Identifying the Amino Acids Important for HIV Rev-Tubulin Interactions

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By

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Abstract

Dukes II, Bruce E. M.S., Department of Biological Sciences, Wright State University, 2015. Identifying the Amino Acids Important for HIV Rev-Tubulin Interactions.

The Rev protein is Human Immunodeficiency virus’s “switch” from events occurring early in infection to later events. Early in infection, the 13 KDa Rev protein begins to accumulate in the host cell nucleus. Once enough Rev is produced, Rev stimulates a switch in viral gene expression by multimerizing onto nuclear viral RNAs and stimulating their export into the cytoplasm. Multimerization occurs on an RNA structure called the Rev Response Element (RRE). Several Rev monomers bind the RRE and once that threshold is met the Rev-RRE complex is exported out of the nucleus. Once out of the nucleus the Rev-RRE complex dissociates and Rev imports back into the nucleus for another cycle of export. Rev’s unique function makes it a theoretically ideal target for inhibiting viral replication. Consequently, understanding the three-dimensional structure of Rev will promote drug design.

Obtaining structural information is difficult because Rev aggregates. While trying to find solutions conditions for crystallography, Watts et al. (2000) discovered Rev depolymerizes microtubules in vitro forming bilayered rings called Rev-Tubulin Toroids (RTTs). RTTs also form when Rev is mixed with tubulin heterodimers. Similar rings form when MCAK and other members of the Kinesin 13 family of microtubule-associated proteins (Kin-13) are mixed with tubulin. The similar primary and secondary structure of Rev (amino acids 34-57) and MCAK (amino acids 506-530) has prompted Watts et al. to hypothesize that the two proteins interact with tubulin and microtubules by a shared mechanism. Studies have shown mutating amino acids within this shared region
has a detrimental affect on Kin-13 ability to depolymerize microtubules and form spindles. Therefore, Rev may serve as a model to further the understanding how Kin-13 proteins function.

To test Rev’s ability to be used as a model for Kin-13 interaction with tubulin point mutations were introduced into the shared region, (A37D, R42A, E47A, and E57A). Then purified proteins were mixed with tubulin heterodimers to see if RTTs form. The A37D, E47A, and E57A mutations do not have any meaningful affect on Rev structure. All were able to form hollow filaments at high concentrations comparable to filaments formed with wtRev. When mixed with tubulin, A37D, E47A, and E57A form RTTs with similar ring diameter and thickness as wild-type rings. These results suggest that the mutated amino acids are unimportant for Rev-tubulin interactions. These data are somewhat consistent with data published for MCAK. The A→D substitution in MCAK also has no affect on MCAK activity. The glutamic acid corresponding to E47 has not been tested in MCAK and this warrants testing. The glutamic acid corresponding to E57 in MCAK behaves differently. This residue is essential for MCAK activity whereas it appears to have no effect on Rev-tubulin interactions. These data suggest that Rev and MCAK may work by different mechanisms.

Mutation of RevR42 had significant effects on RTT formation. R42A does not interact with tubulin heterodimers and RTTs do not form. Moreover, the mutation affects the thickness of Rev filaments suggesting that this amino acid is important for Rev-Rev and Rev-tubulin interactions. Mutating the corresponding amino acid in MCAK will be an interesting test of the hypothesis that Rev and MCAK act by a shared mechanism.
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Introduction

Microtubules (MTs) play essential roles in intracellular transport, cell polarity, cell movement and cell division. MTs are 24 nm thick polymers comprised of α and β tubulin heterodimers. Heterodimers are arranged head-to-tail to form protofilaments and thirteen protofilaments associate laterally to form an individual MT. MTs are intrinsically dynamic structures that spontaneously polymerize and depolymerize. This property allows the cell to remodel MT arrays rapidly as is seen during mitosis. Cells regulate MT behavior through the function of microtubule-associated proteins (MAPs). Some MAPs stabilize MT by reducing dynamics while other MAPs promote growth and/or depolymerization. Alteration of MT dynamics can trigger cell death, a property exploited by many anticancer regimens (e.g., taxol and vinca alkaloids) (Ogawa et al., 2004; Shipley et al., 2004).

Watts et al. (2000) demonstrated the HIV protein Rev could depolymerize MT in vitro. This raises the prospect that Rev might affect MT dynamics in HIV infected cells. The analysis of Rev-MT interactions may also provide insight into how MT dynamics are regulated.

Rev Function in HIV Infection

Rev (regulator of expression of virion proteins) plays an essential role of regulating the shift to early and late viral gene expression (Pollard et al., 1998). Early in HIV infection, proviral transcription leads to the nuclear accumulation of ~9 kb, ~4 kb, and ~2 kb mRNAs. Only fully spliced 2 kb mRNAs are transported into the cytoplasm. They encode three proteins including Rev. The longer RNAs are retained and degraded owing to the presence of unspliced introns. After its translation, Rev’s primary function
is to stimulate the expression of proteins encoded by these underspliced mRNA by promoting their export into the cytoplasm.

Rev has several functional motifs. One is an arginine-rich motif (ARM) encompassing amino acids 34–57. The ARM contains a nuclear localization signal (NLS) recognized by importin β that confers competence for nuclear import (Truant et al., 1999). The ARM also has the ability to bind the ~9 kb and ~4 kb mRNAs (Pollard et al., 1998, Hope et al., 1999). Its binding site is found in the 3’ intron called the Rev Response Element (RRE) (Pollard et al., 1998, Hope et al., 1999). The RRE is ~351 nt long and is characterized by double stranded stems and single stranded loops. Rev binds a specific stem loop (IId) with nanomolar affinity site (Malim et al., 1991; Brice et al., 1999; Meyer et al., 1994; Malim et al., 1989). The binding of a monomer stimulates the binding of additional Rev monomers onto the RRE (Malim et al., 1991; Brice et al., 1999; Meyer et al., 1994; Daly et al., 1989; Van Ryk et al., 1999). The capacity for multimerization is mediated by sequences on both sides of the ARM (amino acids 18-56 and 75-83) (Thomas et al., 1998; Malim et al., 1991). Twelve or more additional monomers may bind a single RRE in vitro (Brice et al., 1999; Van Ryk et al., 1999). Following multimerization, Rev-RRE complexes are exported into the cytoplasm. Nuclear export is conferred by a nuclear export sequence (NES) found toward the C-terminus (amino acids ~68-78) that binds the host cell export factor CRM1 (Malim et al., 1991; Fornerod et al., 1997). Multiple Rev monomers must be bound to a single RRE before export can occur ensuring that sufficient concentrations of Rev are present before there is a commitment to late gene expression (Malim et al., 1991). In the cytoplasm, Rev-RRE complexes dissociate, the ~9 kb and ~4 kb mRNAs are translated into Gag.
Pol, Env, accessory proteins, and Rev re-imports into nucleus for another cycle of RNA export. The overlapping NLS and RNA binding functions of the ARM prevent re-import of Rev-RRE complexes back into the nucleus.

**Rev-MT Interactions**

Mutations that inhibit Rev function also inhibit HIV infection illustrating the importance of Rev (Thomas *et al.*, 1998; Malim *et al.*, 1991; Wingfield *et al.*, 1991; Heaphy *et al.*, 1991). While most mutations are recessive, several transdominant mutations have been identified that inhibit virion production even in the presence of wtRev (Malim *et al.*, 1989; Dayton *et al.*, 2000). The most potent transdominant mutation is M10 that inhibits nuclear export of Rev-RRE complexes (Malim *et al.*, 1989). This demonstrates that Rev is a potential clinical target for inhibiting viral production. Many labs are actively using a variety of approaches to stop Rev function (Dayton *et al.*, 2000). These include ribosome targeting RRE-Rev interactions, antibiotics targeting Rev-RRE binding, RNA inhibiting Rev function, and transdominant mutations (Malim *et al.*, 1989; Dayton *et al.*, 2000; Ptak *et al.*, 2002).

Recent work by DiMattia *et al.* (2010) and Daugherty *et al.* (2010) have provided significant insight into Rev structure. They were able to obtain structural information by blocking Rev’s ability to multimerize, a property that promotes Rev aggregation (Watts *et al.* 1998). DiMattia *et al.* used a Rev specific fragment antigen-binding (Fab) to block Rev multimerization. Daugherty *et al.* mutated residues L12S and L60R that are important for multimerization. Rev persists as a dimer in the crystals obtained by both groups and the first 70 amino acids could be resolved.
Figure 1 shows the relationship between two Rev monomers in a homodimer (Daugherty et al., 2010). Each monomer assumes an anti-parallel helix-loop-helix conformation. The N-terminal helix, amino acids 9-23, is separated from a C-terminal helix, amino acids 34-62, by a proline-rich loop. Monomers interact with each other via hydrophobic interaction formed by amino acids I59-I19 and I52-L22 (Figure 2). The monomers are oriented in a V shape with the loop and ARM at the distal ends. Structure obtained by DiMattia et al. confirms the structure obtained by Daugherty et al.; however, the angle that separates the distal ends of the dimer is different 140˚ vs. 120˚, respectively. Both models suggest the distal ends of the dimer are separated by approximately 4-8 nm. With this structural information Rev may become incorporated into the highly active anti-retroviral therapy (HAART) used to combat HIV infection (Pollard et al., 1998).

Early attempts to find solution conditions necessary to study the structure of Rev, Watts et al. (1998) developed a method whereby high concentrations of Rev would not aggregate and precipitate. Under these conditions, Rev form stable hollow filaments that retain biological activity. Unfortunately, these filaments were still not amenable for crystallographic studies. In an attempt to extend these results, they looked for reagents that blocked aggregation and precipitation (Watts et al., 2000). Since Rev is a basic protein (pI 9.2) with an affinity for polyanions, the addition of polyglutamic acid improved Rev’s solubility. Noting an improvement but not a solution, they speculated that the acidic proteins α and β tubulin might provide even better results.
Figure 1: Model of Rev Dimer

The figure below illustrates how two Rev monomers (green and yellow) form a dimer. Describing the figure starting with the smaller N-terminal α helix (amino acids 9-23) followed by the proline rich loop ending with the larger C-terminal α helix (amino acids 34-62). The distal ends are approximately 4–8 nm apart. The bars indicate the Arginine-Rich Motif (ARM). The highlighted blue areas are point mutation A37D, R42A, E47A, and E57A.
Figure 2: Hydrophobic Region I59-I19 and I52-L22

A) Shows a vertical view of the Rev dimer (green and yellow monomers). The blue highlights are representing amino acids I59, I19, I52, and L22. These amino acids are the hydrophobic region of Rev. B) Shows a top-down view of the hydrophobic region. C) And D) Indicate magnification of the vertical and top-down views respectively. These specific amino acids I59 – I19 and I52 – L22 interact to keep the structure of the monomer in plane and allow the addition of another monomer to form the dimer. Only the first 70 amino acids are seen within the crystal.
A) Vertical View of Hydrophobic Region  

B) Top-Down view of Hydrophobic Region

C) Magnification of Vertical View  

D) Magnification Top-down View
α and β tubulin each possess a polyglutamic acid rich C-terminus, colloquially called an E-hook, they speculated it would bind Rev’s ARM while the globular cores of tubulin would sterically block aggregation. Indeed, such an approach worked a decade later (DiMattia et al., 2010, Daugherty et al., 2010). By combining Rev filaments with tubulin polymerized into MTs, they found that Rev no longer aggregated. Unexpectedly, however, they found Rev filaments had depolymerized and polymeric rings or Rev and tubulin persisted. These rings, called Rev-Tubulin Toroids (RTTs), are 3 to 4 MDa large and bilayered. Each RTT consists of 14, 15, or 16 tubulin heterodimers surrounding 28, 30, or 32 Rev monomers per ring.

These data suggest that Rev depolymerizes MT via ringed intermediates. Tubulin rings and other curved tubulin structures have been seen, usually under conditions promoting MT depolymerization (e.g. tubulin loaded with GDP [Muller-Richert et al., 1998] cold-treated MTs [Mandelkow et al., 1991, Bai et al., 1995], MTs treated with certain antimitotic drugs like Dolastatin 10 [Watts et al., 2000; Boukari et al., 2003; Moores et al., 2008], and MTs treated with kinesin 13 proteins [Moores et al., 2003; Niederstasser et al., 2002; Ovechkina et al., 2002; Bai et al., 1996]). Moreover, “bent” tubulin is diagnostic of MT depolymerization (Nogales et al., 2001). Intriguingly, RTTs also form when Rev filaments are mixed with colchicine-treated tubulin heterodimers (Watts et al., 2000) showing that the polymerization state of tubulin is unimportant.

Rev is not likely to bind tubulin at colchicine and taxol binding sites since RTTs form in the presence of the destabilizing MT drug colchicine or MT stabilizing drug taxol. However, RTT formation is inhibited by the antimitotic drug maytansine, which binds tubulin at or near the vinca site (Watts et al., 2000; Sackett et al., 1995; Moores et
Drugs that bind here often form MT rings (dolastatin 10) and/or spirals (vinblastine) (Bai et al., 1996; Lobert et al., 2000). The formation of RTTs also requires Mg\(^{2+}\) ions, which can withstand the addition of moderate salt concentrations and changes in pH (Watts et al., 2000). Taken together, these observations indicate that Rev-tubulin interactions are specific and has the potential to perturb MT function in HIV infected cells.

**Microtubules**

MTs are intrinsically dynamic structures (Tournebize et al., 2000; Gardner et al., 2013). They spontaneously and stochastically switch between periods of polymer growth and shrinkage, a process called dynamic instability. Reversals of growth are called catastrophes. Reversals of depolymerization are called rescues. Purified MTs undergo periods of polymerization, catastrophe, depolymerization and rescue. These dynamics are a consequence of the events occurring during MT polymerization.

MTs are made of obligate heterodimers of α and β tubulin. Tubulin possesses GTPase activity but within the heterodimer or the MT, only β tubulin can be bound to GTP or GDP (Nogales et al., 2001). α tubulin is always in a GDP state because the nucleotide is trapped between the interface of the α and β subunits (Nogales et al., 2001). The three-dimensional conformation of the heterodimer is dependent upon the nucleotide bound to the β subunit (Nogales et al., 2001). When bound to GTP, the heterodimer assumes a “straight” conformation amenable for polymerization at MT ends. In the GDP bound state, the heterodimer assumes a curved conformation seeing MT peels during MT depolymerization. When heterodimers are added onto a MT polymer, the GTPase activity of underlying β subunits is stimulated. Consequently, only the end of the
growing MT is bound to GTP. The lattice has hydrolyzed its GTP and is bound to GDP. Therefore the MT is poised to depolymerize were it not for the GTP cap at the end, which keeps the tensioned MT intact. Loss of the cap exposes the GDP-tubulin that releases the energy of GTP hydrolysis, causing heterodimer curvature and the MT depolymerizes. The stochastic gain and loss of the cap explains the phenomenon of dynamic instability in which MTs spontaneously grow and shrink regardless of the polymerization state of neighboring MTs.

Whereas purified MTs exhibit dynamic instability, cellular MTs are even more dynamic owing to cellular proteins that stimulate and suppress polymerization and depolymerization (Cassimeris et al., 1988). These factors include stabilizing microtubule-associated proteins (MAPs) and destabilizing depolymerases like XMAP215 and MCAK, respectively (Howard et al., 2007; Hunter et al., 2003; Moores et al., 2002; Moores et al., 2003; Niederstasser et al., 2002; Ovechkina et al., 2002; Hertzer et al., 2003; Gardner et al., 2013). XMAP215 promotes polymerization by either recruiting free tubulin to the polymer within the cell or promoting a straight MT conformation to facilitate polymerization (Howard et al., 2007). It does not have catalytic activity but promotes polymerization when tubulin concentrations are above the critical concentration required for polymerization. When the amount of free tubulin is below MT critical concentration it promotes depolymerization. Conversely, MCAK (Mitotic Centromere Associated Kinesin) is an ATPase that depolymerizes MTs (Hunter et al., 2003; Gardner et al., 2013; Nogales et al., 2001). When MCAK dimers bind the ends of the MT, they promote and stabilize a bent protofilament conformational (Hunter et al., 2003). Curiously, nucleotide hydrolysis is not required for depolymerization as MCAK
stimulates depolymerization even in the presence of non-hydrolyzable AMPPNP (Hunter \textit{et al.}, 2003). ATP hydrolysis is required to release the heterodimer and continue depolymerizing the MT (Ogawa \textit{et al.}, 2004).

Collectively, the balance of MAP and depolymerase activities is important for regulating MT dynamics. They function in many different ways including altering lateral contacts between protofilaments, affecting GTP hydrolysis and/or nucleotide exchange, and physically altering MT structure. Tournebize \textit{et al.} (2000) hypothesized that the stabilizing MAP, XMAP215, antagonizes the activity of MCAK, the most potent depolymerizing member of the Kinesin 13 family. They proposed that XMAP215 stabilizes MTs and even promote growth in the presence of MCAK in interphase cells. However, upon entry into mitosis, XMAP215 is phosphoralated and dissociates from the MT such that it can no longer oppose MCAK-mediated depolymerization (Howard \textit{et al.}, 2007).

The importance of MT dynamics is illustrated by examining mitotic spindles, the microtubule apparatus that segregates chromosomes into daughter cells during cell division. The failure to regulate MT dynamics can promote spindle dysfunction, mis-segregation of chromosomes and ultimately cell death (Tournebize \textit{et al.}, 2000; Sorger \textit{et al.}, 1997). Alteration of MT dynamics can promote cell death and is the basis for anti-cancer drugs (e.g., Taxol, colchicine, dolastatin, and vinca alkaloids). Both, low concentrations of Taxol that stabilize higher MT concentrations and higher concentrations that depolymerize MT stimulates apoptosis (Downing \textit{et al.}, 2000; Nogales \textit{et al.}, 1999). Taxol has a binding site found on the inside of the MT that stabilizes the “M” loop, a tubulin motif important for maintaining protofilament
interactions. Colchicine binds at a different site and works in the different manner but has the same outcome-cell death. At low concentrations, colchicine binds free tubulin heterodimers and blocks the their ability to polymerize into MTs (Dorleans et al., 2009; Stanton et al., 2011). Colchicine appears to bind by a biphasic manner. It first binds the \( \beta \) subunit and then buries itself within the intradimer groove to cause structural changes on the other side of tubulin.

**Mechanism of Rev-MT interactions**

The medical importance of anti-microtubule agents illustrates the importance of understanding how different proteins such as Rev affect MT dynamics (Ogawa et al., 2004). Therefore, the mechanism by which Rev binds and depolymerizes MTs is important. Clues about Rev’s mechanism, comes from the MT motor proteins kinesins. There are three types of kinesins depending upon the location of the motor domain. The ~350 amino acid motor domain may be located near the N-terminus, the C-terminus, or internally (Ogawa et al., 2004). When the motor domain is at either end, the kinesin has the ability to translocate on MT protofilaments in an energy (ATP) dependent manner. In contrast, kinesins with an internal motor domain, previously called KinI or KinM because of the “internal” or “middle” location of the motor domain and now formally reclassified as Kinesin-13’s (Kin-13). Kin-13s lack motor ability and instead depolymerize MTs from either end without requiring ATP hydrolysis. Kin-13s include MCAK, XKCM1, and Kif2C. Their abilities to depolymerize MTs residues in four “motifs” not found in motile kinesins: a polycationic neck, a polycationic-rich helix, a Kin-13 specific region called the “KVD finger,” and sequence differences in an \( \alpha \)-helix involved in MT-binding.
The mechanism Kin-13s use to bind and depolymerize MT has been outlined (Shipley et al., 2004; Woehlke et al., 1997). MCAK binds MTs as a dimer and has a higher affinity for binding MT ends than the lattice. Once bound to the lattice, it can diffuse across the MT lattice until it reaches either end. The neck allows diffusion by sterically hindering tight binding to the lattice. However, this hindrance is removed at MT ends as the neck can insert between and then splay protofilament ends. At the end, MCAK stabilizes a curved conformation at the ends and stimulates protofilament peeling and heterodimer dissociation. Stabilization requires residues in loop11-α4-loop12 and a ‘KVD finger’ is essential for depolymerization. The KVD finger is a domain unique to Kinesin 13 proteins that is absent in motile kinesins (Ogawa et al. 2004).

Depolymerization can occur even when MCAK is loaded with AMPPNP showing that ATP hydrolysis is not needed. ATP hydrolysis however allows for MCAK to dissociate from the released tubulin for another cycle of depolymerization (Shipley et al., 2004; Woehlke et al., 1997; Friel et al., 2011). The loop11-α4-loop12 is particular intriguing with respect to Rev-tubulin interactions. Watts et al. (2000) suggest that this region they have identified having sequence similarity suggest that some of the residues found in Rev may be act like the ones found in MCAK. They both have comparable structures (α helix) and the shared ability to depolymerize MT from the end. They both have the ability to form rings therefore these similarities suggest there is a shared mechanism (Watts et al., 2000) (Figure 3).
**Figure 3: Sequence Similarity to the Loop11-α4-Loop12 in Kinesin-13's**

The below sequence similarity comparison is between Rev to X1KCM1/MCAK and a consensus sequence of all the Kinesin-13 proteins. The difference in colors are as follows: red color means the same amino acid, green color means a conservative amino acid substitution, and blue indicates analogous amino acid substitutions.
<table>
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<th>Protein</th>
<th>Accession</th>
<th>Length</th>
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<td></td>
</tr>
<tr>
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**Specific Aims**

To test the hypothesis that Rev interacts with MT by a mechanism shared with Kin-13, I studied the ability of Rev harboring point mutations to bind colchicine treated tubulin (CT) and to form RTTs. Specifically, I examined the effects of alanine substitutions at amino acids locations 37, 42, 47, and 57. These residues are a subset of amino acids shared with MCAK. Alanine substitutions should minimize alterations in protein structure (e.g., disruption of α helices) (Mailim *et al.*, 1991). These residues were chosen because they are shared with the Kin-13 loop11-α4-loop12 of the Kin-13 motor domain.

In general, there are three predicted ways the mutants can effect Rev-tubulin interactions. The mutation may have no effect, inhibit tubulin binding, or inhibit RTT formation without inhibiting tubulin binding. I aim to compare the interactions of tubulin and mutant Rev with the interactions of tubulin and wild-type Rev using TEM to monitor Rev’s ability to form high molecular weight complexes including RTTs when mixed with tubulin heterodimers. After brief incubations, mixtures of CT tubulin heterodimers and Rev were fixed and subjected to transmission electron microscopy (TEM) confirming the presence or absence of RTTs.

**Predictions**

Mutating amino acids that are unimportant for Rev-tubulin interactions are expected to produce filaments of purified Rev that when mixed with CT, will form RTTs similar to those found in mixtures of CT and wild-type Rev. Mutating amino acids that inhibit tubulin binding should prevent RTT formation and Rev filaments should remain intact because tubulin is no longer able to disrupt the equilibrium between Rev polymer
and Rev monomer. Mutations that retain the ability to bind tubulin and depolymerize Rev filaments but are unable to form RTTs might be explained by a conformation change that precludes ring formation. Such a conformational change might be reflected in a change in the diameter of Rev filaments that occurs due to differences in how monomers pack. The results from this study should allow for comparisons to Kin-13:tubulin interactions.
Materials and Methods

Rev Tubulin Toroids formation assay:

Aliquots of purified tubulin are resuspended in MEM (100 mM MES, 2 mM MgCl₂, and 1 mM EGTA), supplemented with 5 mM GDP, and incubated on ice for 15 minutes. The sample is subjected to centrifugation at 360,000 x g in a TLS optima miniultracentrifuge for 5 minutes. The supernatant is supplemented with 50 mM colchicine (6 µM) and then mixed with an equimolar concentration of Rev resuspended in 100 mM MES, 2 mM MgCl₂. The mixture is incubated at 22°C for 10 minutes.

TEM of Rev-tubulin mixtures:

Aliquots (5µl) of mixtures described in the sedimentation assays are spotted onto a carbon coated mica grid. After 1 minute, excess sample was wicked away and the sample was fixed with 2% uranyl acetate for 20 seconds. The sample was stained with 2% uranyl acetate for 1 minute, wick dried and visualized with Philips EM 208S electron microscope at minimum 56,000 x 10 magnification.

Rev Purification:

The gene encoding Rev subcloned into a pET11D expression vector by Blanco et al. (2001) was kindly gifted by Dr. Blanco. Point mutation was introduced into the wild-type gene by Dr. Mill Miller using overlap PCR. BL21DE3 cells transformed with Rev subcloned into PET11d were grown in 50 ml shaker cultures of LB broth supplemented with 1µl/1ml ampicillin overnight at 37°C. The next day, the entire culture was diluted in 950 ml LB Broth and cells were grown to log phase by monitoring growth measuring the optical density of the culture at 600 nm light. Then isopropyl β-D-1-
thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1µl/1ml. The culture incubated an additional 2 hrs. Cells were collected by 6,000 rpm centrifugation at 4°C for 10 minutes using a Sorvall RC-5B centrifuge.

Rev was purified using the methods of Karn et al. (1995). The cell pellet was resuspended in ice-cold buffer A (400 mM NaCl, 50 mM Tris-HCL pH 8.0, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and sonicated using a Fisher-Scientific Sonic Dismembrator Model 100. The sample was sonicated three times for 30 s each with 30 s ice incubations in between. The sample was frozen for 5 min, thawed on ice, and sonicated 3 times more. The lysate was clarified by 10 minutes centrifugation at 12,000 rpm in a Beckman Avanti centrifuge using a Beckman JA 30.50 rotor chilled to 4°C. The supernatant was recovered and stored at -80°C until Rev was purified by FPLC.

Supernatant is fractionated using FPLC (GE Life Sciences, NJ). The sample was applied to a Q-Sepharose column (GE Life Sciences, NJ) equilibrated with buffer A (50 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM EDTA, and 5 mM PMSF with an additional 400 mM NaCl). Rev-RNA complexes eluted with buffer B (buffer A supplemented with 800 mM NaCl). Eluted fractions containing Rev are applied Heparin-Sepharose column (GE Life Sciences, NJ) equilibrated in buffer B. The column is then washed with buffer C (buffer A supplemented with 1200 mM NaCl) removing RNA that was bound to Rev. Next, the column is subjected to buffer D (buffer A supplemented with 2 M NaCl). This allowed Rev to be release from the Heparin column and collected into 5ml fraction sizes. The purity of Rev was confirmed by SDS-PAGE using coomassie brilliant blue staining and/or Rev specific antibodies to confirm Rev’s presence.
Purified Rev was refolded into filaments and concentrated according to published procedures (Watts et al., 1998). Briefly, Rev was denatured in 6 M Urea and sequential dialyzed against buffer 1 (pH 7.0, 50 mM sodium phosphate, 150 mM NaCl, 600 mM ammonium sulfate, 50 mM sodium citrate, and 1 mM EDTA), buffer 2 (pH 7.0, 50 mM sodium phosphate, 150 mM NaCl, 50 mM sodium citrate, and 1 mM EDTA) and buffer 3 (pH 7.0, 20 mM Hepes, 100 mM NaCl, 50 mM sodium citrate, and 1 mM EDTA). After dialysis Rev filaments were collected by centrifugation at 320,000 x g for 4 hours (TLA100 rotor). Concentrations of Rev were determined using ImageJ to densitometrically measure the amount of coomassie brilliant blue staining. BSA as a standard. Protein concentration was also determined spectrophotometrically (ε=8.31 mM⁻¹cm⁻¹). R42A was purified using similar methods as described above however, R42A was not bound to RNA therefore it was eluting into the Q-Sepharose flow through. It was noted that the Q-Sapharose flow through samples would have a light refraction property to it, I hypothesized that the flow through sample that had the most of this property had the most R42A proteins in it. Continuing, the flow through sample that was deemed to have he most R42A was directly applied to the Heparin column for purification.

**Statistical Methods:**

A sample size of 20 filaments and rings produced by each protein were subjected to statistical analysis including one-way ANOVA and statistically significant differences samples were subjected to t-Tests with Bonferroni corrections.
**SDS-PAGE:**

Protein samples were prepared by mixing 10 µl of protein with 5 µl of SDS-PAGE protein sample buffer (1X = 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.025 % BFB), and boiled for 5 minutes. The samples are subjected to 5 sec centrifugation using a tabletop microfuge and the entire sample was subjected to SDS-PAGE (%T=15%). Proteins were resolved using 30 mA constant current () for 45-60 min. Proteins were stained with 0.1% Coomassie Brilliant Blue, 50% MeOH, 10% HoAC overnight and destained with 35% MeOH, 10% glacial acetic acid. Images of each gel were acquired using Adobe Photoshop.

**Tubulin Purification:**

Tubulin purification was performed from following established methods of Desai et al. (2001). Concentration of tubulin was determined spectrophotometric ($\varepsilon^{280} = 100,000 \text{ M}^{-1}\text{cm}^{-1}$) and densitometric using ImageJ with bovine gamma globulin as standard.

**Results**

This study aims to test the hypothesis that Rev and Kin-13 proteins interact with tubulin by a shared mechanism. This study examines only four of the shared amino acids because they could be purified. Large amounts of functional protein are needed to test the role of Rev and mutants in CT binding and RTT formation. Wild-type Rev is routinely purified from transformed E. coli cells as using the methods of Karn et al. (1995) as described in the “Materials and Methods.” Figure 4 shows typical results of BL-21(DE-3) cell lysate fractionated by Q-Sepharose chromatography. Because Q-Sepharose has a high affinity for RNA, RNA-Rev complexes bind the Sepharose column.
while most cationic proteins are removed in the flow-through fractions. RNA-Rev complexes are eluted from the column with the addition of 800mM salt.
Figure 4: Purification of wtRev

Rev was purified as described in the Material and Methods. Briefly, whole cell extract (WCE) were resolved by Q-Sepharose chromatography. Proteins that are not binding the column are denoted by (QF). Protein complexes binding and eluting from the Q-Sepharose column are denoted by (QE). Rev containing fractions were subjected to Heparin-Sepharose chromatography. Proteins that are not binding to the column are denoted as (HF). Rev protein binding and eluting from the Heparin column are denoted as (HE). Representative fraction were resolved by 12% SDS-PAGE and stained with coomassie brilliant blue. Representative results are shown below. Left hand column depicts molecular weights x 10^3 Da. Arrow indicates purified wtRev. 10µl protein solution aliquots were prepared using equal volume of protein sample mixed with 2x sample buffer (1X = 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.025 % BFB). All aliquots were boiled for 5 minutes and subjected to flash centrifuge before loading: (WCE) Whole cell extract, (QF) Q-Sepharose flow through [Na+] = 400mM, (QE) Q-Sepharose Elution [Na+] = 800mM, (HF) Heparin-Sepharose flow through [Na+] = 1200mM, (HE) Heparin-Sepharose elution [Na+] = 2 M, purified mutant E57A, and purified wtRev.
wtRev

<table>
<thead>
<tr>
<th>WCE</th>
<th>QE</th>
<th>HF</th>
<th>HE</th>
<th>HE</th>
<th>Wt</th>
</tr>
</thead>
</table>

[Image of a gel with bands at 72, 55, 40, 33, 24, 17, and 11]
The Q-Sepharose fractions containing Rev are pooled and applied to Heparin column. Heparin column has a high affinity for Rev and other cationic proteins. Purified Rev is then denatured, refolded and concentrated as described in the “Materials and Methods”. These steps ensure Rev fold into 14 nm thick cylindrical tubes that retain biological activity (Watts et al., 1998). The final purified product is highly purified Rev protein (Figure 5). The identity of the purified protein is confirmed to be Rev by its electrophoretic mobility and by immunoblotting (data not shown).

To confirm Rev activity, Rev was mixed with equal molar concentrations of colchicine-treated tubulin (CT). If active, Rev and CT should interact to form bilayered rings with 36-44nm diameters (Watts et al., 2000). Figure 6 and Table 1 confirms the protein used in this study is active. The data in Table 1 confirms filament formation that is 12.9 (± 1.1) nm wide. The table also shows that wild-type Rev indeed forms RTTs, with outer diameter 46.5 (± 2.6) nm and a ring thickness of 10.8 (± 0.9) nm, this data is comparable to known filamentous/RTT data.
Figure 5: Electron Micrograph of Purified Refolded Rev Filaments

Below is a representative picture of purified refolded hollow wild-type Rev filaments. Magnification bar is 100 nm.
Figure 6: RTT formation mixing wild-type Rev and Colchicine Treated Tubulin

Formation of RTTs when mixing equal molar concentration of wild-type Rev and colchicine treated tubulin. Magnification bar is 100 nm.
Table 1: Characteristics of Rev-Tubulin Interactions

The table below characterizes Rev filaments and Rev-tubulin RTTs. All formed filaments as previously described. Filament width of A37D (p < 0.05) was significant compared to wild-type Rev and R42A (p < 0.05 or 0.01) was significantly different than wild-type Rev. The outer diameters of A37D’s RTTs are significant compared to the RTTs of wild-type Rev. E42A had no RTT formation.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Filament Width (nm ± S.D.)</th>
<th>RTT Outer Diameter (nm ± S.D.)</th>
<th>RTT Thickness (nm ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WtRev</td>
<td>12.9 ± 1.1</td>
<td>46.5 ± 2.6</td>
<td>10.8 ± 0.9</td>
</tr>
<tr>
<td>A37D</td>
<td>14.2 ± 1.63</td>
<td>50.9 ± 2.84</td>
<td>12.5 ± 0.9</td>
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<tr>
<td>R42A</td>
<td>16.3 ± 1.65</td>
<td>n/a4</td>
<td>n/a7</td>
</tr>
<tr>
<td>E47A</td>
<td>13.7 ± 2.1</td>
<td>59.3 ± 5.5</td>
<td>12.1 ± 1.2</td>
</tr>
<tr>
<td>E57A</td>
<td>14.0 ± 1.5</td>
<td>57.6 ± 3.5</td>
<td>10.6 ± 2.1</td>
</tr>
</tbody>
</table>

1 Filament sample size of each protein N = 20.
2 Ring sample size of each protein N = 20.
3 Filament width of A37D (p < 0.05) is significantly different from wt Rev.
4 RTT thickness of A37D (p < 0.05 and p < 0.01) is significantly different from that of wtRev.
5 Filament width of R42A (p < 0.05) is significantly different from that of wtRev, E47A, and E57A. R42A (p < 0.01) is significantly different from all other proteins.
6,7 R42A was not applicable (n/a) because it did not form RTT’s.
Three mutant proteins E57A, E47A and A37D were successfully purified by the same methods used to purify wild-type protein. Similar purity and yields were obtained (Figures 7-12; Table 1). Each mutant formed hollow filaments (Figure 13). Mutants A37D and R42A both formed filaments that were significantly different from filaments formed from wild-type Rev (p < 0.05). Moreover, R42A filaments are significantly thicker than all other Rev proteins used in this study (p < 0.01). Differences in filament width might reflect a structural change in Rev that affects how Rev packs into the filament. R42 is located near the distal ends of the α helices in the Rev dimer (Figure 2).

R42A did not bind to the Q-Sepharose column (Figure 12). This suggests R42A does not bind RNA well. This amino acid is present in the ARM that binds the RRE so this is not entirely surprising. Moreover, the M6 mutation that includes R42 (^{41}RRRR\rightarrow DL) also has a reduced affinity for Q-Sepharose (Sharma, 2009). During R42A purification, several flow-through fractions strikingly refracted light in a way that was atypical. Hypothesizing the refraction patterns were owing to these fractions possessing R42A, these flow-through fractions were pooled and subjected to heparin affinity chromatography. As is seen in Figure 14, R42A bound the column and was eluted similar to wild-type Rev. This protein was denatured and refolded into hollow filaments (Figure 13). The mean diameter of these filaments was significantly thicker than wild-type Rev filaments (Table 1).
Figure 7: Purification of A37D

Mutated Rev was purified as described in Figure 4 and in the Material and Methods. Briefly, whole cell extract (WCE) were resolved by Q-Sepharose chromatography. Proteins that are not binding the column are denoted by (QF). Protein complexes binding and eluting from the Q-Sepharose the column are denoted by (QE). Rev containing fractions were subjected to Heparin-Sepharose chromatography. Proteins that are not binding to the column are denoted as (HF). Rev protein binding and eluting from the Heparin column are denoted as (HE). Representative fraction were resolved by 12% SDS-PAGE and stained with coomassie brilliant blue. Representative results are shown below. Left hand column depicts molecular weights x 10^3 Da. Arrow indicates purified wild-type Rev. 10µl protein solution aliquots were prepared using equal volume of protein sample mixed with 2x sample buffer (1X = 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.025 % BFB). All aliquots were boiled for 5 minutes and subjected to flash centrifugation before loading: (WCE) Whole cell extract, (QF) Q-Sepharose flow through [Na+] = 400mM, (QE) Q-Sepharose Elution [Na+] = 800mM, (HF) Heparin-Sepharose flow through [Na+] = 1200mM, (HE) Heparin-Sepharose elution [Na+] = 2 M, purified mutant E57A, and purified wild-type Rev.
Figure 8: Q-Sepharose Fractionation of E47A

As described in Figure 7 Rev samples are subjected to protein fractionation and representative results are shown below. Left hand column depicts molecular weights x $10^3$Da. Arrow indicates purified wild-type Rev. (WCE) Whole cell extract, (QF) Q-Sepharose flow through $[\text{Na}^+] = 400\text{mM}$, (QE) Q-Sepharose Elution(s) 1-4 $[\text{Na}^+] = 800\text{mM}$, purified mutant E57A, and purified wild-type Rev.
Figure 9: Heparin-Sepharose Purification of E47A

As described in Figure 7 Rev samples are subjected to protein fractionation and representative results are shown below. Left hand column depicts molecular weights x $10^3$Da. Arrow indicates purified wild-type Rev. (HF) Heparin-Sepharose flow through $[\text{Na}^+] = 1200\text{mM}$, (HE) Heparin-Sepharose Elution(s) 1-4 $[\text{Na}^+] = 2\text{ M}$, and purified wild-type Rev.
Figure 10: Q-Sepharose Fractionation of E57A

As described in Figure 7 Rev samples are subjected to protein fractionation and representative results are shown below. Left hand column depicts molecular weights x 10^{-3}Da. Arrow indicates purified wild-type Rev. (WCE) Whole cell extract, (QF) Q-Sepharose flow through [Na+] = 400mM, (QE) Q-Sepharose Elution(s) 1-5 [Na+] = 800mM, and purified wild-type Rev.
Figure 11: Heparin-Sepharose Purification of E57A

As described in Figure 7 Rev samples are subjected to protein fractionation and representative results are shown below. Left hand column depicts molecular weights x $10^{-3}$Da. Arrow indicates purified wild-type Rev. (HF) Heparin-Sepharose flow through [Na+] = 1200mM, (HE) Heparin-Sepharose Elution(s) 1-4 [Na+] = 2 M, and purified wild-type Rev.
Figure 12: Purification of R42A

R42A was purified as described in the Material and Methods. Briefly, whole cell extract (WCE) were resolved by Q-Sepharose chromatography. Proteins that are not binding the column are denoted by (QF). Rev protein binding and eluting from the Heparin column are denoted as (HE). Representative fraction were resolved by 12% SDS-PAGE and stained with coomassie brilliant blue. Representative results are shown below. Left hand column depicts molecular weights x 10^3 Da. Arrow indicates purified wild-type Rev. 10µl protein solution aliquots were prepared using equal volume of protein sample mixed with 2x sample buffer (1X = 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.025 % BFB). All aliquots were boiled for 5 minutes and subjected to flash centrifugation before loading: (WCE) Whole cell extract, (QF) Q-Sepharose flow through [Na+] = 400mM, (HE) Heparin-Sepharose elution [Na+] = 2 M, and purified wild-type Rev.
R42A

<table>
<thead>
<tr>
<th>WCE</th>
<th>QF</th>
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<td>11</td>
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Arrow pointing to band at 17.
Figure 13: Mutant A37D, R42A, E47A, and E57A filaments

The EM pictures below shows a sample representation of purified refolded mutant Rev filaments A) A37D, B) R42A, C) E47A and D) E57A. Each picture has its own magnification bar at 100 nm.
Figure 14: Comparison of R42A purity vs. wild-type Rev purity

The below SDS-PAGE gel shows that even with the slight modification during purification of R42A the purity is comparable to wild-type Rev.
The above data suggest that wild-type Rev and all mutants can be highly purified and that the purified protein retains the ability to form hollow filaments. With functional proteins in hand, the ability of each mutant to dissociate Rev filaments and form RTTs was measured.

The results (Figures 15) show that wild-type, A37D, E47A, and E57A are all able to bind CT and form RTTs (Table 1). RTTs formed with A37D, E47A, and E57A are significantly larger than wild-type Rev (Table 1). By subtracting the inner diameter from the outer diameter it is possible to calculate RTT thickness. A37D rings were significantly thicker than the rings formed by the other proteins (Table 1). In contrast, R42A did not form RTTs when mix with CT (Figure 16). Moreover, Rev filaments were visible in these mixtures. This suggests that changing arginine to an alanine in R42A at this amino acid site is detrimental to Rev’s binding ability to tubulin and creation of RTTs.
Figure 15: WtRev and Mutants are able to form RTT's

The figure illustrates representative RTT samples of mixing equal molar concentration of A) wild-type Rev, B) A37D, C) E47A and D) E57A with colchicine treated tubulin. The magnification bar is 100 nm.
Figure 16: No RTT formation when mixing Mutant R42A and CT

This picture shows when mixing R42A with CT no RTT formation occurs and the filaments persist. The magnification bar is 100 nm.
Discussion

The purpose of these experiments is to identify the amino acids that mediate tubulin binding and RTT formation. When CT is mixed with wild-type Rev, Rev filaments dissociate. Shortening can occur by two pathways. First, CT may directly bind filaments and liberate Rev monomers (or oligomers) from the filament. Rev-CT dimers (or heterooligomers) are then free to form RTTs. Alternatively, CT may bind Rev monomers and disrupt the equilibrium that exists between Rev monomers and polymers. Consequently, the addition of CT will sequester Rev monomers and shift the equilibrium favoring filament dissociation. Liberated Rev is free to form RTTs. Therefore, when amino acids important for Rev-Tubulin interactions are mutated, Rev filaments should disappear and RTTs may not form. Filament dissociation indicates Rev-CT binding but does not guarantee RTT formation. Rev mutants may retain the ability to bind CT while changing the structure of Rev-tubulin complexes in a manner that prohibits RTT formation. In this case, it seems likely the mutated amino acid alters Rev structure.

The experimental data in this study suggest that the A37D mutant protein behaves similarly to wild-type Rev. Both A37D and wild-type Rev readily formed comparable hollow filaments (Figure 13) and when mixed with CT, formed comparable RTTs (Figure 15). These data suggest that A37 is not critical for Rev-tubulin interactions. The fact A37D rings were larger than those formed by wild-type Rev suggest that this amino acid plays a minor role. This data is consistent with observations that show the comparable A509D substitution in xMCAK does not affect MT binding and depolymerization (Walzczak et al., 2002, Walzcak pers. comm.). It is therefore unlikely that residue A37 plays an important role in Rev-tubulin interactions.
The data pertaining to E57A provides an interesting contrast to data obtained studying the comparable mutation in xMCAK, E529A (Ems-McClung et al., 2007). E57A forms hollow filaments like wild-type Rev and when mixed with CT forms RTTs (Figures 13 and 15). RTTs formed by E57A are significantly larger than those formed by wild-type Rev and A37D. This suggests that E57 like A37 is not critical for Rev-tubulin interactions but may play a minor role.

However, the comparable amino acid in MCAK E529 is critical for MCAK activity. The comparable mutation E529A reduces MT depolymerase activity 4.5 fold (Ems-McClung et al., 2007). Moreover, this amino acid is a part of a tripeptide (KEC) that is essential for MCAK activity (Ogawa et. al., 2004). Our data suggest that MCAK and Rev interact with tubulin by a different mechanism. The results of this study are qualitative and did not quantify rates of ring formation relative to wild-type Rev. Additional assays such as sedimentation assays capable of quantifying ring formation are needed. Moreover, the assays of Ems-McClung et al. were different from those used in this study. It is important that MT depolymerization rates of E57A be measured. One may yet conclude that Rev and MCAK share a mechanism if E57A reduces MT depolymerization rates. Moreover, one would also predict that MCAK E529A retains its ability to bind CT. These experiments should be preformed to better compare these amino acids.

This study also examined E47A, which corresponds to E519A in MCAK. E47A is similar to wild-type Rev with its ability to form filaments that dissociate following dilution or addition of salt (data not shown). E47A and wild-type Rev both readily form RTTs when mixed with CT, which suggests that E47A is not critical for Rev-tubulin
interactions based on these results alone. E47A rings are significantly larger than wild-type Rev and A37D rings suggesting it may have minor role. The corresponding amino acid in MCAK E519A has not been studied. If E519 has similar function as E47, then one might predict that E519 is relatively unimportant for MCAK activity.

The R42A mutant behaves differently from the other mutants studied. First, R42A filaments formed during the refolding are significantly thicker than wild-type Rev (Figure 13, Table 1). This mutation probably changes the number of monomers present per turn within the Rev filaments. This phenotype suggests this mutation affects some subtle structural change that is reflected by a different arrangement of monomers packed within a filament. This is somewhat unexpected, as the alanine substitution is not predicted to affect α helical nature in this region of the protein. However, like wild-type Rev, these filaments dissociated normally upon dilution or addition of salt (data not shown) showing that these filaments are not aggregated protein. This is confirmed by experiments where R42A is mixed with CT–no RTTs are seen but short Rev filaments persist, which are the predicted results when Rev is diluted during the course of these experiments (Figure 16). These data strongly suggest that R42A filaments dissociate and functional protein is available to interact with tubulin. Since R42A filaments persist after the addition of CT, it is likely that the R42A mutation reduces Rev’s ability to bind tubulin. If R42A where able to bind CT, the Rev filaments should not be observed. These data are consistent with Woehlke et al., (1997) which showed mutation of basic amino acids in motile kinesins reduces the overall affinity of motile kinesin to bind MT.
The corresponding mutation in MCAK R514A has not been examined. If Rev and MCAK share a common mechanism, R514A should have a greatly reduced affinity for tubulin and that depolymerase activity should drop precipitously.

The R42A mutation is interesting in another context. R42 is one of four amino acids mutated in M6 mutation in which four arginines are replaced with an aspartic acid and a leucine RRRR_{41-44}→DL. The M6 mutation introduces a net deletion of two amino acids introduces a hydrophobic amino acid and changes the charge of this region from +4 to -1. Despite the extreme nature of this mutation, M6 retains the ability to bind CT and form RTTs. It does however lack the ability to depolymerize MTs *in vitro* (Sharma, 2009). This raises the question as to why does the more extreme mutation of M6 have a more normal interaction than R42A. The reason is not traceable to the unusual fractionation of R42A with Q-chromatography because M6, like R42A, also has a reduced affinity for Q-Sepharose. It is likely that the M6 deletion brings an amino acid into the putative tubulin binding site that is capable of compensating for R42. There are arginine residues adjacent to the M6 mutation that may suffice. Certainly, any model that explains Rev-tubulin interactions must account for this observation. Future experiments should examine the ability of amino acids adjacent to R42 to bind tubulin.

There are several possible suggestions why RTTs do not form when mixing R42A with CT. The presence of R42A filaments suggests that the affinity for tubulin is reduced. Explanations of reduction in affinity are several fold. One being that having an arginine in this specific location is essential for Rev-tubulin interaction. Another explanation is the type of amino acid change that causes a subtle structure change within Rev. This structure change would most likely be found in α helix and that helix has
dispositioned itself which is not allowing for Rev-tubulin interactions. Examining the ring forming ability of R42K might be informative. Alternatively, there could be some new intermolecular Rev-Rev interaction present in this mutation that precludes Rev-tubulin interactions. In order to determine which suggestion is correct we would need a structural model that predicts Rev-tubulin interactions. This would allow us to see how these specific amino acids changes Rev-tubulin interactions. Computer modeling using docking programs like HEX will be informative.

Rev has a sequence similarity to Kin-13 proteins. This similarity is quite high and statistically significant (Watts et al. 2000). If Rev and Kin-13 share a mechanism when interacting with tubulin then using the shared sequence similarity as a foundation for future studies is idea. Some of the point mutations (A37D, E47A, and E57A) suggest that there is no essential interaction. However, these mutants formed RTTs that were larger than normal. The increase in size is mostly do to the incorporation of additional tubulin heterodimers. These rings may be larger because these mutations do not bend tubulin heterodimers as much as wild-type Rev. Alternatively the larger rings production may be do to Rev-Rev interactions, which is impossible to determine which conclusion is correct with out high-resolution structural data. Since E47 and E57 are close to amino acids important for dimerization and multimerization it seems likely that the later conclusion is correct.
References


Friel, C and Howard, J. 2011. The kinesin-13 MCAK has an unconventional ATPase cycle adapted for microtubule depolymerization. The EMBO Journal. 30:3928-3939.


