe promoter that depolymerizes MTs to produce tubulin rings, or inhibit rescue of MTs. Rev, like XKCM1, causes disassembly of MTs from both ends (Watts *et al*., 2000).

Mechanism of KinI-mediated MT depolymerization

Much is known about how KinI kinesins bind and depolymerize MTs. Moores and colleagues (2002) supported that the N-terminal domain of KinI's mediates localization to the centromere; the C-terminal domain is required for dimerization of the

KinI although monomers are functional (Maney *et al*., 1998; Hertzer *et al*., 2006), and

that these properties can be completely separated from the KinI internal motor domain

necessary for depolymerization activity. The "neck" domain, a region adjacent to the

motor domain (head), confers directionality and motor processivity in motile kinesins

(Case *et al*., 1997; Henhingsen and Schliwa, 1997; Endow and Waligora, 1998; Romberg

et al., 1998). The neck itself does not possess depolymerizing activity for MCAK

(XKCM1 human homologue) (Maney *et al*., 2001). The neck region may be important in

stabilizing the interaction between the KinI and the MT, and not necessarily required for

depolymerization. The "neckless" KIF2C can bind to the side-wall of MTs but

depolymerization activity is weak (Ogawa *et al*., 2004). A "neckless" pKinI, MCAK,

and KIF2C exhibit some depolymerization activity; however, the depolymerization is

enhanced 5-fold with the addition of the neck region (Ovechkina et al., 2002; Maney *et al*., 2001; Moores *et al*., 2002; Ogawa *et al*., 2004).

There are three other regions in XKCM1 within the motor domain that are unique

to KinI proteins and may therefore play a role in binding and depolymerization of MT's:

(1) the Tu-C Loop, (2) the Lys/Arg Loop, and (3) the L11-α4 helix-L12 region of the

catalytic domain (Rev homology region) (Figure 3).

(1) The Tu-C Loop (L2) is a positively charged chain of amino acids that is

predicted to lie near the negatively charged C-terminus of tubulin (Niederstrasser *et al*.,

2002). This loop contains a cluster of class specific residues known as the "KVD finger"

(Ogawa *et al*., 2004; Shipley *et al*., 2004). This finger is thought to lie next to the MT

surface, and when mutated depolymerization activity is greatly reduced (Ogawa *et al*.,

2004; Shipley *et al*., 2004). The KVD finger was indicated to not be involved in MT

binding, but plays an important role in MT depolymerization (Ogawa *et al*., 2004).

       (2) The Lys/Arg Loop (L8) might affect the affinity of XKCM1 to tubulin or

contribute to the destabilization of the MT lattice. It may serve as a sensor for ATP

hydrolysis at the end of the MT (Ogawa *et al*., 2004; Shipley *et al*., 2004). When the

KinI reaches the end of the MT and loses ADP, the switch II cluster (L11-α4) makes

contact with the MT, and L8 along with loop 12 are possible anchor points on the MT

around the switch II domain (Ogawa *et al*., 2004; Shipley *et al*., 2004). L8 may then be

involved in the tugging and bending of the tubulin protofilament underneath (Ogawa *et

al*., 2004; Shipley *et al*., 2004). As a result of the curved conformation of tubulin, ATP

hydrolysis is triggered.

       (3) The atomic structure of the L11-α4 helix-L12 region is not known for

XKCM1, but using structural information available for Kif1a, a motile kinesin, and Kif2c

to model XKCM1-MT interaction, the shared domain of XKCM1 and Rev may consist of

a long α-helix that may bind the MTs in the space between the α and β-tubulin subunits

(highlighted in Figure 3) (Niederstrasser *et al*., 2002; Woehlke *et al*., 1997). It is the

main MT binding region for Kif2c (Ogawa *et al*., 2004). When the Kif2c makes full

contact with the MT, the α4 helix is inserted in the intradimer grove (Ogawa *et al*., 2004).

       Shipley *et al*. (2004) presented a 1.6 angstrom crystal structure of pKinI motor

core with no nucleotide present. Mutated residues R242A (in L11), R272A (in α4),

KEC268,269,270AAA (in α4) indicated reduced binding and ATPase activity, with the

KEC mutant indicating almost no depolymerization activity (Shipley *et al*., 2004). The

R272 and KEC residues are indicated to be adjacent to the intradimer interface of the α/β-

tubulin heterodimer, and play an essential role in pKinI/MT interaction (Shipley *et al*., 2004). EM images showed that the KEC mutant barely decorated the MT, indicating that MT binding was reduced, and R272 indicated similar results (Shipley *et al*., 2004). R242A, however, can still bind the MT although to a lesser extent, but may play a greater role in ATP hydrolysis (Shipley *et al*., 2004). XKCM1 contains the KEC domain, and Rev has a homologous E amino acid at the same position. In XKCM1, when the E of the KEC mutant is mutated, XKCM1 is depolymerization deficient (M. Miller, personal communication). This residue may prove essential to the Rev/MT interaction. The L11-α4 helix-L12 region is important in both MT binding and depolymerization with the α4 helix region being more involved in binding.

There is a precise curvature of the pKinI-AMPPNP-tubulin ring produced by depolymerization of MTs by pKinI's. Through contour mapping and gray scale imaging, the pKinI motor appeared to sit in a more centered position with respect to the tubulin dimer than does motile KinN protein (Moores *et al*., 2002). There seemed to be a deformation of the tubulin ring as a result of the binding AMPPNP. Due to the fact that this ring conformation was not noticed in other stabilized MTs under the same experimental conditions, Moores *et al*. (2002) proposed that the pKinI binding is entirely responsible for the bent tubulin conformation.

Ogawa et al. (2004) proposed a model of MT depolymerization using crystallographic data of Kif2c (a KinI) bound to ATP or ADP. They propose that Kif2c binds at the end of the MT, making full contact with the protofilament by injecting its α4 helix (Rev homology region) into the intradimer groove. Creating a curved tubulin confirmation, the other regions of the KinI (neck and TuC Loop) bind to the MT and

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stabilize the curved conformation, destabilizing the lateral interaction. Then more KinI's

bind to the curved protofilament, which changes MT dynamics enough to cause

depolymerization (Ogawa *et al*., 2004) (Figure 3).

There is also a proposed mechanism for MT depolymerization by KinI Kinesins

(Shipley *et al*., 2004; Niederstrasser *et al*., 2002; Moores *et al*., 2002). The KinI reaches

the end of the MT by lattice diffusion (Helenius *et al*., 2006; Hunter *et al*., 2003) and

exchanges its ADP nucleotide. Then, ATP binding at the β-tubulin tail causes a

conformational change; this results in a bent confirmation of the tubulin dimer. Due to

the curved tubulin conformation, the tubulin dimer is released from the protofilament,

and hydrolysis of ATP is triggered. After ATP hydrolysis, the KinI dissociates and is

free to bind to another MT (Helenius *et al*., 2006). Although Rev is not using ATP

hydrolysis to depolymerize MTs, there is still useful information obtained from the

consideration of KinI's.

Comparison of depolymerizing activities of Rev and KinI proteins (Table 1)

The motor core is highly conserved among the kinesin superfamily (Ogawa *et al*.,

2004), and in KinI kinesins is responsible for MT depolymerization. The pKinI motor

core appeared to sit in the middle to the tubulin subunits (Shipley *et al*, 2004;

Niederstrasser *et al*., 2002; Moores *et al*., 2002; Ogawa *et al*., 2004), which is important

due to the similarity of this motor core with Rev activity. Kinesins are ATPases that

undergo nucleotide-dependent conformational changes (Moores *et al*., 2002). Since Rev

does not bind nucleotide, Rev is unlikely to mimic the nucleotide-dependent

conformational changes achieved by KinI proteins. However, KinI-mediated MT

depolymerization is not dependent on ATP hydrolysis (Moores *et al*., 2002). When

pKinI motor core is added to MTs, depolymerization of the MT occurs in the presence of

ATP or AMPPNP (non-hydrolysable ATP). By using other nucleotide analogs to mimic

the different states of the ATPase cycle and taxol-stabilized MTs, Moores *et al*. (2002)

and Niederstrasser *et al*., (2002) demonstrated that ATP binding to the KinI motor core

was needed for MT depolymerization, but not hydrolysis. They also established that the

C-terminal tail of tubulin is essential for KinI depolymerization, as depolymerization did

not occur with subtilisin-treated MTs. However, Helenius *et al*. (2006) showed that the

decrease in depolymerization of subtilisin-treated MTs by KinIs is due to the decrease in

end targeting caused by the lack of C-terminal-tail-mediated diffusion along the MT. In

addition, Moores *et al*. (2002) and Niederstrasser *et al*. (2002) demonstrated that the β-

tubulin C-terminus is essential for depolymerization. However, Rev has been indicated

to still depolymerize subtilisin-cleaved MTs, lending support to the argument that the

Rev/MT interaction is more specific than an electrostatic interaction (Watts *et al*., 2000).

Specific Aims

Due to their sequence similarity and shared ringed-intermediates during

depolymerization, it is hypothesized that Rev binds and depolymerizes MTs by a

mechanism similar to that of the KinI kinesins. The long term goal is to demonstrate

which Rev residues are important for KinI activity by mutating residues to inhibit Rev's

ability to bind and/or depolymerize MTs *in vitro*. To test this hypothesis, the following

point mutations have been placed in Rev: T34A, A37D, R39A, R42A, E47A, R50A,

E57A, and E47A/E57A. To address the hypothesis, the following aims must be

addressed:

Aim 1) Express Wild-type and mutant Rev proteins in *E.coli* and purify.

Aim 2) Purify tubulin.

Aim 3) Complete MT depolymerization/binding assays to compare the depolymerization/binding ability of wild-type Rev versus mutants.

Expected Results

Basic Residues (R39, R42, R50)

Rev's basic residues may be important since they are likely to bind the acidic MT residues. The Rev residues R39A and R42A are highly conserved among KinI's (Ogawa *et al*., 2004). Both R39A and R42A are also involved in binding the RRE and, therefore, should be accessible for MT binding. Rev R50A is predicted to be close to the MT/kinesin interface, as indicated by the homologous residues highlighted on the Kif2c crystal structure (Figure 4). This residue lies in between the M6 and M7 mutants, responsible for RNA binding and multimerization of Rev, therefore, also accessible for MT binding. Consequently, limited MT depolymerization activity as compared to wild type Rev is expected because of reduced binding of these basic residue mutants to the MT during the MT binding assay.

Acidic Residues (E47, E57)

Rev's acidic residues are expected to be more important during MT depolymerization than binding. The Rev E57A residue is found in all KinI's and may be the most important residue for depolymerization activity (Shipley *et al*., 2004; Ogawa *et al*., 2004). In Rev, E57 appears one amino acid before the M7 mutant, which is responsible for Rev structure and multimerization and considered accessible for MT binding. The E in the KEC mutant of the pKinI aligns with E57 (Shipley *et al*., 2004).

This residue mutated in XKCM1 was indicated to have markedly reduced depolymerization activity (unpublished data, Dr. Mill Miller).

Rev E47A is common to most KinI's (Ogawa *et al*., 2004). It appears three amino acids after M6, which is responsible for RNA binding. This acidic residue may be partly responsible for destabilizing the MT's in order to depolymerize them similar to E57A. The E47A/E57A mutant combines the activity of both the acidic residues in Rev.

Reduced Priority Residues (T34, A37)

Rev T34A residue is conserved among the KinI's (Ogawa *et al*., 2004). T34 is found near the M5 mutant, which has a role in RNA binding. Therefore, I expect this mutant to have reduced MT binding versus wild type Rev, and should have less MT depolymerization activity versus wild type Rev due to its inability to bind the MT.

Rev A37D is conserved in most KinI's (Ogawa *et al*., 2004). A37 is near Rev's multimerization domain. I expect this mutant to bind the MT similar to that seen with wild type Rev binding assay. However, I expect multimerization to be important to MT depolymerization by Rev, so this mutant should indicate reduced depolymerization activity as compared to wild type Rev. In XKCM1, the homologous residue to A37D has been mutated and had no effect on depolymerization activity of XKCM1 (K. Hertzer & C.E. Walczak).

To test the hypothesis that Rev will bind and depolymerize MT's in a mechanism similar to KinI's, wild-type Rev and mutants will first be expressed, purified, and re-folded in an active state. Then binding and depolymerization assays capable of measuring their ability to bind and depolymerize MTs *in vitro* need to be validated. This data will not only provide structural information about Rev, but due to the sequence

similarity with KinI kinesins, this information may also provide insights into which

residues are necessary for KinI kinesin depolymerization of MTs, which could aid in

further mechanistic discovery of MT depolymerization by KinI's.

Materials and Methods

Protein Expression / Large Scale Cultures

The wild-type Rev gene was previously ligated into the pET11d vector (Blanco *et al*., 2001). Wild-type and mutant Rev constructs are perpetuated in DH5α cells (Sambrook *et al*., 1989). For protein expression, pET-Rev constructs were transformed into chemically competent BL21 (DE-3) cells (Invitrogen). Transformed cells were grown and selected on LB plates with 100 µg/ml ampicillin overnight at 37˚C. One transformed colony was used to inoculate a 10 ml LB broth-100 µg/ml ampicillin. This culture, incubated at 37ºC overnight with 250 rpm shaking, was used to inoculate a 1 L LB-100 µg/ml ampicillin culture. When cell density reached the log growth phase (OD ≈0.6), IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. Cells were collected 2 hours later by centrifugation at 10,000 rpm (SS-34 rotor) for 10 minutes and stored at -20ºC.

FPLC: Rev Purification

Method 1, Karn *et al*., (1995)

Frozen cells were lysed by sonication, 3 times for 30 seconds, in 11 ml buffer A (400 mM sodium chloride, 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 8.0) per 1 L culture. Sonicates were frozen and thawed and sonicated as before. Sonicates were clarified by 10,000 rpm for 10 minutes in a SS-34 rotor. The supernatant was applied to a Q-sepharose column (HiPrep QXK16). After eluting with 2 column volumes of buffer A, Rev was eluted with buffer B (800 mM sodium chloride, 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.0) and applied to a Heparin-sepharose column (HiPrep Heparin Sepharose 6FF). After the column was washed with Buffer C (1 M

sodium chloride, 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.0) such that the optical density of the eluant was background, Rev was eluted with buffer D (2 M sodium chloride, 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.0). Aliquots of Q-sepharose and Heparin-sepharose columns were resolved by SDS-PAGE and detected by coomassie staining.

Protein concentration was determined using Fuji Multigauge software and the coomassie stained SDS-PAGE gels mentioned above before refolding. All aliquots containing Rev were pooled and denatured in 6M urea, and refolded properly by sequential dialysis steps in dialysis buffers 1-3 overnight as described by Watts *et al*. (1998). Dialysis buffer 1 contained 50 mM sodium phosphate, 600 mM ammonium sulfate, 150 mM sodium chloride, 50 mM sodium citrate, 1 mM EDTA, pH 7.0. Dialysis buffer 2 contained 50 mM sodium phosphate, 150 mM sodium chloride, 50 mM sodium citrate, 1 mM EDTA, pH 7.0. Dialysis buffer 3 contained 20 mM Hepes, 100 mM sodium chloride, 50 mM sodium citrate, 1 mM EDTA, pH 7.0, and also served as the storage buffer for Rev at 4°C. Rev is refolded into filaments and stored in this form.

Method 2, Wingfield *et al*., (1991)

Mutant proteins that did not purify well with Method 1 were purified by Method 2. Cell pellets from 1 L cultures were resuspended in 11 ml of buffer E (100 mM Tris-HCl, 5 mM DTT, 5 mM EDTA, 5 mM benzamide, 1 mM PMSF, pH 8.0) and sonicated as above. Sonicates were clarified by centrifugation at 10,000 rpm for 10 min. as above. The supernatant was centrifuged at 60,000 x g for 1 hour in an SW-27 rotor. The supernatant was diluted 3-fold with buffer F (50 mM Tris-HCl, 1 mM sodium azide, pH 8.0) and applied to the Q-sepharose column equilibrated with buffer F. Select fractions

from the Q-sepharose column were dot-blotted using 3 μl from each fraction, and developed using polyclonal Rev antibody. The flow-through fractions containing Rev were collected, pooled and denatured with the addition of urea to 6 M. This extract was diluted 2-fold with buffer G (20 mM sodium phosphate, 2 M urea, 1 mM DTT, 1 mM EDTA, pH 6.5), and the pH was adjusted to 6.5 using H_3PO_4. The solution was then applied to the Heparin-sepharose column equilibrated with buffer G and when the baseline absorption was zero, Rev was eluted with a linear gradient of buffer G + 1 M NaCl with Rev eluting at approximately 0.5 M NaCl. SDS-PAGE gels of aliquots from Q-sepharose and Heparin-sepharose fractions were coomassie stained. Rev containing fractions were pooled and denatured in 6 M urea and dialyzed as described above.

Tubulin Purification

Tubulin was purified from bovine brain (http://mitchison.med.harvard.edu/ protocols/tubprep.html). Three brains supplied by Copey's Butcher Shop (Medway, Ohio) were transported to the lab in a cooler with 3 L pre-cooled 1.5% NaCl and 1 large bag of ice. The meninges were stripped at 4ºC and brains homogenized with PB (Pipes Buffer, 0.1 M Pipes, pH 6.8, 0.5 mM $MgCl_2$ 2 mM EGTA, 0.1 mM EDTA, 0.1% B-ME, 1 mM ATP) to 1.75 L. The homogenate was spun at 22,680 x g for 75 minutes at 4˚C in a GSA/SS-34 rotor. 570 ml supernatant was supplemented with 50% volume of glycerol warmed to 37˚C, ATP to 1.5 mM, GTP to 0.1 mM, and $MgCl_2$ to 3.5 mM. Extracts were swirled to mix incubated at 37ºC for 60 minutes, and subjected to centrifugation in a Type 50.2 Ti rotor at 13,000 rpm for 160 minutes.

Recovered pellets were placed on ice and resuspended in 20 ml of cold PB, 1 mM ATP and 0.1% 2-mercaptoethanol. Pellets were homogenized using a dounce

homogenizer and placed on ice for 40 min. Extracts were subjected to centrifugation using a Type 50.2 Ti rotor at 42,000 rpm for 30 minutes at 4ºC.

The supernatants were recovered and supplemented with 50% volume of pre-warmed glycerol, GTP to 0.5 mM, and $MgCl_2$ to 4 mM. The supernatant was incubated at 37ºC for 40 minutes and spun in a Type 50.2 Ti rotor at 47,000 rpm for 30 minutes at 37ºC. Recovered pellets were resuspended in ice-cold 50 ml of CB (Column Buffer, 50 mM Pipes, pH 6.8, 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 0.1 % B-ME, 1 mM ATP) using a dounce homogenizer. After incubation at 4˚ C for 40 min., the sample was spun in a Type 50.2 Ti rotor at 42,000 rpm for 30 minutes.

The supernatant was applied to a phosphocellulose column (PC) (approximately 200 ml volume) equilibrated with CB. The flow rate was set at 2 ml/min, and 3 ml fractions were collected. Bradford assays were performed and fractions that indicated greater than 1 mg/ml tubulin were collected and combined (fractions 36-60, 125 ml). The final concentration was determined spectrophotometrically ($\varepsilon_{280} = 115,000$ $M^{-1}cm^{-1}$) to yield 125 ml of 14.2 µM tubulin.

The column was washed with three volumes of 1 M KCl in CB to elute the microtubule associated proteins (MAPs). MAPs eluted after 200 ml of 1 M KCl in CB were added, and fractions greater than 1 mg/ml were collected. The MAPs were frozen in liquid nitrogen and stored at –80˚C. The column was equilibrated with 10 volumes of CB + 0.1% NaN_3 for storage.

Tubulin Cycling

Two 7 ml aliquots of phosphocellulose purified (PC) tubulin were thawed on ice and cold water. Aliquots were supplemented with 5X BRB80, 1M $MgCl_2$, and 100 mM

GTP to final concentrations of 0.5X, 4 mM, and 1 mM, respectively. The tubulin mixture was incubated on ice for 5 minutes, and then shifted to 37ºC for 2 minutes. Ten percent (1.4 ml) DMSO was added to the tubulin mixture and this was incubated at 37ºC for 40 minutes. A 60% glycerol cushion containing 1.6 ml 5X BRB80, 12 ml 100% glycerol, and 6.4 ml sterile water was added in 10 ml aliquots to two 50.2Ti rotor tubes. The tubulin mixture was layered on top of the cushion and spun at 50K, 35ºC for 35 minutes.

The pellets were washed twice with 1X IB (10X IB contains 500 mM K+ glutamate, 50 mM $MgCl_2$, pH 7.0 with KOH), resuspended in 500 µl of ice cold 1X IB using an 18 g needle and incubated 30 min. on ice. Sample was then subjected to centrifugation at 2ºC for 90K for 15 minutes in the Sorvall, 30° fixed angle, Beckman type 50.2 Ti pre-cooled rotor. The supernatant was collected, aliquoted, and frozen at -80ºC. The concentration of tubulin was determined spectrophotometrically as above.

MT Depolymerization Assay

Cycled tubulin was polymerized at 37ºC for 30 minutes in the presence of 10 mM GMPcPP and BRB80/DTT (1X BRB80, 1 mM DTT) and collected by centrifugation (TLA100 at 35ºC, 90K, for 5 minutes). The pellets were resuspended in 20 µl of BRB80/DTT at room temperature. The tubulin concentration was determined spectrophotometrically as above. The microtubule stock was diluted to 3 µM with BRB80/DTT. Rev protein was diluted to concentrations ranging from 50 nM to 3 µM with Dialysis Buffer 3. Rev protein is stored in the filamentous form, and becomes partially oligomeric and monomeric during the assay when the concentration is lowered.

The reactions were set up so that the total volume of each reaction was 40 µl. Four microliters of Rev protein were added to 16 µl BRB80/DTT and 20 µl of 3 µM MTs

were added last. The reactions occurred within 1.5 ml eppendorf tubes at room

temperature (22ºC). After 10 minutes, 20 µl of each reaction was removed and spun

down in a TLA100 rotor at 22ºC for 5 min at 90,000 rpm. The remaining 20 µl of each

reaction were removed and spun down in the same manner 20 minutes from MT addition.

The supernatants were collected and added directly to 20 µl of 2X SDS-PAGE sample

buffer and boiled. Twenty microliters of 2X SDS-PAGE sample buffer was added

directly to the pellet and resuspended, then 20 µl of BRB80/DTT was added and the

pellet sample boiled. All samples were resolved on a 15% SDS-PAGE gel and coomasie

stained or transferred and a western blot was performed.

Visual MT Binding Assay

Cycled tubulin was polymerized at 37ºC for 30 minutes in the presence of 10 mM

GMPcPP and BRB80/DTT (200 µl 5X BRB80, 2 µl 0.5 M DTT, 796 µl dH$_2$0) and

collected by centrifugation (TLA100 at 35ºC, 90K, for 5 minutes). The pellets were

resuspended in 20 µl of BRB80/DTT at room temperature. The tubulin concentration

was determined spectrophotometrically as above. The microtubule stock was diluted to 3

µM with BRB80/DTT. Rev protein was diluted to 125 nM in dialysis buffer 3.

Four non-stress fractured chocks were added to each spin down tube (Kimble HS,

No.45500-15), and then a poly-L-lysine coated coverslip was added to each tube. 5 ml of

1X BRB80 was added to each spin down tube and they were balanced. 2 µl 5X BRB80,

0.6 µl KCl, 1.4 µl H$_2$0, 5 µl 3 µM MTs and 1 µl 125 nM Rev protein were added together

and incubated at room temperature for 15 min. Each spin down tube was underlain with

2 ml BRB80/10% glycerol. Three microliters of reaction mixture was fixed with 30 µl

BRB80/1% paraformaldehyde. Each reaction was then diluted with 800 μl BRB80, mixed gently by inversion, and 50 μl was placed on top of BRB80 in spin down tube.

Reactions were sedimented for 70 min at 8,000 rpm (10,000 x g) in an HB4 rotor at 20˚C. The coverslips were post fixed with -20˚C methanol for 5 minutes, and then placed in TBS-T. Coverslips were then moved to a Petri dish, washed with TBS-T (8.8g NaCl, 0.2g KCl, 3g Tris, 500 μL Tween-20, dH$_2$0 to 1L, pH 7.4) three times, and immunostained. After blocking with 75 μl 2% BSA/TBS-T, coverslips were incubated for 30 min. in 20 μl of Monoclonal Anti-α-Tubulin Clone DM1A (1:5000) and Sheep anti-HIV-1 Rev (1:500). Coverslips were washed 1 time (short wash for 20 seconds) and then 2 times (long washes for 1 minute) with TBS-T, and then incubated for 30 min. in 30 μl of Fluorescein (FITC)-conjugated Donkey Anti-Sheep IgG (1:500) and Rhodamine (TRITC)-conjugated Goat Anti-Mouse (1:500) in 2%BSA/TBS-T. The coverslips were washed as above, and mounted on slides using 1.5 μl Vectashield, sealed with nail polish, and examined. The slides were stored at 4˚C.

Alternatively, after fixing the reaction mixtures, 50 μl of the reaction mixture were spotted directly onto coverslips and incubated for 4-6 hours. The reaction mixture was removed and coverslips were washed once with TBS-T. The coverslips were then post-fixed with –20 °C methanol for 5 minutes and then placed in TBS-T. Coverslips were immunostained and stored as indicated above.

Statistical Analyses

Dr. DeAnne C. French, Statistical Consultant at the Wright State University Statistical Consulting Center, completed all analyses with SPSS v. 15.0.1.

Results

Rev Purification (Specific Aim 1)

If biochemical assays previously used to study KinI proteins are to be used to study Rev/MT interactions, then large amounts of purified, biologically active proteins are required. Previous studies have shown that recombinant Rev expressed in *E.coli* is biologically active (Karn *et al.*, 1995; Brice *et al.*, 1999; Watts *et al.*, 1998, 2000; Wingfield *et al.*, 1991). Using the pET11 vector where the Rev gene is under control of the lac promoter (Blanco *et al.*, 2001), preliminary experiments indicated that the two-hour treatment of log phase cultures with 1 mM IPTG gave optimal expression of full length Rev with minimal accumulation of truncated Rev (data not shown).

Initial purification of Rev followed the procedures by Karn *et al.* (1995) owing to the availability of chromatographic equipment. Cells are sonicated, and clarified by low speed centrifugation. The supernatant, containing Rev, is sequentially chromatographed over Q-sepharose and Heparin-sepharose columns. Rev binds the positively charged Q-sepharose column indirectly through Rev's interaction with nucleic acids that bind to the column. After eluting Rev with 800 mM NaCl, Rev is bound to the negatively charged Heparin-sepharose column. After washing the column with 1 M NaCl, Rev is eluted with 2 M NaCl. Purified Rev is then refolded through sequential dialysis steps described in "Materials and Methods."

Typical results of the purification of wild-type Rev are represented in Figure 4. Rev co-elutes with a single peak of proteins released from the Q-sepharose column by 800mM NaCl (Figure 4 A/B). It elutes from Heparin-sepharose column as a pure protein in a single peak (Figure 4 C/D). Purified Rev is refolded and dialyzed, indicated in

Figure 4 E. This procedure yields 1-1.5 mg of total protein per liter culture at concentrations ranging from 1-1.7 mg/ml.

Rev R42A was also purified using this method. Rev R42A co-elutes with a single peak of proteins released from the Q-sepharose column by 800 mM NaCl (Figure 5 A/B). It is important to note that a large amount of Rev R42A that does not bind the Q-sepharose column. This is most likely due to the decreased affinity of this mutant to nucleic acids, as this residue is present in the region that mediates RRE binding (Malim *et al*., 1989). Rev R42A then co-elutes with a single peak of proteins released from the Heparin-sepharose column by 2 M NaCl (Figure 5 C/D). This mutant protein is not as pure as WT Rev as indicated by coomasie stained SDS-PAGE (compare Figures 4 E and 5 E).

Attempts to purify additional mutants Rev E57A, Rev R39A, and Rev A37D using this method failed. In each instance, Rev did not bind the Q-sepharose column (Figure 6). The inability of Rev E57A to bind the Q-sepharose column may be due to a structural change in the protein from this mutation, as this residue lies at the end of the multimerization domain and is considered a structurally important amino acid (Brice *et al*., 1999). E57A is so close to the RNA binding domain (residues 35-50) may change the folding of Rev, and not present the arginine residues needed for RRE binding (Brice *et al*., 1999). Rev R39A is located in the ARM (arginine rich motif), this region contains the RNA binding domain and a mutation in this domain reduces the proteins ability to bind nucleic acids. Rev A37D is located near the RNA binding domain, however A37 is not thought to be important for RNA binding, but this residue is thought to lie in a hydrophobic patch that hold helix 1 and helix 2 together (Thomas *et al*., 1998).

These failures necessitated a need for a second purification scheme. To this end, the methods of Wingfield *et al*. (1991) were adapted to the available FPLC columns. Designated as Method 2, is the method that uses an anion exchange column to separate nucleic acids from Rev. The former binds the Q-sepharose column whereas Rev does not. Since most of the mutant Rev is found in the flow through fractions, this method is chosen.

Results of the purification of wild-type Rev through Method 2 are represented in Figure 7. Rev co-elutes with a single peak of proteins in the flow through of the Q-sepharose column (Figure 7 A/B). Approximately half of the wild-type Rev binds to the Q-sepharose column, although this is discarded in the second peak (Figure 7 A/B). Once the flow through is denatured with 6 M urea and diluted to 4 M urea, it is chromatographed over the Heparin-sepharose column. Wild-type Rev then elutes with a single peak of proteins released from the Heparin-sepharose column by 1M NaCl and 4 M urea (Figure 7 C/D). Wild-type Rev is re-folded through sequential dialysis steps.

Results of the purification of Rev E57A through Method 2 are represented in Figure 8. Rev E57A primarily co-elutes with a single peak of proteins in the flow through of the Q-sepharose column (Figure 8 A/B), although 20% or less of Rev E57A is bound to the column and discarded (Figure 8 A/B). The remaining flow-through fractions containing Rev E57A are pooled and denatured with 6 M urea, diluted to 4 M urea, and then chromatographed over the Heparin-sepharose column. Rev E57A then co-elutes with other proteins in a single peak released from the Heparin-column by 1 M NaCl and 4 M urea (Figure 8 C/D). An additional 10% or less of Rev E57A is lost in the flow

through on the Heparin-sepharose column (Figure 8 C, Peak 11-15). Rev E57A is re-folded through sequential dialysis steps (Figure 8 E).

The disadvantage of this method is that the protein is not as pure as that purified by Method 1 (compare Figure 4 E vs. 8 E). Whether these contaminants will affect Revs ability to bind and/or depolymerize MTs is not clear. To generate appropriate negative controls for the MT assays, BL21 cellular proteins were subjected to Method 2 purification. Figure 9 shows the negative control obtained from Method 2 purification. There were no visible proteins on a coomasie stained SDS-PAGE gel after the Heparin-sepharose column; therefore, to obtain a negative control for Method 2, the flow-through fractions were pooled after the Q-sepharose column. The proteins visible on a coomassie stained SDS-PAGE gels (Figure 9) are similar to the contaminating proteins in Figure 8 E, although, there are more proteins present in the negative control.

Tubulin Purification (Specific Aim 2)

Tubulin was purified from bovine brain as described in Materials and Methods. Briefly, tubulin was sequentially polymerized, depolymerized, and centrifuged repeatedly, then passed over a phosphocellulose column to separate it from the MAPs. Tubulin was collected and stored at -80ºC. The concentration of tubulin, 14.2 μM, is much lower than the expected 227 μM tubulin (http://mitchison.med.harvard.edu/ protocols/tubprep.html). Purified tubulin resolved by coomasie stained SDS-PAGE is represented in Figure 10. MAPs were also eluted from the column and stored at -80ºC.

MT Depolymerization Assay (Specific Aim 3)

Previous assays showing that Rev binds and depolymerizes MTs have relied on TEM. To study the biochemical properties of the Rev/MT interaction, assays used to

measure the KinI/MT interaction might be adapted to study Rev's mode of action. Now

that the proteins are purified, assays capable of testing Rev's ability to bind and

depolymerize MTs needs to be optimized.

One common assay, described in Materials and Methods, measures the amount of

tubulin released from MT polymers following addition of a depolymerizing agent

(Niederstrasser *et al*., 2002; Moores *et al*., 2002; Moores *et al*., 2004; Desai *et al*., 2001).

In brief, polymerized MTs are incubated with wild type or mutant Rev for various time

periods. Then the reaction mixture is subjected to high-speed centrifugation to separate

MTs from liberated tubulin heterodimers. The supernatant and pellet are resolved by

SDS-PAGE and the percentage tubulin released into supernatants is determined.

There are several variables to be considered during the optimization of the

depolymerization assay. First, the assay has to be completed with tubulin alone. As a

positive control, tubulin should stay in the pelleted fraction until a depolymerizing agent

is added to the reaction mixture. Second, completing the assay with Rev alone will help

indicate depolymerization because Rev that does not sediment to the pellet should be

greater with MTs in the reaction mixture than with Rev alone. Third, the molar ratio of

Rev to tubulin will provide information about the concentration dependence of MT

depolymerization by Rev. Fourth, a time dependence of MT depolymerization by Rev

has to be considered.

MTs need to be demonstrated not to depolymerize on their own in order to

indicate that there is an interaction with Rev that causes the MTs to depolymerize. When

3 μM MT's are used alone in the depolymerization assay, a range from 30-50 percent of

tubulin ends up in the supernatant over 60 minutes (Figure 11, Figure 12, 0 nM Rev). A

shortcoming to the depolymerization assay is that this percentage is too high based on

assays completed with KinI proteins (Niederstrasser *et al*., 2002; Moores *et al*., 2002;

Desai *et al*., 2001; personal communication, M. Miller). A more acceptable percentage

would be around 10%.

In addition to tubulin, Rev concentrations can also be confirmed during this assay

by western blotting. If the amount of Rev present in the supernatant is greater when Rev

is mixed with MTs than with Rev alone, then an interaction of Rev with MTs in indicated

due to the movement of Rev. When Rev is used alone during the depolymerization

assay, it remains predominately in the pellet at 10 and 20 minutes, approximately 23%

and 30% in the supernatant respectively (Figure 13). When 3 µM MT's are added, the

amount of Rev in the supernatant increases at 10 and 20 minutes, approximately 40% and

62% in the supernatant respectively, with the latter being statistically significant (p=.037)

(Figure 13).

The optimal stoichiometry for MT depolymerization by Rev needed to be

determined. The MTs were always kept at 3 µM. Figure 11 and 14 represents results

from adding different concentrations of Rev for 10 and 20 minutes incubations at each

concentration. Differences in linear regressions for concentration dependence are not

statistically significant (p=.389); however, there is a statistically significant difference in

the linear regression for 10 and 20 minute treatments (p=.021) with more

depolymerization at 20 minutes (Figure 11). This trend could be present because MTs

themselves depolymerize more over time and not because of the addition of Rev. At

equimolar amounts of Rev to tubulin (3000 nM Rev), Rev has been shown to produce

ringed structures or intermediates (Watts *et al*., 2000). The ability of these rings to spin

down into the pellet at the speed used in this assay could be presenting an additional problem. This would represent depolymerized tubulin in the pellet instead of the supernatant as expected.

To consider the time variable, MTs were incubated with 30, 300, and 3000 nM Rev for 10, 20, 40, and 60 minutes (Figure 12). There is a statistically significant ($p<.001$) increase in depolymerization at increasing reaction time. At 10 minutes, there is approximately 26% to 37% MT depolymerization over all Rev concentrations. At 60 minutes, the percentages increase to 48% to 68% depolymerization over all Rev concentrations. At 10 minutes, the rate of MT depolymerization is approximately 3% per minute and at 60 minutes is approximately .9% per minute, indicating a decrease in the rate of depolymerization over time.

Visual MT Binding Assay (Specific Aim 3)

Equivocal results in the depolymerization assays necessitated the visual MT binding assay next to determine if Rev is even binding MTs. If Rev is depolymerizing MTs via a mechanism shared with KinI kinesins, then it is hypothesized that Rev will preferentially bind the ends of MTs to depolymerize them. To test this hypothesis, the visual MT binding assay was completed as described in Materials and Methods (Desai *et al*., 2001). In brief, GMPcPP stabilized MTs are polymerized and diluted to 3 μM. MTs and varying concentrations of Rev are mixed, incubated, and then fixed. The reaction mixtures are then spun down onto coverslips and immunostained for the presence of Rev and tubulin.

Rev alone and MTs alone were spun down onto coverslips initially because the immunostaining conditions for Rev and MTs had to be optimized. Rev spun down

uniformly onto the coverslips; however, MTs did not (data not shown). There were small MT subunits all over the coverslip, but no MTs of measurable length. To confirm the presence of MTs, a small aliquot of the reaction mixture was spotted onto coverslips for immunostaining, which indicated that the difficulty with this assay lies with the MTs. The rest of the assay was completed with the alternative method as described in "Materials and Methods."

In addition to observing Rev and MT colocalization for the visual MT binding assay, the length of MTs were measured at different times to determine if MT length was shortening, demonstrating MT depolymerization (Figure 15). The MT length was measured for over 130 different MTs after 5 minutes incubation and then 60 minutes incubation for MTs alone and MTs mixed with equimolar amounts of Rev (3 µM). As indicated in Figure 16, there is a greater decrease in the average length of MTs when mixed with Rev for 60 minutes than for 5 minutes. Also noted is a qualitative result of more densely packed MTs at 5 minutes than at 60 minutes for MTs alone and MTs mixed with Rev (Figure 16). It is unclear whether this is subject to handling error in the experiment, or indicative of the inability of the MTs to remain stable over the 60 minute reaction time.

Discussion

Protein Purification (Specific Aim 1 & 2)

This study attempted to develop strategies to purify wild type and mutant Rev proteins and then use these proteins to develop assays that would measure their abilities to bind and depolymerize MTs. Two published methods were successful in purifying wild type Rev protein, but to different degrees of purity. Wild-type Rev appears as a single band on coomassie-stained SDS-PAGE gels using Method 1. This method appears more useful for purifying wild type Rev protein. Method 2 proved more useful in purifying mutant Rev proteins, although contaminating proteins are more prevalent. It is unlikely these contaminating proteins affect MT polymerization; however, conditions for preparing suitable controls for these contaminants were identified so that MT depolymerization and visual MT binding assays could be used (Figure 9). A buffer control should suffice for wild-type Rev purified by Method 1. Rev R42A purified by Method 1 will require a negative control by passing BL21 *E.coli* cellular proteins over the Q-sepharose and Heparin-sepharose columns as described by Method 1. If proteins after the Heparin-sepharose column fall below detection on coomassie-stained SDS-PAGE, then proteins present in the bound fractions from the Q-sepharose column can be used.

Method 1 was used to purify traditional mutant M7 (data not shown). Brice *et al*. (1999) also used this method to purify M7 and M4. Rev E57A, which lays one amino acid after M7, theoretically should purify with Method 1, also. However, this mutant, along with R39A and A37D, would not. Rev E57A is in a region of the protein that is considered important for protein folding (Brice *et al*., 1999). This mutation may disrupt

the conformation of Rev and not allow the arginine rich residues important for Method 1 purification to be exposed (Brice *et al*., 1999). It will be important that the activity of this mutant purified by Method 2 be accessed before it is used in assays.

There are procedural changes that may improve purification of mutant proteins, including changing the salt concentration of the flow-through from the Q-sepharose column of Method 2 (0 mM NaCl) to equal that of the eluate of the Q-sepharose column of Method 1 (800 mM NaCl). Raising the salt concentration of the solution before subjecting it to the Heparin column would allow more contaminating proteins to flow through instead of binding the column. Method 2 buffer F contains no salt. Method 1 buffer B contains approximately 800 mM NaCl. Method 1 purification works more efficiently and without as many protein contaminants. If the flow through from Method 2 is brought to 800 mM NaCl before subjected to the Heparin-sepharose column, to be similar to that of the eluate in Method 1, then the Heparin-column may run more effectively. It may also prove beneficial to use a gradient elution versus a step elution when attempting to purify mutants with Method 1. This would allow a slower increase in salt concentration and may provide fractions that contain mutant Rev with less protein contaminants.

MT Depolymerization Assay (Specific Aim 3)

To test the hypothesis that Rev depolymerizes MTs in a mechanism similar to that of KinI Kinesins two assays were developed following KinI methods and not those provided by Watts *et al*. (2000). The first assay focuses on wild-type and mutant Rev's ability to depolymerize MTs. This assay tests whether Rev can depolymerize MTs. The long-term goal is to compare mutant with wild-type Rev protein.

The first step was to use wild type Rev in this assay to have a comparison for the mutants. During the assay, Rev predominately stayed in the pelleted fraction when it was assayed alone. The location of Rev alone is important because it will be used as a comparison when MTs are added to the reaction mixture. Upon the addition of MTs, more Rev shifted to the supernatant fractions. This initial finding was the first evidence that Rev is binding and interacting with MTs.

At 3 μM MTs, 20-50 percent of the tubulin was in the supernatant fractions. GMP-cPP stabilized MTs are supposed to be in the pelleted fraction and remain there over time because GMP-cPP is a non-hydrolyzable form of GTP. The depolymerization of GMP-cPP stabilized MTs requires multiple rounds of action, and is useful to analyze the reaction mechanism (Desai *et al*., 2001). There is a small percentage (10-20%) of tubulin expected to be in the supernatant at approximately the same amount over time (Desai *et al.*, 2001; Moores *et al.*, 2002; Niederstrasser *et al*., 2002; personal communication, M. Miller). There were increasing amounts of tubulin (20-50 percent) in the supernatant over time. This indicates that the MTs are depolymerizing on their own; therefore, the increase in tubulin in the supernatant cannot be solely attributed to Rev depolymerizing MTs. A possible explanation for tubulin depolymerizing on its own is that the tubulin used in the assay was not recycled. Tubulin needs to be cycled to ensure that only active tubulin is being used in the assay.

When MTs and Rev were mixed, the concentration of Rev and the time of the reaction had to be measured as experimental variables. It was evident early on that there was a time dependency for MT depolymerization (Figure 11, 12). However, there was little evidence of a Rev concentration dependence. The later finding was surprising. To

solve the problem of MTs depolymerizing on their own, more stable MTs need to be prepared using taxol plus GMP-cPP and a higher starting concentration of recycled tubulin. Taxol is another stabilizer of MTs that works by eliminating the GTP hydrolysis destabilization of tubulin (Desai *et al.*, 2001).

The MT depolymerization assay indicated that there is an increasing trend for wild-type Rev to depolymerize more MTs over time, but the critical concentration that is needed of Rev to depolymerize MTs cannot be determined (Figures 11 and 12). This concentration will need to be determined in order to increase the efficiency of the assays. If the mutants indicate a similar graded result of more MT depolymerization over time and there is no critical concentration determined, then there is no sensitivity to the assay.

A potential shortcoming in the depolymerization assay is that the ringed intermediates may pellet, which would indicate depolymerized tubulin in what we are considering polymerized tubulin. The rings are approximately 3.8 MDa with a diameter of approximately 40 nm (Watts *et al.*, 2000).

There has been recent improvement of the depolymerization assay that has proven useful (personal communication, A. Sharma). A protocol based on Watts *et al.* (2000) has been implemented using MES buffer with Rev and MEM buffer with MTs instead of dialysis buffer 3 and BRB80, and a higher concentration (20 μM instead of 3 μM) of Rev and MTs. It is known that citrate (present in dialysis buffer 3) chelates Mg^{2+} ions (needed for MT polymerization). Using the MEM/MES buffering system and higher concentrations of proteins has provided reproducible results with the depolymerization assay indicating that wild type Rev is depolymerizing MTs.

Visual MT Binding Assay (Specific Aim 3)

A visual MT binding assay was used to address the hypothesis that Rev binds the ends of MTs to depolymerize them. However, it also produces a means for visually confirming MT length as a function of Rev activity. While end binding is addressed, depolymerization of MTs can also be measured during this assay.

End binding was addressed by visualizing colocalization of Rev on the MT. MTs were immunostained red and Rev immunostained green, colocalization was indicated by a yellow color, the combining of the two. Yellow ends of MTs were noted (data not shown), indicating preferential end binding. There were also many instances where the entire MT was decorated with Rev, or colored yellow. This later finding could be a result of the high concentration of Rev used, along with the fact that the MTs were not spun down onto the coverslip, but instead were aliquoted directly to the coverslip. Directly aliquoting the reaction onto the coverslip makes the background much higher and immunostaining more difficult.

The MTs were spotted alone, then MTs incubated with varying amounts of Rev for 5-60 minutes were spotted and MTs under each condition measured. There was a greater decrease in length of MTs treated with Rev at 60 minutes than at 5 minutes, implying a time dependence of the depolymerization activity of Rev. There was a noticeable trend in the number of MTs present in the Rev treated reactions. At 60 minutes there were only a fraction of the number of MTs present at 5 minutes. This trend was present for MTs alone, also, which presents a problem with the finding with the Rev treated reactions. The MTs are obviously not stable over the 60 minute time period used.

Using GMP-cPP and taxol, doubly stabilized MTs may allow MTs to be stable long enough for the assays to be completed.

There are shortcomings to this assay. It is important to develop a way to spin the reaction mixtures down in order to eliminate small particles that can contribute to aesthetics of the coverslip (Hertzer *et al*., 2006; Desai *et al*., 2001). Using the MEM/MES buffers in this assay may provide useful as BRB80 is present so the problem still exists with citrate (dialysis buffer 3) chelating Mg^{2+} (BRB80). Also, the length of the MTs used needs to be increased by increasing the concentration of tubulin during the polymerization step. Lengthening MTs may contribute to more reliable measurements of MT length and allow MTs to be measured over a longer period of depolymerization time. Also, using taxol and/or taxol plus GMP-cPP stabilized MTs may make the MTs stay intact and spin down onto the coverslip because the taxol and GMP-cPP inhibit the hydrolysis of GTP and promotes MT polymerization.

Taken together, these two assays have indicated that there is an increasing trend in MT depolymerization by Rev over time, although this is clouded by the observation that MTs alone shorten with time. This result was confirmed in both assays. There is a need for a higher starting recycled tubulin concentration for both assays in order to have active tubulin that will polymerize into longer MT's and/or more stable MTs with taxol and/or taxol plus GMP-cPP. The new MES/MEM buffering system needs to be continued and implemented in the visual MT binding assay, along with the higher protein concentrations (Watts *et al*., 2000). It is also important to consider the functionality of purified Rev in these assays.

Activity of Rev

Preliminary steps to test Rev activity relied on injecting purified wild-type Rev into the cytoplasm of frog oocytes. If Rev is folded properly and active, once injected into the oocyte, it should readily localize to the nucleus. Oocytes incubated for 0 to 6 hours to overnight after injection were placed in TCA to fix the proteins, and the nucleus was removed. The nucleus and cytoplasmic portions were combined and then resolved by SDS-PAGE and western blotted for Rev. No Rev was detected on any of the blots. Potential reasons for this outcome include Rev adhering to the injection needle and not being injected into the egg. To solve this problem, the needle can be blocked with BSA prior to the experiment to stop any non-specific binding by Rev to the glass. Alternatively, Rev was degraded upon entry into the oocyte. This seems unlikely because oocytes are designed for storage of proteins for use after fertilization, not to degrade them, unless Rev is not folded well. Another means of confirming Rev activity is clearly required. A potential assay is the transfection of Rev into Hela cells followed by immunostaining. If Rev is folded properly and active, it should localize to the nucleus of the Hela cells. Another way to assess functionality of Rev is to do a gel retardation assay to determine if Rev is binding the RRE and multimerizing (Brice *et al*., 1999; Thomas *et al*., 1998). If Rev is not folded properly or is inactive, then the purification and/or dialysis method will have to be altered to promote proper folding of Rev.

Hypothesis Revisited

I hypothesized that Rev is depolymerizing MTs in a shared mechanism with KinI Kinesins. Rev could be binding the end of the MT, multimerizing along the length of the MT, causing a bent conformation in the MT, and subsequently causing depolymerization.

The residues important to Rev function were not studied (mutations), however, the initial/optimization experiments with wild-type Rev were completed. The mentioned hypothesis is still unanswered, but there are reliable methods for protein purification of wild type and mutant Rev and tubulin. Initial work with the MT depolymerization and visual MT binding assays has also been completed. Early studies indicate that Rev is interacting with MTs and there is a trend in MT depolymerization in a time dependent manner. These results are clouded by the tendency of the MTs to depolymerize on their own, for which corrective measures have been discussed including the use of MES/MEM buffers (Watts *et al*., 2000). The initial results are consistent with the hypothesis by Watts *et al*. (2000) that Rev is binding and depolymerizing MTs. The future work with mutants in this project will provide information into the mechanism by which Rev is depolymerizing MTs.

Mechanistic and structural information about Rev and KinI's ability to depolymerize MTs may be useful in the future study for treatments for HIV/AIDS and cancer. Since MT polymerization and depolymerization are necessary events for cellular division, a MT depolymerizing agent (peptides derived from Rev) may be used to alter cell division. This technology would be useful in the treatment of cancer where uncontrolled cell growth is the problem, and HIV where stopping infected cells from multiplying is imperative. Tubulin derivatives may be designed to interact with Rev and detour Rev into unproductive reactions. This data will also be useful to provide structural information about Rev protein.

Table 1: Comparison of HIV-1 Rev and KinI Kinesin Characteristics

Characteristics	HIV-1 Rev	KinI Kinesins
Sequence homology with	KinI's	Rev
Multimerization required for depolymerization of MTs	Unknown	No
Depolymerizes MTs forming rings	Yes	Yes
ATPase activity	No	Yes (not required for MT depolymerization)
Depolymerize subtilisin-treated MTs	Yes	No
Depolymerize from end of MT	Yes	Yes

Figure 1: HIV-1 Rev mutant amino acid sequences and diagram. **(A)** Traditional Rev mutants (red), and Rev:XKCM1 homology mutants (black) sequences. The positions of the mutants are below the sequence and how the sequences are changed listed above. The XKCM1 homology mutants are named by their substitution listed on the top of the sequence. **(B)** Schematic diagram of Rev domains with Rev:XKCM1 point mutations on top and traditional mutations on bottom.

(A)

```
       D   DL                  DL        DL                      ΔS ΔS                              DL
22  L Y Q S N PPPNPEGT R Q A RR N RRRR WRERQRQIH S I S ERILSTYLGRSAEPVPLQLPP LE RL  81
         M4                   M5        M6                        M7                                M10
```

```
                    A   D A   A       A   A        A
22  LYQSNPPPNPEG T RQ A RR NR R RRWR E RQ R QIHSIS E RILSTYLGRSAEPVPLQLPPLERL  81
                  34  37 39  42      47  50       57
```

(B)

```
                                    34 37 39   42   47 50    57
1                                   *  *  *    *    *  *     *                                      116
```


| | Multimerization 1 | RNA Binding | Multimerization 2 | | Activation Domain | |

Helix 1   Poly Proline   Helix 2

```
         *                    *   *           *                         *
        M4                   M5  M6          M7                        M10
```

41
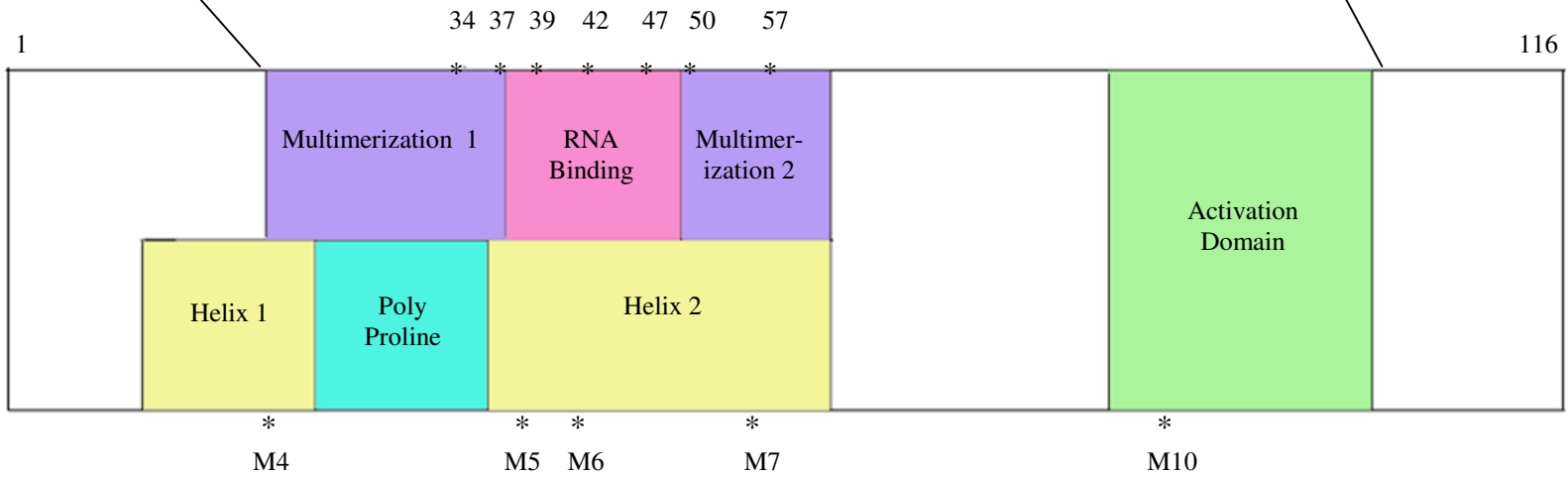
Figure 2: Rev and KinI Kinesin Sequence Alignments Amino acid alignment of Rev, XKCM1, Kif2c. For XKCM1 and Kif2c residues identical to Rev are indicated in red, conservative substitutions in green, and similar substitutions in blue. The sequence alignment of Kif1A, a motile kinesin, is shown for comparison.

```
REV      34          TRQARRNRRRRWRERQRQIHSISERILSTYLGRSAEP
XKCM1    500 NERGVDTASADRITRMEGAEINRSLLALKECIRALGQNKSHTPFRESKLTQ
Kif2c    492 NERGADTSSADRQTRMEGAEINKSLLALKECIRALGQNKAHTPFRESKLTQ

Kif1A    252 SERADSTGAKGTRLKEGANINKSLTTLGKVISALAEMDSGPNKNKKKKTDF
```

Figure 3: Structure of Kif2c with Rev Homology Domain **(A)** The structure of Kif2c in ADP bound state (PDB, 1V8J). **(B)**The structure of Kif2c in AMP-PNP bound state (PDB, 1V8K). The Rev homology domain is highlighted in cyan, and residues are colored to match the XKCM1 homology, identical residues are red, conservative substitutions are green and similar substitutions are blue. Kif2c residues and loops are labeled for orientation. Amino acids 498, 501, 503, 506 (B only), and 534 are not present in the structure of Kif2c.

(A)

L2 (Tu-C)

L11

E521

α4

E511

L8 (Lys/Arg)
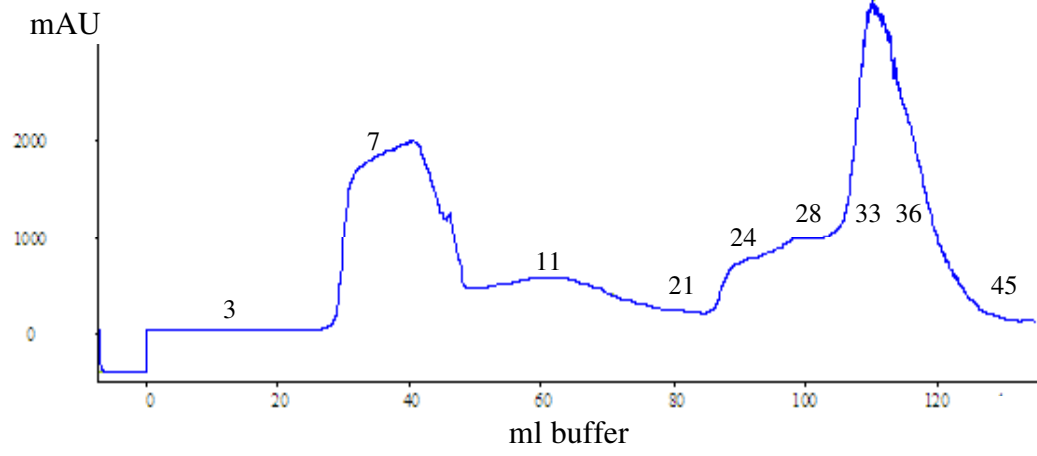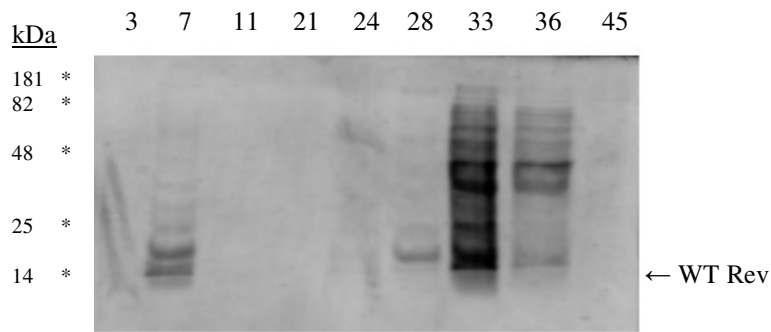
(B)

L2 (Tu-C)

L11

E521

α4

E511

L8 (Lys/Arg)
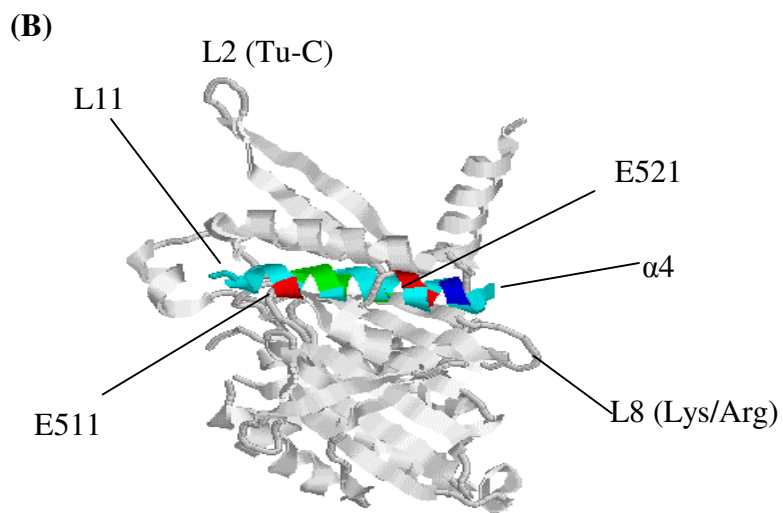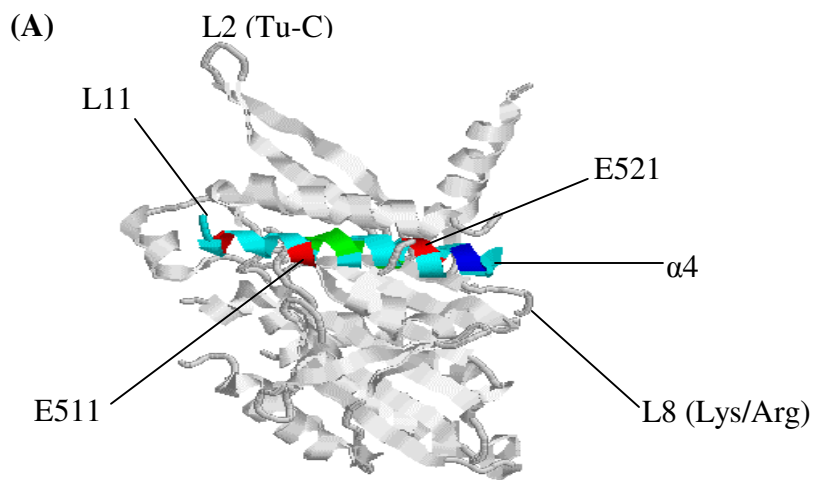
Figure 4: Method 1 Purification of WT Rev Sonicates clarified by 10,000 x g were subjected to sequential Q-sepharose **(A)** and Heparin-sepharose **(C)** columns as described in Materials and Methods, x-axis represents milliliters of buffer (ml) and y-axis represents milli absorbance units (mAU). Representative f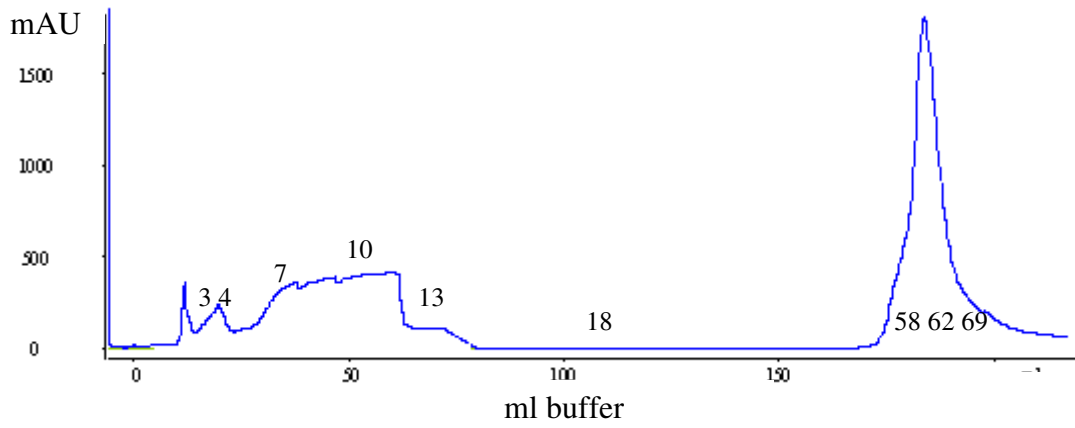ractions for each column were resolved by SDS-PAGE **(B,D)**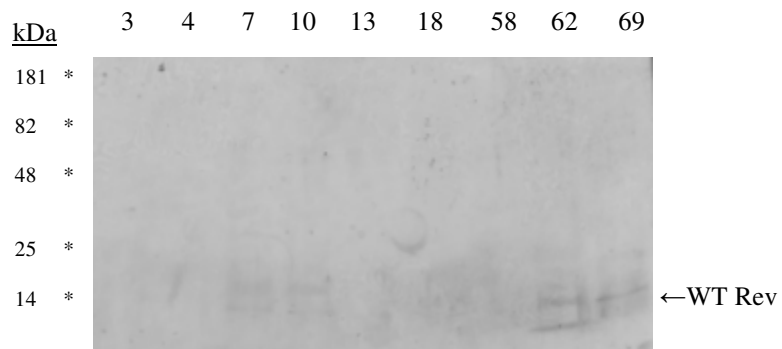. Rev protein (10 μg) was re-folded by sequential dialysis steps as described in Materials and Methods **(E)** and resolved by SDS-PAGE.

(A)

(B)

(C)

47

(D)

kDa	5	13	29	30	31	38

181 *
82 *
48 *

25 *

14 *

←WT Rev

(E)

kDa

181 *

82 *

48 *

25 *

14 *
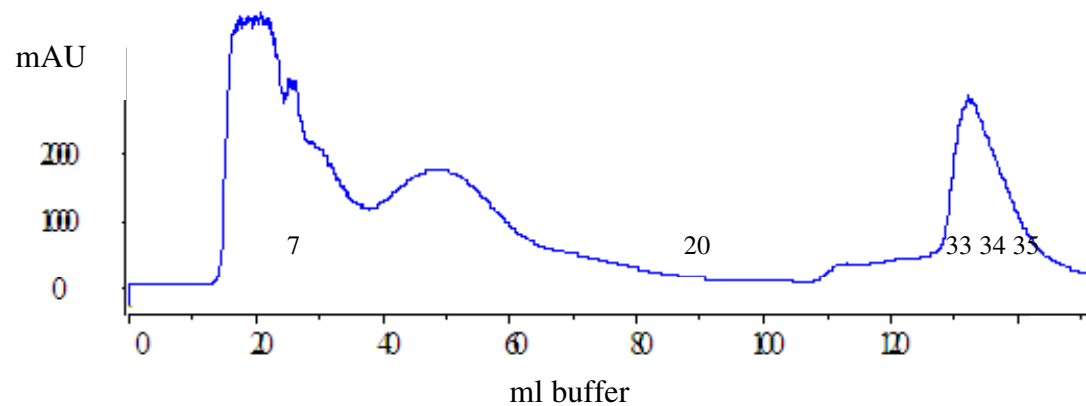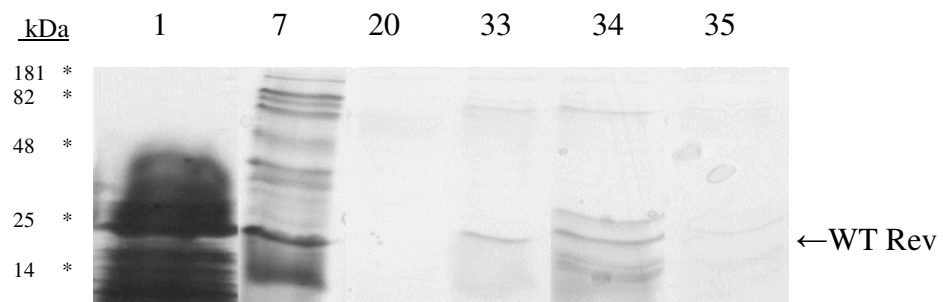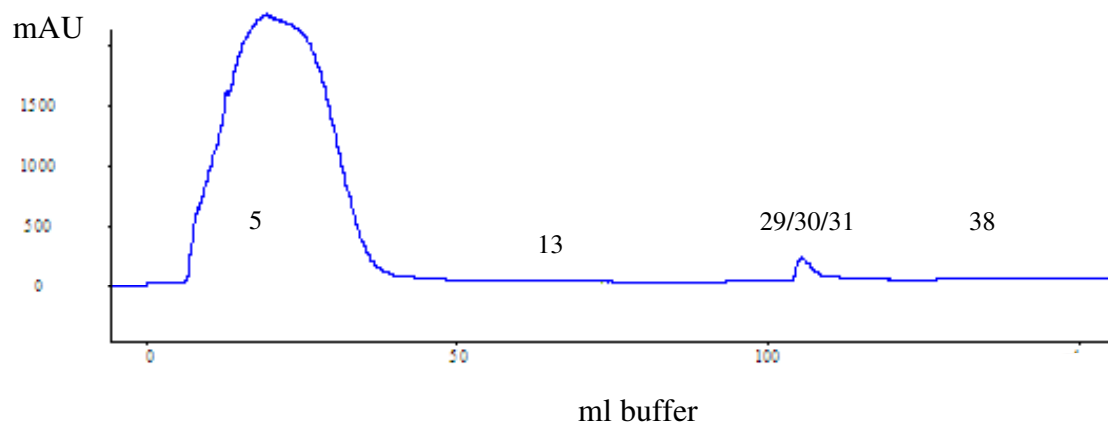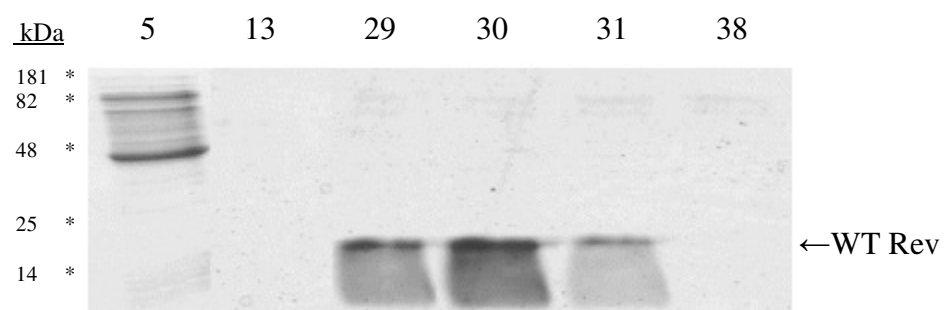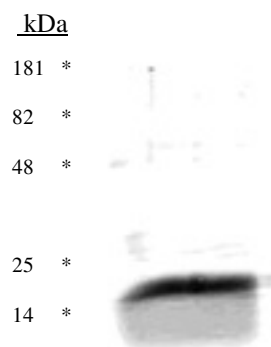
Figure 5: Method 1 Purification of R42A Sonicates clarified by 10,000 x g were subjected to sequential Q-sepharose **(A)** and Heparin-sepharose **(C)** columns as described in Materials and Methods, x-axis represents milliliters of buffer (ml) and y-axis represents milli absorbance units (mAU). Representative fractions for each column were resolved by SDS-PAGE **(B,D)**. R42A Rev protein was re-folded by sequential dialysis steps as described in Materials and Methods **(E)** and resolved by SDS-PAGE.

(A)

mAU

800mM NaCl

ml buffer

(B)

← R42A

(C)

mAU

2M NaCl
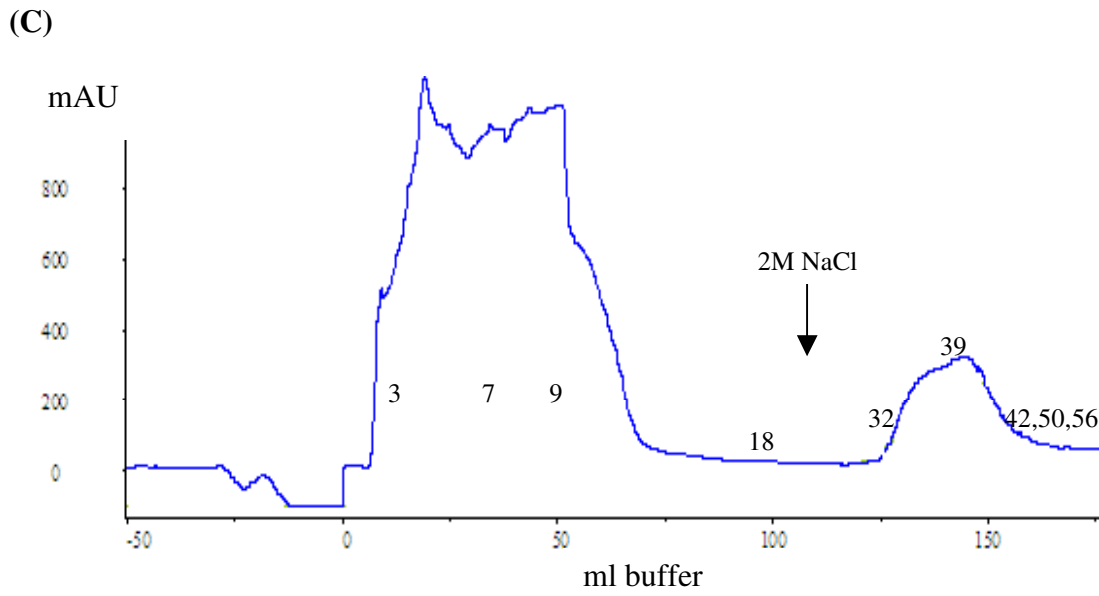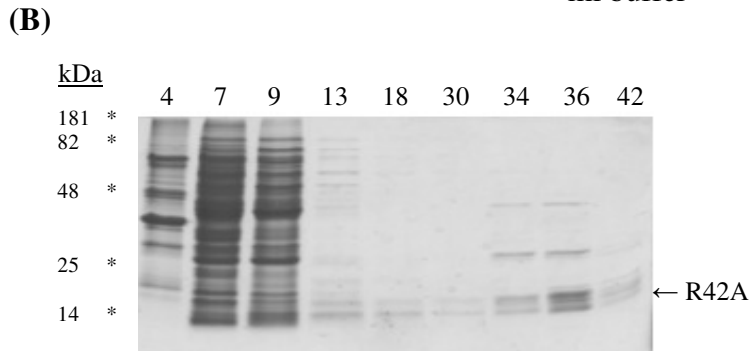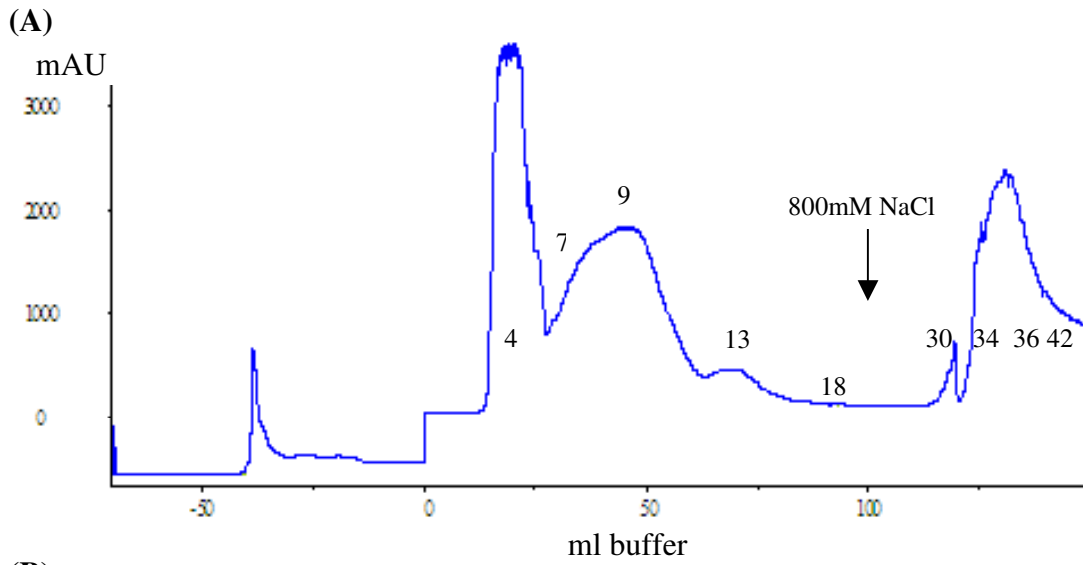
ml buffer

(D)
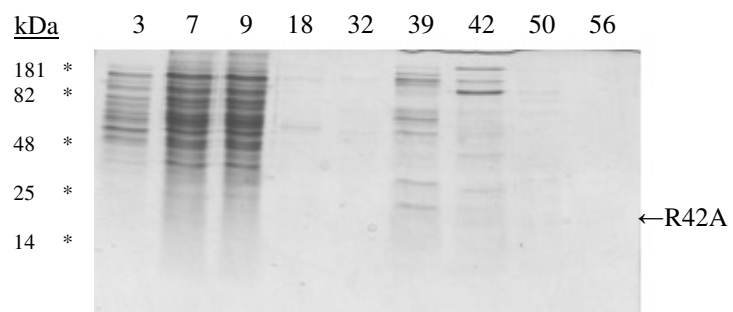
(E)
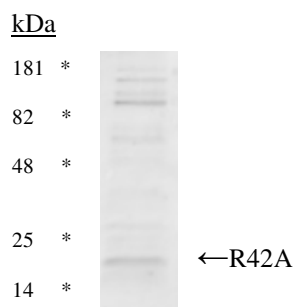
Figure 6: Attempted Purification of Rev R39A Using Method 1   Sonicates clarified by 10,000 x g were subjected to sequential Q-sepharose **(A)** column as described in Materials and Methods, x-axis represents milliliters of buffer (ml) and y-axis represents milli absorbance units (mAU). Representative fractions were resolved by SDS-PAGE **(B)**.

(A)
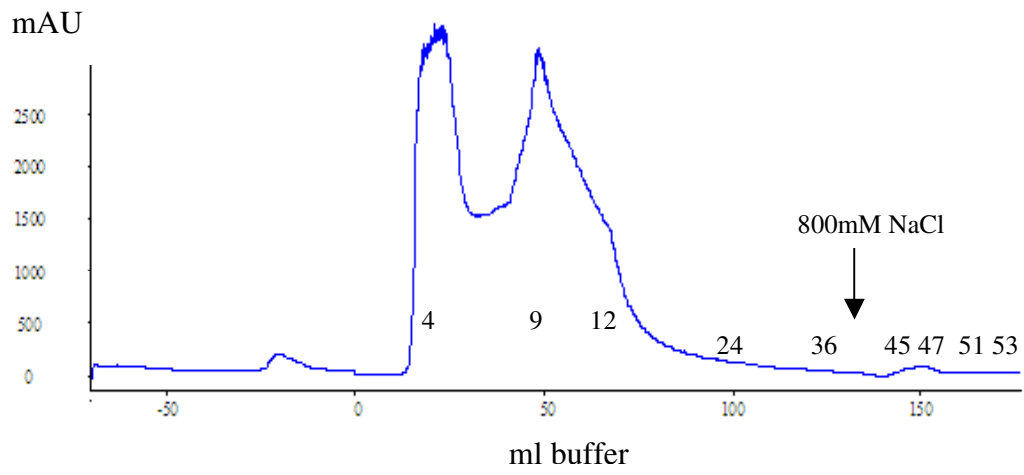
(B)
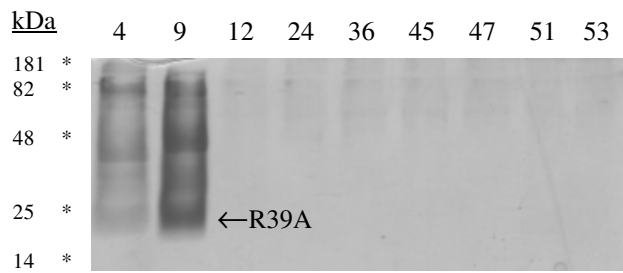
Figure 7: Method 2 Purification of WT Rev Sonicates clarified by 10,000 x g were subjected to sequential Q-sepharose **(A)** and Heparin-sepharose **(C)** columns as described in Materials and Methods, x-axis represents milliliters of buffer (ml) and y-axis represents milli absorbance units (mAU). Representative fractions for each column were resolved by SDS-PAGE **(B,D)**.

(A)

(B)

(C)

(D)

Figure 8: Method 2 Purification of E57A Sonicates clarified by 10,000 x g were subjected to sequential Q-sepharose **(A)** and Heparin-sepharose **(C)** columns as described in Materials and Methods, x-axis represents milliliters of buffer (ml) and y-axis represents milli absorbance units (mAU). Representative fractions for each column were dot blotted **(B)** and resolved by SDS-PAGE **(D)**. E57A Rev protein was re-folded by sequential dialysis steps as described in Materials and Methods **(E)** and resolved by SDS-PAGE.

(A)
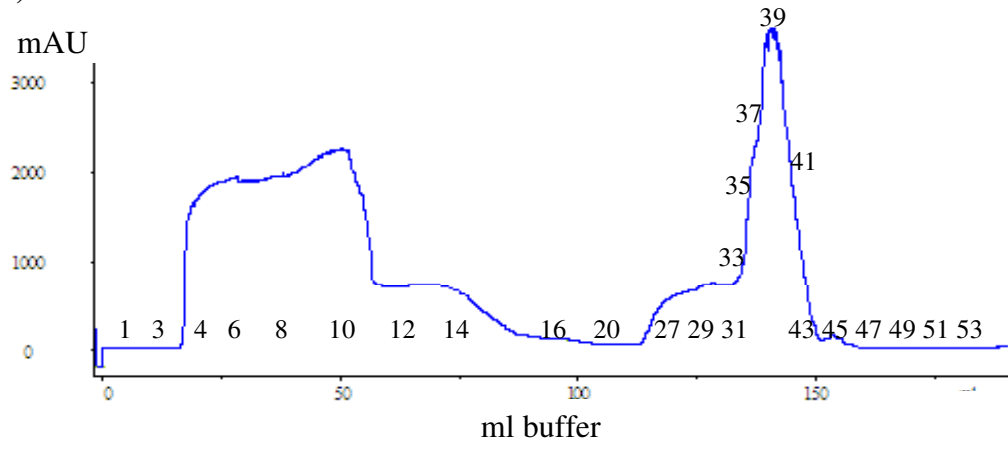
(B)
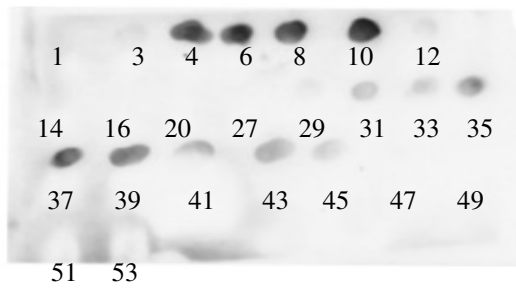
(C)
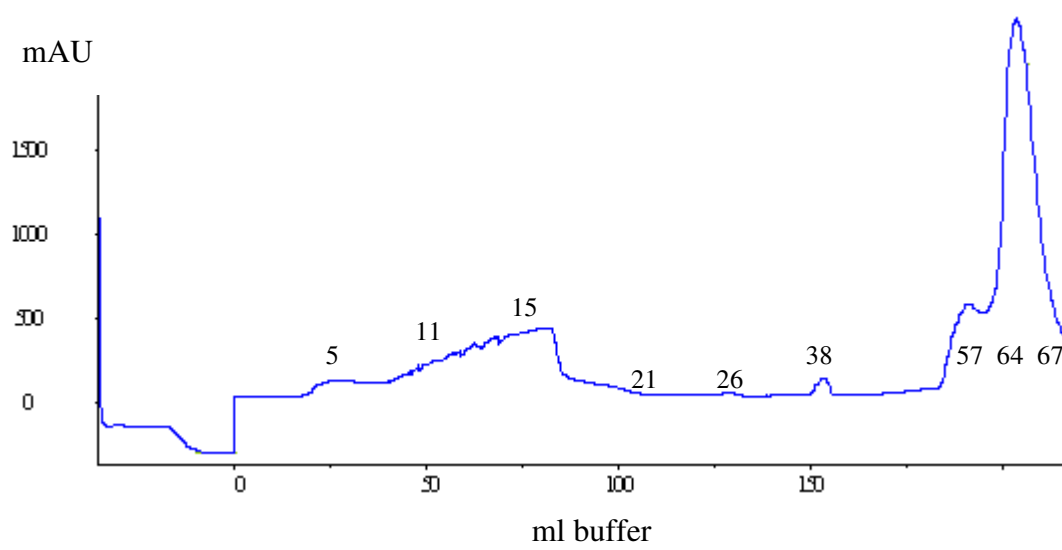
(D)
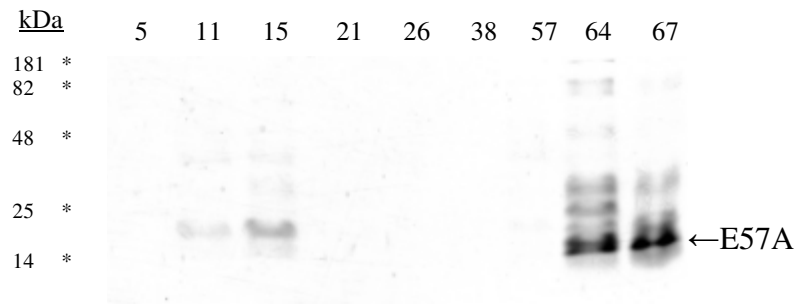
(E)
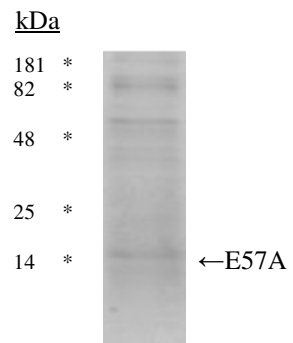
Figure 9: Negative Control Purification Method 2 10 µl protein sample purified by Method 2. Sample taken from end of Q-sepharose column (BL21 cells only). Contains Method 2 contaminating proteins and no Rev.
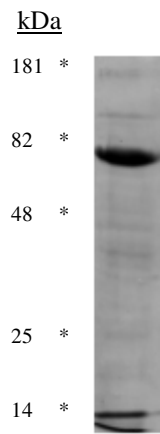
Figure 10: Purified Bovine Tubulin Protein Purified bovine tubulin resolved by coomasie stained SDS-PAGE. Tubulin is a 55 kDa protein. Lane 1 contains 1.56 µg tubulin, and lane 2 contains 4.68 µg tubulin.
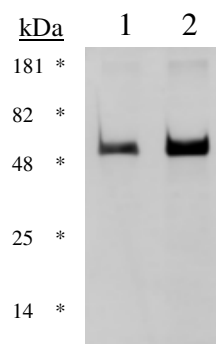
Figure 11: Depolymerization of Tubulin as a Function of Rev Concentration Green diamonds indicate 10 minute incubation and red squares indicate 20 minute incubation of Rev with MTs. X-axis indicates log of nanomolar concentration of Rev mixed with 3 µM MT's. The y-axis depicts the percentage of tubulin released into the supernatant, indicative of MT depolymerization. Averages and standard deviations computed on 3 independent experiments for 10 nM, 80 nM, 100 nM, and 800 nM Rev at 10 and 20 minutes. Averages and standard deviations computed on 7 independent experiments at 30 nM and 300 nM Rev, 8 independent experiments at 3000 nM Rev at 10 and 20 minutes, and 9 independent experiments at 0 nM Rev at 10 and 20 minutes.

Equation for Green Line: $y = .0022x + 22.3$ $R^2 = .0513$
Equation for Red Line: $y = .0002x + 36.0$ $R^2 = .0011$

Analysis of variance indicates that differences in linear regressions for concentration dependence are not statistically significant (p=.389). However, analysis of variance indicates that differences in linear regressions for 10 and 20 minute time points are statistically significant (p=.021), and this is confirmed by one-way ANOVA between 10 and 20 minute data points which show statistically significant differences (p=.008).
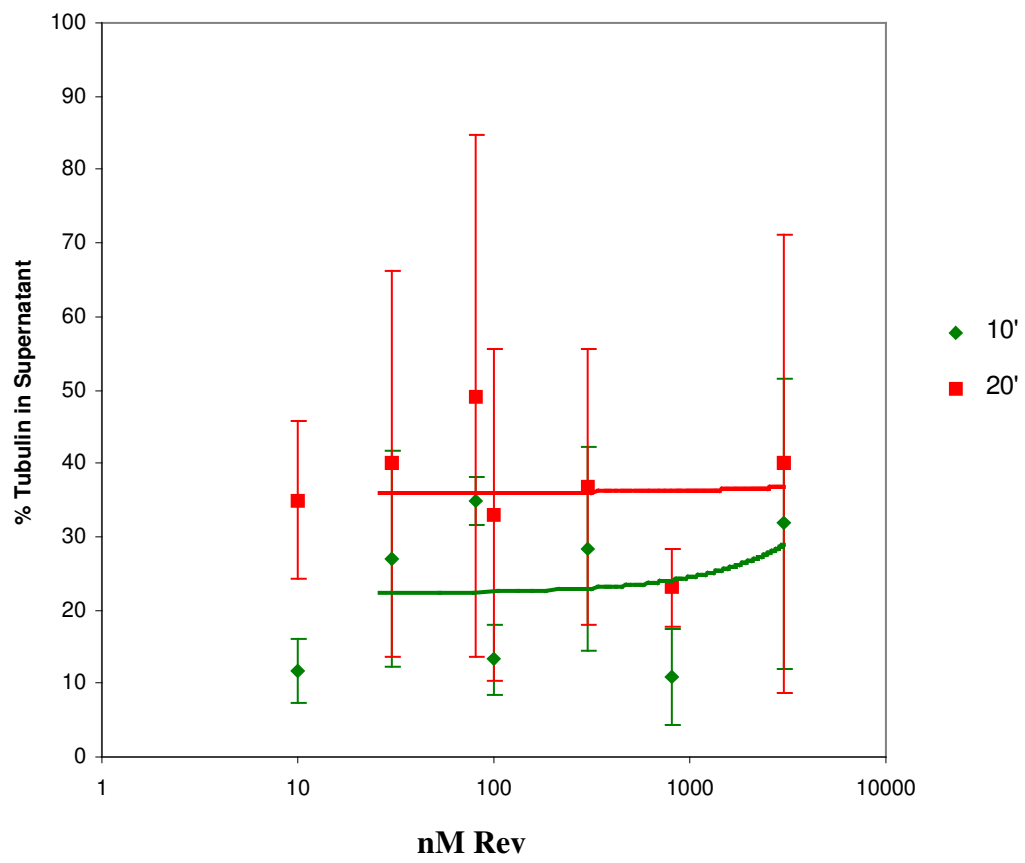
<u>Figure 12: Depolymerization of MT's by Rev Over Time</u> Blue diamonds indicate 0 nM Rev incubation, pink squares indicate 30 nM Rev incubation, green triangles indicate 300 nM Rev incubation, red x's indicate 3000 nM Rev incubation. X-axis indicates time (min) Rev mixed with 3 μM MTs, and y-axis indicates % of tubulin released into the supernatant (indicative of MT depolymerization). Averages and standard deviations computed on 7 independent experiments at 30 nM and 300 nM Rev at 10 and 20 minutes, and 3 independent experiments at 40 and 60 minutes. Averages and standard deviations computed on 8 independent experiments at 3000 nM Rev at 10 and 20 minutes and 3 independent experiments at 40 and 60 minutes. Averages and standard deviations computed on 9 independent experiments at 0 nM Rev at 10, 20, 40, and 60 minutes.

Equation of Blue Line: $y = .262x + 29.5$ $R^2 = .420$
Equation of Pink Line: $y = .630x + 21.1$ $R^2 = .736$
Equation of Green Line: $y = .837x + 22.4$ $R^2 = .853$
Equation of Red Line: $y = .288x + 34.4$ $R^2 = .274$

Analysis of variance indicates that differences in linear regressions for concentration dependence are not statistically significant ($p=.686$). However, analysis of variance indicates that differences in linear regressions for time dependence are statistically significant ($p<.001$), and this is confirmed by two-way ANOVA between 10, 20, 40, and 60 minute data points which show statistically significant differences ($p=.009$).
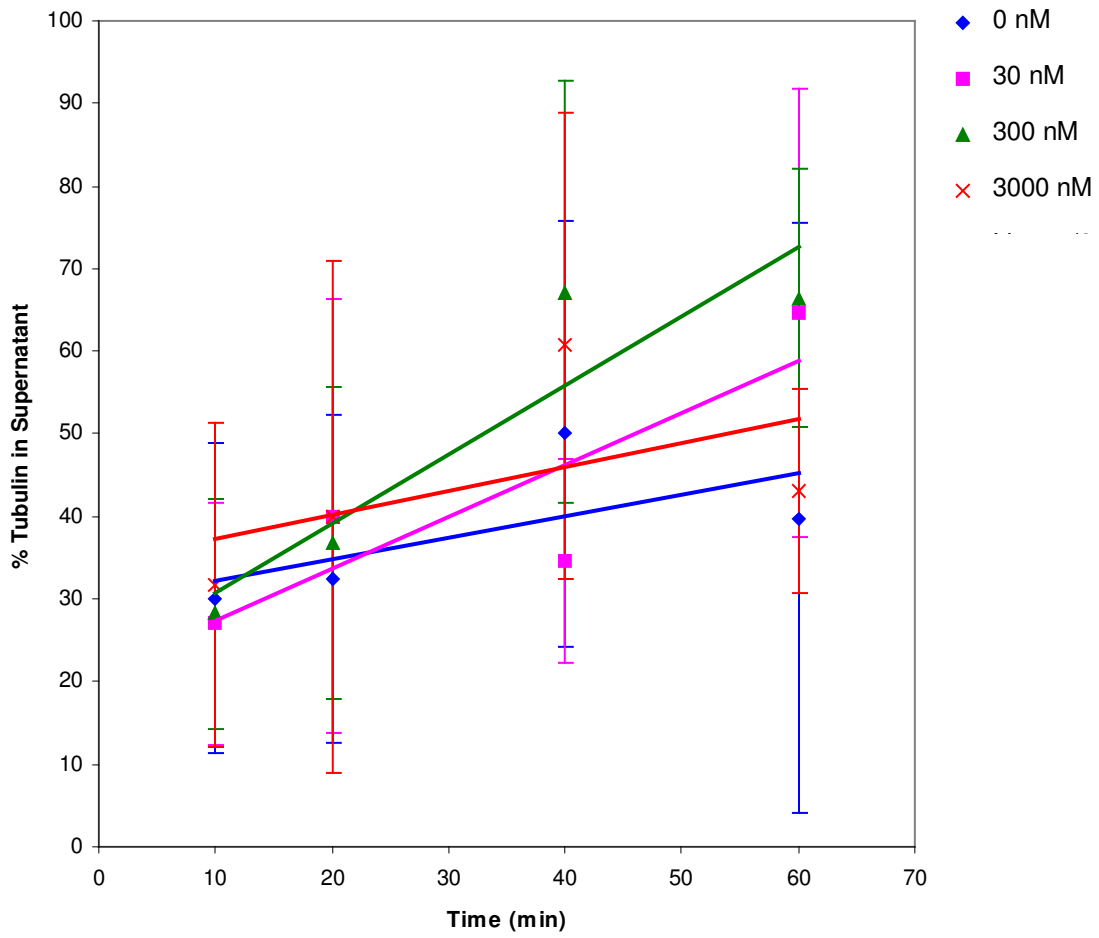
Figure 13: Amount of Rev Released into 90,000 rpm Supernatant Blue bars indicate 3 µM Rev alone. Red bars indicate 3 µM Rev mixed with 3 µM MTs. X-axis indicates time in minutes and y-axis indicates percentage of Rev present in the supernatant. Averages and standard error of the mean computed on 3 independent experiments at 10 minutes and 2 at 20 minutes for Rev alone (blue bars). Averages and standard deviation computed on 4 independent experiments at 10 and 20 minutes for Rev and MTs (red bars).

There is no statistically significant difference in the amount of Rev in the supernatant with the addition of MTs at the 10 minute time period (p=.388), however statistically significant differences are noted at the 20 minute time period (p=.037).
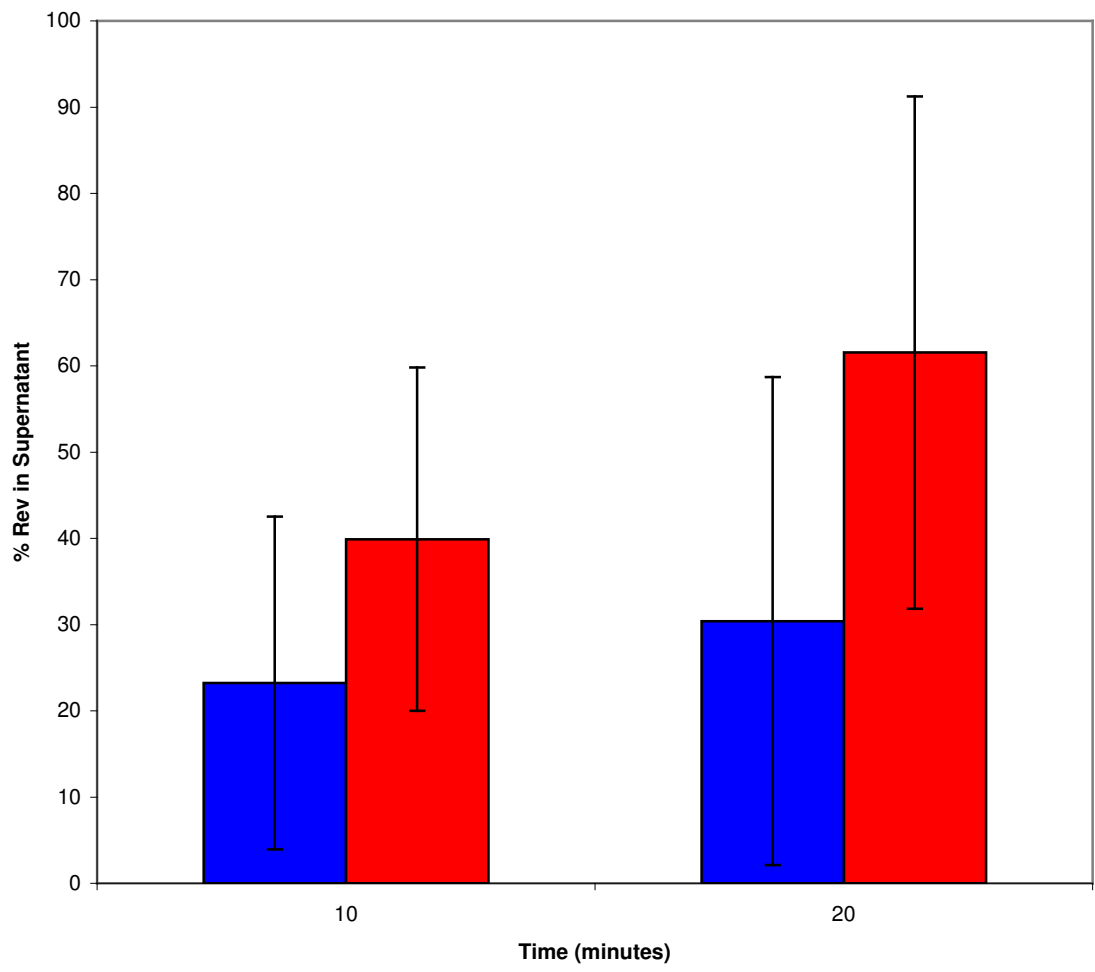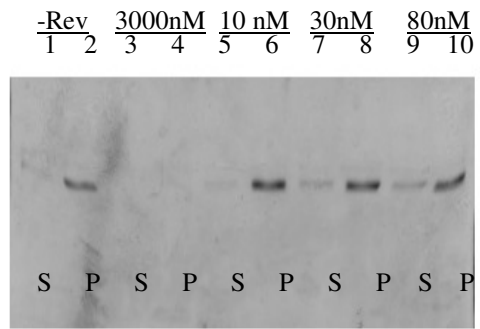
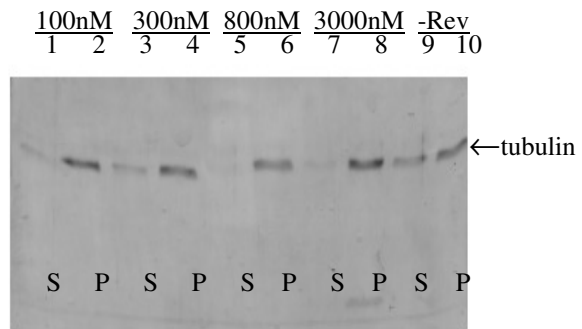**Figure 14: Coomasie stained SDS-PAGE gels of MT Depolymerization Assay**
Representative gels from MT depolymerization assay as described in Materials and Methods. Each lane contains 10 µl of reaction mixture. All lanes except 3 and 4 (gel 1); and 1 and 2 (gel 3) contain 3 µM MT's plus the amount of Rev indicated above the lane number. All Gel 1 lanes and Gel 2 lanes 1-8 are after 10 minutes. Gel 2 lanes 9-10 and all gel 3 and 4 lanes are after 20 minutes. Lanes 7-10 on gel 4 are blank. (S = supernatant; P = pellet)
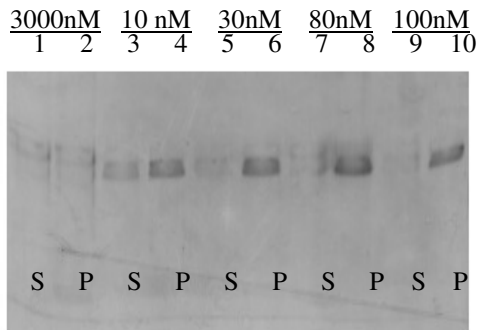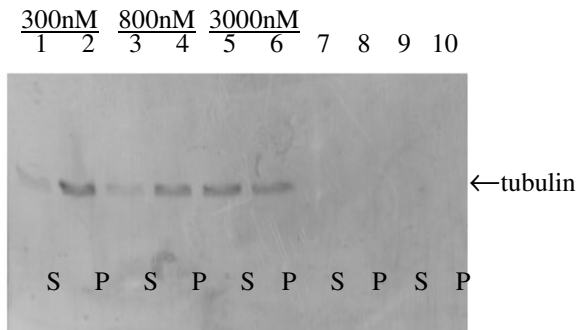
Gel 1

-Rev	3000nM	10 nM	30nM	80nM
1 2	3 4	5 6	7 8	9 10

S P S P S P S P S P

Gel 2

100nM	300nM	800nM	3000nM	-Rev
1 2	3 4	5 6	7 8	9 10

←tubulin

S P S P S P S P S P

Gel 3

3000nM	10 nM	30nM	80nM	100nM
1 2	3 4	5 6	7 8	9 10

S P S P S P S P S P

Gel 4

| 300nM	800nM	3000nM		
1 2	3 4	5 6	7 8	9 10

←tubulin

S P S P S P S P S P

Figure 15: MTs Spotted on Coverslip **(A)** MTs incubated with 300 nM Rev for 10 minutes, fixed, and spotted on a coverslip. MTs are visible and measurable. MTs are red, Rev is green, and colocalization of Rev and MTs is yellow. Similar pictures were obtained for 30 and 3000 nM Rev with MTs. **(B)** MTs alone, fixed, and spotted on a coverslip. Bar 1.78 μm.

(A)
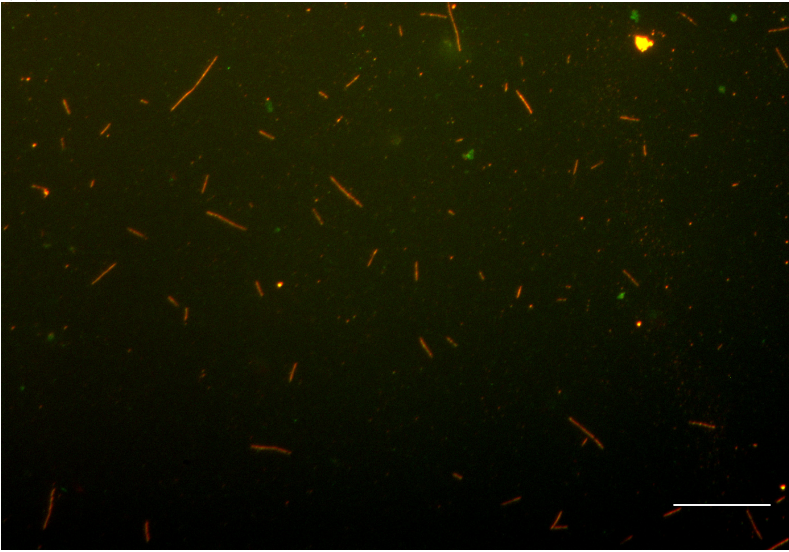
(B)
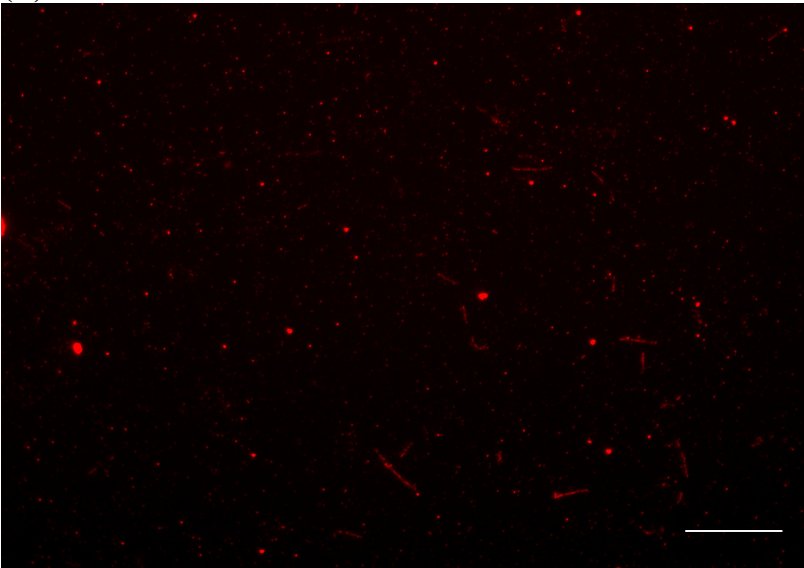
Figure 16: MT Depolymerization by Visual MT Binding Assay **(A)** MT length measured during visual MT binding assay. Red bars are 3 μM MTs alone. Yellow bars are equimolar amounts of Rev and MTs (3 μM). At least 130 MTs randomly measured at each time point to determine averages and standard deviation. **(B-E)** Representative pictures of each reaction mixture with total number of measured MTs listed above. MTs are red, and the measurements are multicolored lines with numbers next to them. **(B)** 5 minutes MT, **(C)** 60 minutes MT, **(D)** 5 minutes MT + Rev, **(E)** 60 minutes MT + Rev. Bar =1.78 μm.

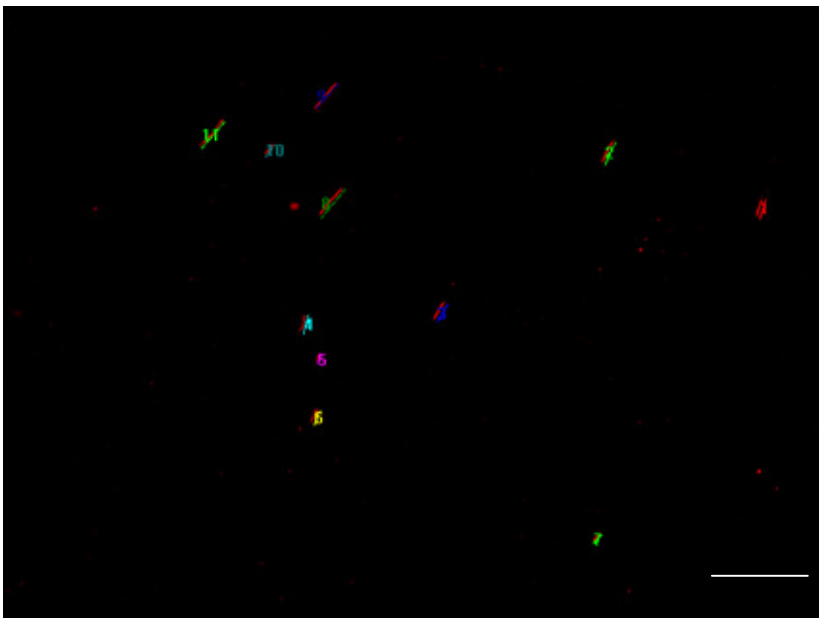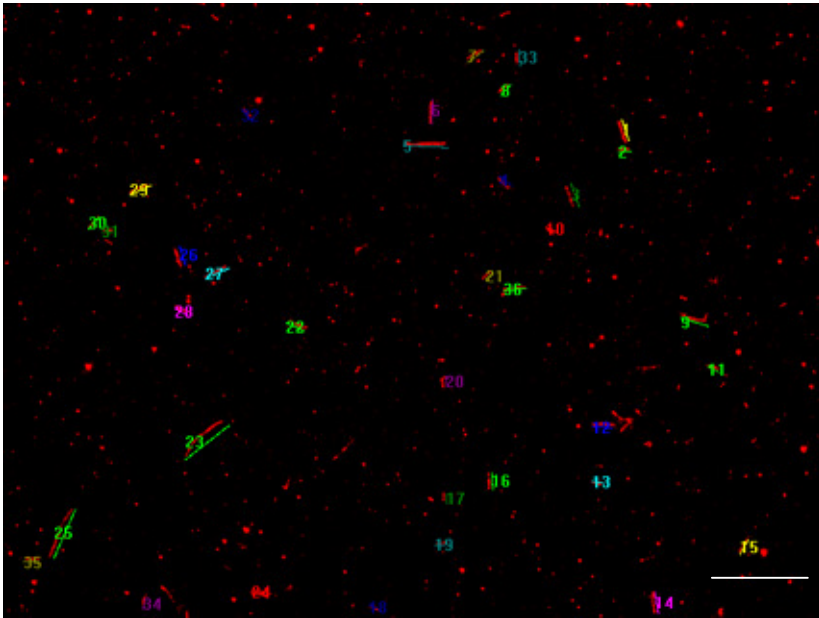No statistical significance (p>.05)
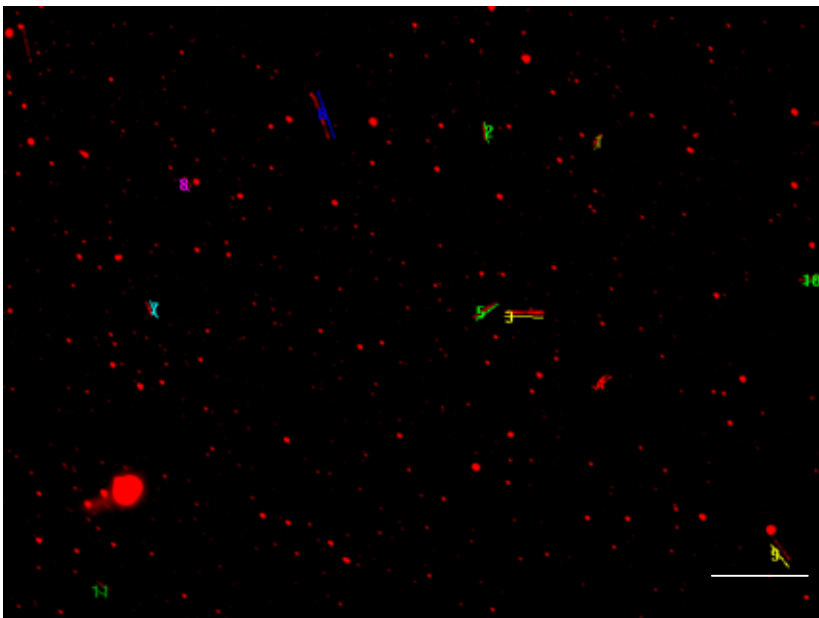
(A)
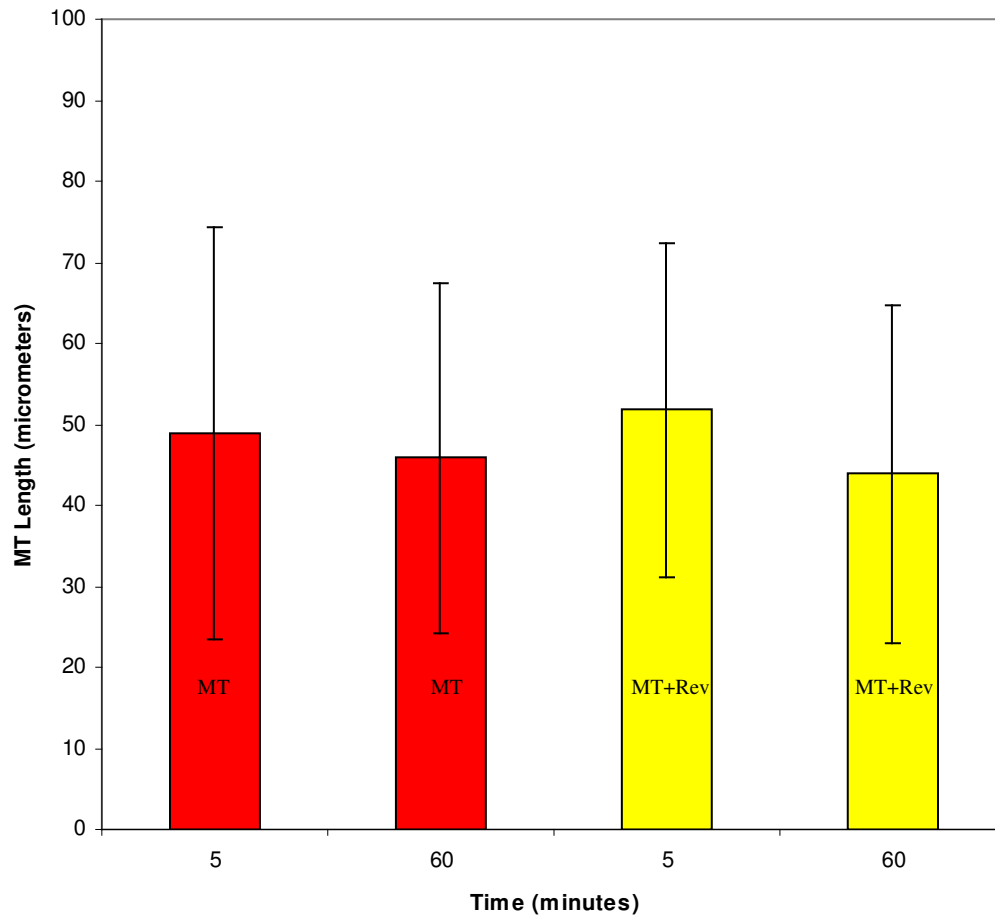
(B) 43 measured MTs
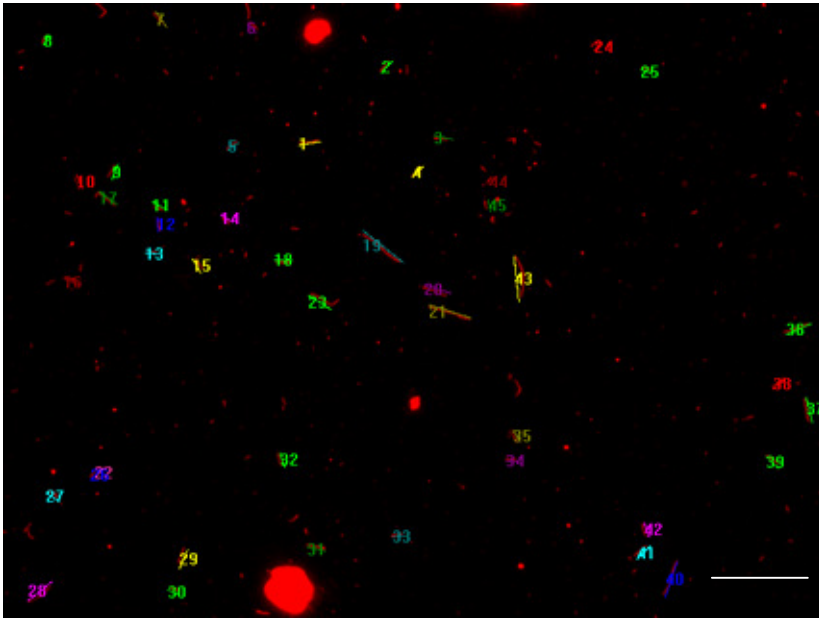
(C) 11 measured MTs

(D) 36 measured MTs

(E) 11 measured MTs

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