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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Identifying the Contributions and Mechanisms of p31^{comet} and TRIP13 Function
During Mitotic Checkpoint Silencing

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in
Biology

by

Kimia Candice Mashouf

Committee in charge:

Professor Don Cleveland, Chair
Professor Gen-sheng Feng, Co-chair
Professor Samara Reck-Peterson

2016

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University of California, San Diego

2016

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ABSTRACT OF THE THESIS

Identifying the Contributions and Mechanisms of p31^{comet} and TRIP13 Function
During Mitotic Checkpoint Silencing

by

Kimia Candice Mashouf

Master of Science in Biology

University of California, San Diego, 2016

Professor Don W. Cleveland, Chair
Professor Gen-sheng Feng, Co-chair

Silencing the mitotic checkpoint requires that new MCC formation is prevented and pre-existing MCC is disassembled. This will release the APC/C-Cdc20 from inhibition so it can resume its activity and promote anaphase onset. Very recent studies

have hypothesized that the joint action of the AAA+ ATPase TRIP13 and an adapter protein p31comet can mediate the catalytic disassociation of the mitotic checkpoint protein Mad2 from Cdc20-Mad2 complexes or from the four protein complex BubR1-Bub3-Mad2-Cdc20 known as the mitotic checkpoint complex (MCC). Furthermore, using purified components in vitro, TRIP13 ATPase-dependent activity has been shown to convert closed, “active” Mad2, capable of binding to Cdc20, into its inactive “open” conformer. This is only in conjunction with p31comet, which is proposed to act as an adapter to facilitate recognition of closed Mad2 and deliver it to TRIP13. Viewing all evidence, key mechanistic questions are unresolved, most specifically the role(s) of TRIP13 and p31comet in mitotic checkpoint silencing. An ATP-hydrolysis-deficient mutant of TRIP13 (E253Q), is shown to stably capture its substrates, as its ATP-hydrolysis activity is required for release and remodeling of its substrates (48). By utilizing this mutant we can view TRIP13 substrate binding and elucidate TRIP13 function. Using all purified components, I performed disassembly assays to identify whether TRIP13 and p31comet can disassemble both the initiator of MCC, the Mad1-Mad2 template, and a pre-existing potent inhibitor of the APC/C-Cdc20, the MCC. We can conclude that the joint activity of TRIP13 and p31comet, in the presence of ATP, do not disassemble Mad2 from the Mad1-Mad2 core complex. Additionally, the MCC disassembly assay shows good potential to allow us to discern if there is a role for TRIP13 and p31comet in disassembling the MCC.

Chapter 1: Introduction

A key event for a cell in mitosis is to faithfully transmit its genome to each of its daughter cells. The mitotic checkpoint is a protective mechanism in mitosis that ensures accurate chromosome segregation occurs by halting anaphase onset until all chromosomes have properly attached to microtubules through a location on the chromosome known as the kinetochore (45, 46). Defects in this checkpoint most often lead to having an abnormal number of chromosomes in a cell, a phenomenon known as aneuploidy that was observed over 100 years ago as a common feature of tumor cells (2). It is now known that aneuploidy is a common feature in solid human tumors, but it is still under debate whether aneuploidy is a cause of tumorigenesis or a consequence of malignancy already present in the cells (3). It is important to understand the mechanisms of the mitotic checkpoint in order to alleviate the occurrence of checkpoint defects and prevent the appearance of these cancerous cells. Using a well-established in-vitro reconstitution of the mitotic checkpoint, the Cleveland lab has demonstrated that unattached kinetochores, multi-protein complexes assembled onto the centromeric region of each chromosome, catalyze the production of a mitotic checkpoint inhibitor. This generation of anaphase inhibitor by unattached kinetochores is termed the “wait-anaphase” inhibitor and was first identified by several researchers (4-6). The formation of this mitotic inhibitor is based on the actions of some essential proteins in mitotic checkpoint signaling, namely Bub1, Bub3, Mad1, Mad2, BubR1, and Mps1 (7-9). The mitotic checkpoint functions to inhibit the anaphase-promoting complex/ cyclosome (APC/C), which requires its co-activator Cdc20 to target the specific mitotic proteins Cyclin B and Securin for degradation, and consequently leading to the activation of anaphase onset and the division of the cell into two (10). In particular, the Mad2 protein is shown to directly bind to the activator of

the APC/C, Cdc20 (11). In vivo and in vitro fluorescence recovery after photo-bleaching experiments (FRAP) have elucidated a model in which Mad2 undergoes conformational change, from open to closed, when recruited to a stably bound Mad1-Mad2 complex localized at unattached kinetochores (12, 13). This catalytic conformational change of a second Mad2 molecule to its active conformation, from its inactive one, is now known to be responsible for amplifying the formation of the BubR1-Cdc20 complex, the final inhibitor of the APC/C (14) (Figure 1).

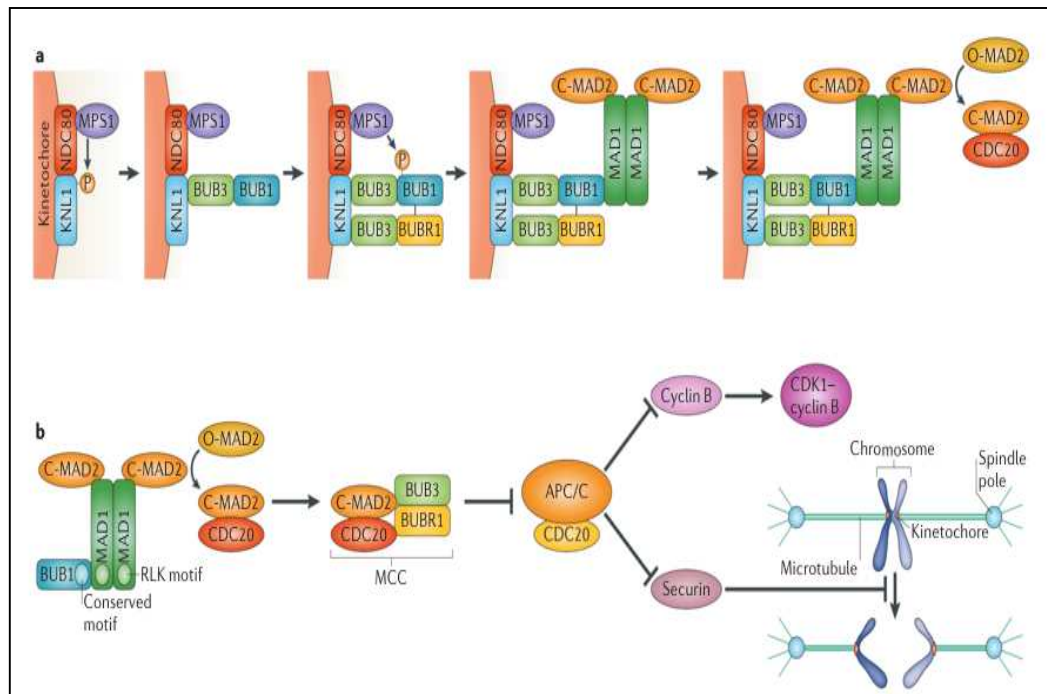


Figure 1. Kinetochores Activation of the checkpoint through hierarchical checkpoint protein recruitment (from London and Biggins, 2014)

Our Lab has worked extensively to identify the nature of the mitotic checkpoint, both in its activation and inactivation. Inactivation of the checkpoint is proposed to be controlled through many different pathways, as reviewed in the following paper (1). Particularly, the protein p31^{comet} has been implicated in this process, in that it not only is it a structural mimic of Mad2, but specifically recognizes and binds to the closed, active conformation of Mad2. Thus, p31^{comet} has been proposed to lead to checkpoint inactivation by binding to Mad1- or Cdc20-bound Mad2, to inhibit Mad2 activation as well as promote the dissociation of Mad2-Cdc20, respectively. (15-17). Also, the p31^{comet} binding site on Mad2 overlaps with the interface of Mad2-BubR1 in the MCC, suggesting that p31^{comet} may compete with BubR1 for Mad2 binding (18,19), another way in which it may stop the formation of MCC. More recently, there has been the discovery of the protein TRIP13 (Thyroid Hormone receptor interactor 13). TRIP13 is a highly conserved AAA+ ATPase that contributes to homologue pairing, synapsis, and recombination during meiosis, (20-23). However, its functions in mitosis are just starting to be studied. AAA-ATPases are classically known to remodel and/or disassemble protein complexes by ATP hydrolysis (24). TRIP13 is also seen to be over-expressed in many cancers and is ranked as a top gene in association with chromosomal instability (CIN) in human cancers (25-28). Interestingly, through data-mining, another group of researchers discovered that TRIP13 not only co-expresses with a group of core centromere/ kinetochore components, but localizes at Mad2 positive kinetochores (47). Additionally, it was found that TRIP13 directly interacts with p31^{comet} in vitro (29). Hershko and associates performed disassembly assays with these proteins and MCC components and claimed it was the joint activity of ATP-dependent TRIP13 and p31^{comet} that caused dissociation of Mad2 from immunoprecipitated Cdc20 and BubR1 complexes in vitro (36,37). Likewise, Corbett

and associates found that ATP-dependent TRIP13, together with its adaptor protein p31^{comet}, produce mitotic checkpoint silencing by inducing a conformational change in Mad2 from closed to open (inactive) form (38). Although the discovery of the AAA-ATPase TRIP13 as a previously unknown potential mitotic checkpoint silencer is exciting, its exact function and role in disassembling the mitotic checkpoint complex still remains unknown.

This study was conducted to further elucidate the function of TRIP13 in regards to mitotic checkpoint silencing. Specifically, I want to identify the targets of TRIP13 and whether TRIP13 and p31^{comet} do in fact work together to enhance disassembly of the mitotic checkpoint components, namely the Mad1-Mad2 core complex and free MCC, a complex made up of Mad2-Cdc20-BubR1-Bub3. From the observation that TRIP13 is an oncogene, and that its over-expression can induce malignancy in non-malignant cells (39), as well as studies identifying TRIP13 as a possible silencer of the mitotic checkpoint (36-38), targeting TRIP13 could prove to be an attractive anti-cancer strategy in the future.

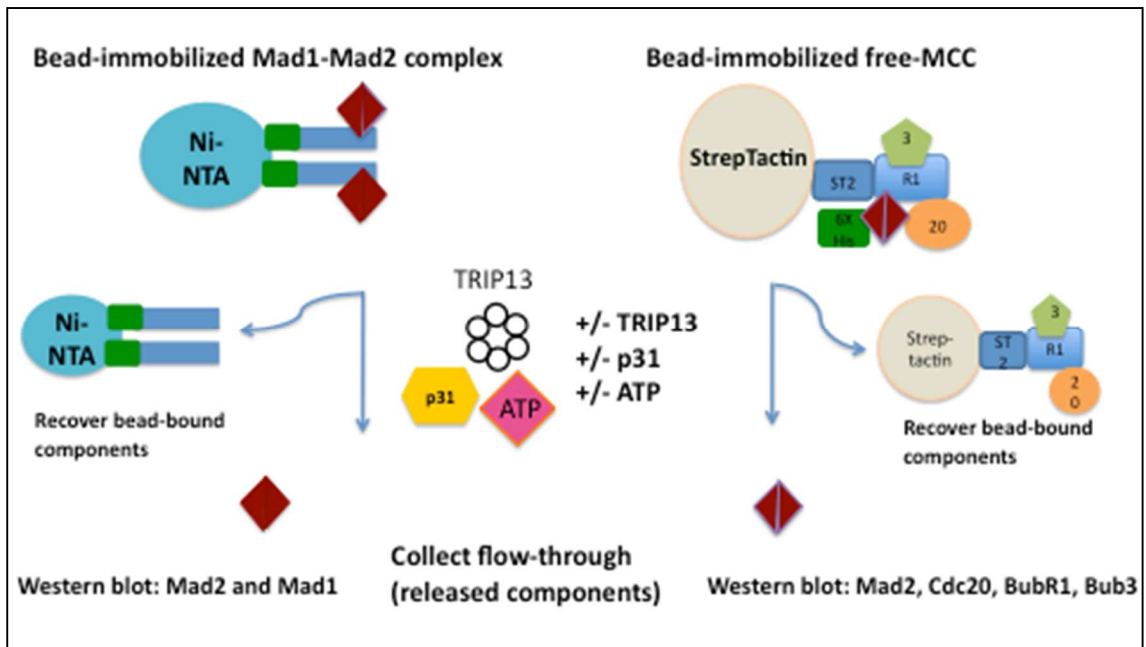


Figure 2. Disassembly approach to identify the functions of TRIP13 and p31^{comet} on the Mad1-Mad2 complex and free-MCC

Chapter 2: Materials & Methods

DNA Constructs. pFL-ST2-BubR1-Bub3 and pFL-Cdc20-6xHis-Mad2 were made using a Gibson assembly kit. ST2-BubR1, Bub3, Cdc20, 6xHis-Mad2 and pFL vector backbones were generated by PCR using the indicated primers (Figure 3).

```
GTACCGCATGCTCACTGAAAGAGCAAAGCCCCAG
GATCACCCGGGATGTGGAGCCATGGTCCCCAGTTCGAAAAAGCGGCGGTGAAGAAG
GAAGGG
GGCTCCACATCCCGGGTGATCAAGTCTTCG
CGAACCGGTCATGGATCCGCGCCCGATGGTGG
GCGCGGATCCATGACCGGTTTGAACGAATTCAAG
CAGGCTCTAGTTACGTGGACTTGGGCTTTGTTTCTG
GTCCACGTAAGTAGAGCCTGCAGTCTCGAC
CTTTCAGTGAGCATGCGGTACCGGGAGATG
GTACCGCATGCTCAGCGGATGCCTTGGTGGATG
GGCTCCACATCTCGAGATCCCGGGTGATC
GATGGTGATGCATGCGCCCGATGGTGGGAC
CATCGGGCGCATGCATCACCATCACCATCACGG
GACTGCAGGCTCAGTCATTGACAGGAATTTGTAGG
GTCAATGACTGAGCCTGCAGTCTCGACAAGC
GGCATCCGCTGAGCATGCGGTACCGGGAGATG
GGATCTCGAGATGGCACAGTTCGCGTTTCGAGAG
GAACTGTGCCATCTCGAGATCCCGGGTGATC
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Figure 3. Primers sets used for Gibson assembly of pFL-ST2 BubR1-Bub3 and pFL-Cdc20-6xHis-Mad2.

Protein Expression & Purification. hTRIP13 WT/EQ variants, p31^{comet} and Mad1(485-718)-Mad2 (plasmid #502) were purified as follows: pET30-hTRIP13, pET28a-hp31^{comet} and pET-2rbs-hsMad2-hsMad1(485-718) were transformed into Rosetta (DE3). A single colony was inoculated into 5 ml LB seed culture and expanded to 100ml LB, which was used the following day to inoculate 6 L of LB for expression. Induction was done with 0.05 mM IPTG at O.D. of 0.5 for overnight. Cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl pH 7.7, 0.1 M NaCl, 20 mM imidazole, 5 mM MgCl₂, and protease inhibitors LPC & PMSF). The lysate was

sonicated on ice at 40% amplitude with 2.5 seconds ON and 8 seconds OFF for 2.5 minutes total. Lysates were centrifuged for 1 hour at 17,000g and the cleared lysate was added to 1 ml of equilibrated Ni-NTA beads for 1 hour of rocking incubation at 4°C. The lysate was incubated with 1 ml of equilibrated Ni-NTA for 1 hour. The resin was transferred to an affinity chromatography column, washed twice with washing buffer 1 (25 mM Tris-HCl pH 7.7, 300 mM NaCl, 0.1% Triton X-100, 20 mM imidazole, LPC, PMSF), and the protein was eluted with 3 ml of elution buffer (20 mM Tris-HCl pH 7.7, 0.1 M NaCl, 20 mM imidazole, 5 mM MgCl₂, and protease inhibitors LPC & PMSF, 250 mM imidazole). PD-10 desalting column (GE Healthcare) was used to remove free imidazole. After desalting, we obtained a protein concentration of 0.42 mg/ml and 0.33 mg/ml and a yield of 2.5 mg and 2.1 mg for TRIP13 WT and EQ, respectively. p31^{comet} protein concentration was 1 mg/ml and yield was 6.4 mg. The MCC was generated using the Multi-bac system (Poly-genetic expression system). For the MCC purification, pFL-MCC (6xHis-Mad2, Cdc20, Bub3, and ST2-BubR1) was transformed into DH10Bac to create the MCC containing Bacmid, which was used to do Baculovirus infection of Sf9 cells. I performed a two-step purification with Ni-NTA and Streptactin beads. The cell lysates were resuspended in lysis buffer (20 mM Tris-HCl pH 7.7, 0.1 M NaCl, 20 mM imidazole, 5 mM MgCl₂, and protease inhibitors LPC & PMSF) and sonicated on ice at 40% amplitude with 2.5 seconds ON and 8 seconds OFF for 2.5 minutes total. Lysates were centrifuged for 1 hour at 17,000g and the cleared lysate was added to 1 ml of equilibrated Ni-NTA beads for 1 hour of rocking incubation at 4°C. The resin was then transferred to a Biorad poly-prep chromatography column and washed three times with washing buffer (20 mM Tris-HCl pH 7.7, 0.3 M NaCl, 0.2% Triton-X 100, 50 mM imidazole, 5 mM MgCl₂, and LPC/PMSF), and then once with

storage buffer (lysis buffer + 10% glycerol). 2 ml of elution buffer (lysis buffer + 250 mM imidazole) was added and incubated for 30 minutes at 4°C before elution. Eluted proteins were mixed with 500 ul equilibrated Streptactin beads for 1 hour and bead suspension was poured into small Biorad column. Resin was washed twice with washing buffer 2 (washing buffer 1 without imidazole), and then incubated for 45 minutes with 1 ml of elution buffer 2 (storage buffer + 10 mM desthiobiotin). Strep-tagged proteins were eluted with a total of 5 ml of elution buffer 2 (1 ml for each elution).

Antibodies. BubR1 (1:10,000) & Bub3 (1:2,000) are homemade antibodies. Mad2-Bethyl laboratories (1:1,000) (Cat #: A300-301A-2). Cdc20-Santa Cruz p55SC8358 (1:1,000) (Cat #: sc-1906). Mad1- used anti-his antibody (1:3,000).

Disassembly Assays. Mad1-Mad2 disassembly: Equilibrate master-mix of 200µl Nickel-NTA beads in wash buffer (25 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.1% Triton X-100, LPC protease inhibitor, and PMSF) to have 20 µl of beads for each reaction. Bind 1.5 µg purified Mad1-Mad2 complex to Mastermix of beads for 1 hour at 4°C. Wash beads three times in harsh wash conditions (25 mM Tris-HCl, 250 mM NaCl, 0.2% TritonX-100) and aspirate all wash buffer. Add 200 µl reaction buffer (25 mM Tris-HCl pH7.6, 5 mM MgCl₂, 1 mM DTT) and aliquot 40 µl of 50% Ni bead slurry for each sample. Add 10 ng GST-TRIP13 WT/EQ lysate, 20 ng p31^{comet}, 0.1 mM ATP and incubate at RT for 2 hours. Collect all of flow-through from beads and separately boil flow-through and beads in protein loading dye for 5 minutes. Run SDS-Page gel for Western blot analysis of MCC components. The MCC disassembly experiment protocol is the same as above, except 1 ml of MCC cell lysate is bound to an equilibrated

master-mix of 200 μ l Streptactin beads. In addition, 10 ng of purified recombinant his-TRIP13 WT and EQ were used instead of TRIP13 lysate.

Chapter 3: Results

Joint activity of TRIP13, p31^{comet}, and ATP do not disassemble Mad2 from the Mad1-Mad2 core complex. Previously published studies show that the joint activity of ATP-dependent TRIP13 and p31^{comet} lead to the disassembly of Mad2 from anaphase-inhibitory complexes (36, 38). p31^{comet} is shown to act as an adaptor for TRIP13 and the two work together to induce a conformational change of Mad2 from its closed-active conformation to its open-inactive conformation (38). In addition, p31^{comet} has been shown to transiently associated with the Mad1-Mad2 core complex and through the “p31-capping model” has been proposed to act as a cap on Mad2 to prevent dimerization of cytosolic O-Mad2 on Mad2 of the Mad1-Mad2 template (35, 16). This model supports the idea that this p31 cap will stop amplification of the closed-Mad2 conformer, and thus MCC formation, to release the APC/C-Cdc20 from inhibition (16). If p31^{comet} acts as an adaptor for TRIP13 to induce a conformational change in substrate proteins, and has been shown to bind Mad2 of the Mad1-Mad2 core complex, then TRIP13 may function with p31 to disassemble Mad2 from the Mad1-Mad2 complex, the template for activation of Mad2 (40). To test this, we performed a disassembly assay in which Nickel bead-immobilized Mad1-Mad2 complex was incubated in the presence of TRIP13WT/ EQ, purified recombinant p31^{comet}, and ATP. After collecting the flow-through of components, as well as recovering the bead-bound components, the amount of Mad1 and Mad2 protein was detected by Western blot (Figure 4). The results show that neither the activity of TRIP13, p31^{comet}, or the combination of the two in the presence of ATP results in disassembly of Mad2 from its counterpart Mad1 of the Mad1-Mad2 complex (Figure 5). The disassembly of this complex has never been tested before and gives us insight into what the exact

substrates of TRIP13 ATPase activity may be. A test that needs to be done to verify that we have a functional TRIP13 is the Mad2 conformational conversion assay performed by the Corbett lab in their studies of TRIP13 (38). If this assay shows that TRIP13, aided by p31^{comet}, is able to function by converting Mad2 to its inactive conformation, this would serve as a positive control to validate that our TRIP13 is functional and that it truly does not function to disassemble the Mad1-Mad2 complex.

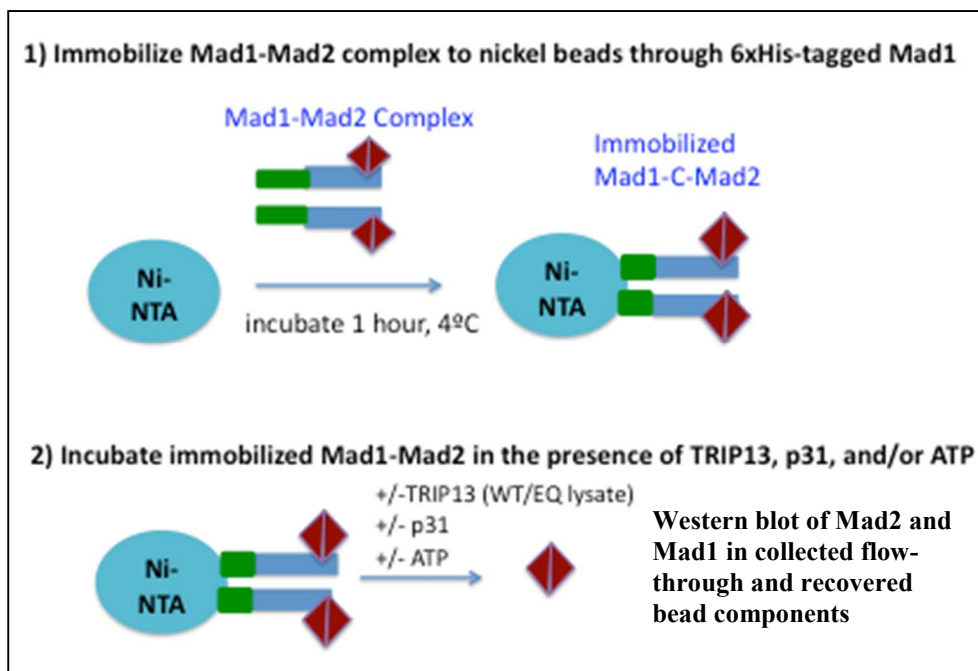


Figure 4. Schematic of Mad1-Mad2 disassembly assay

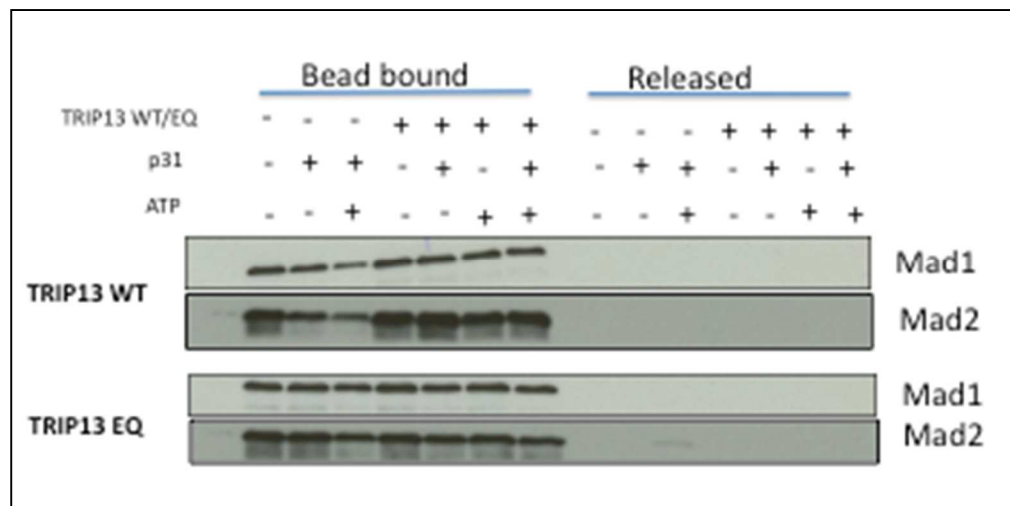


Figure 5. Activity of TRIP13, p31^{comet}, and ATP in the disassembly of Mad2 from the Mad1-Mad2 core complex

Joint activity of TRIP13, p31^{comet}, and ATP on the disassembly of MCC. In the lab of Nobel laureate Avram Hershko, it was proposed that the combined effect of TRIP13 and p31^{comet} on MCC leads to the dissociation of Mad2 from MCC (36). This model suggests that TRIP13 and p31 play a centrally important role in the sequence of events leading to MCC disassembly, and thus checkpoint inactivation, by removal of Mad2 from the MCC. Due to the fact that BubR1, not Mad2, is the essential component of the anaphase-inhibitory complex responsible for APC/C-Cdc20 inactivation (14, 41), this model does not support complete disassembly of MCC and leaves a BubR1-Bub3-Cdc20 complex to continue inhibition of the APC/C-Cdc20. My plan is to create an MCC disassembly assay that clearly shows the component of the MCC that is being targeted by TRIP13 and p31^{comet} for removal. The design of this experiment is similar to the Mad1-Mad2 disassembly experiment, with the exception that free-MCC is first

immobilized to beads by incubation of MCC lysate with Streptactin beads, then after several washes of the beads, they are subsequently incubated with all recombinant purified GST-TRIP13 WT/EQ, his-p31^{comet}, and ATP (Figure 6). Upon analyzing the flow-through of the beads and the remaining bead-bound components by Western blot, we observe the release of all four MCC components, with BubR1 and Bub3 most noticeably releasing off Streptactin beads (Figure 7). This poses a problem for the disassembly assay due to the fact that our immobilized component of the MCC, BubR1, is not stably bound and is leeching off the beads. As a result of this, the presence of Bub3 in the flow-through is expected as its main role is as a BubR1-binding-protein (42). Although we see some disassembly of both Cdc20 and Mad2 as well, no clear conclusion can be made at this time on whether this disassembly is indeed resulting from the enzymatic activity of TRIP13 in conjunction with its binding-partner p31^{comet}. Again, our TRIP13 and p31^{comet} will need to be assessed first for proper function and this will be done using the Mad2 conformational conversion assay utilized by the Corbett lab (38), showing TRIP13 activity in changing Mad2 conformation. In addition, it will be ideal to be able to fully purify recombinant free-MCC to use for the experiment. Altogether, these results give us a reasonable starting point for future work and the following “Discussion section” elucidates our work-in-progress to modify our MCC disassembly assay approach.

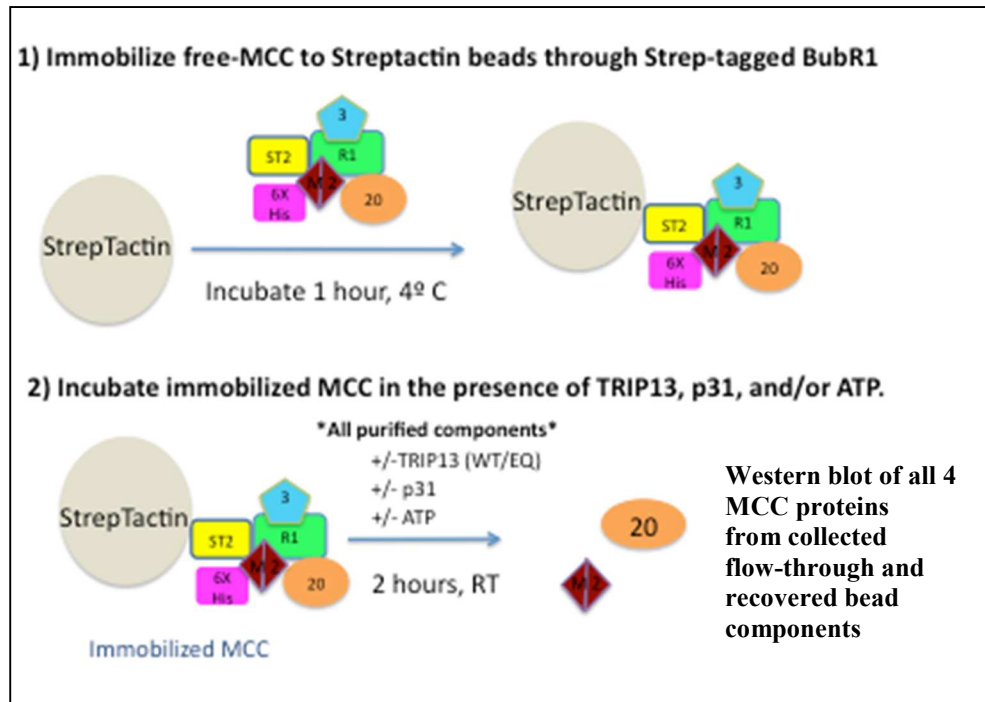


Figure 6. Schematic for MCC Disassembly Assay

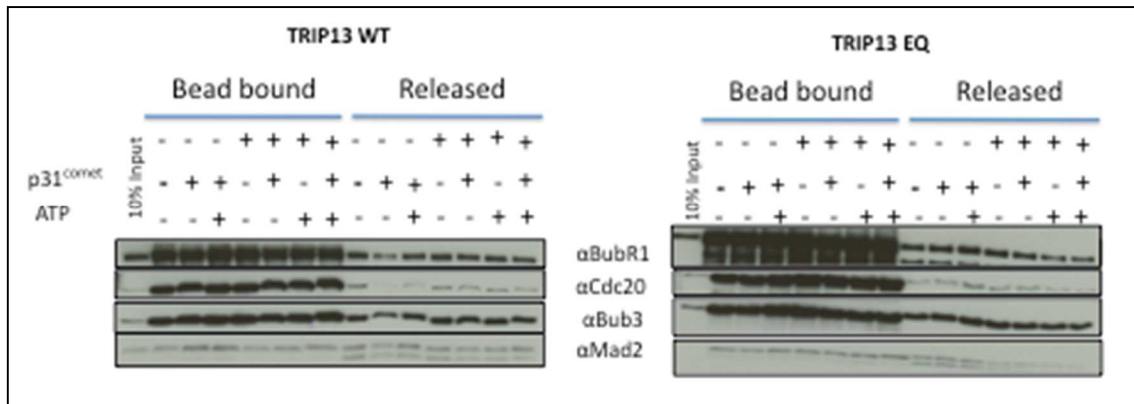


Figure 7. Preliminary MCC Disassembly Assay Results

Chapter 4: Discussion

Inactivation of mitotic checkpoint signaling requires two steps: 1) termination of mitotic checkpoint signaling and, thus MCC formation and 2) disassembly of pre-existing MCC. p31^{comet} interacts with C-Mad2 in both the MCC and the C-Mad2-Mad1 complex, and has been proposed to play a role in silencing of both complexes (15, 33, 35). In addition, the identification of the AAA+ ATPase TRIP13 as a novel checkpoint silencing protein (29) led to further studies that identified TRIP13 and p31^{comet} as directly interacting proteins (43). The most convincing evidence showing that TRIP13 and p31^{comet} play an active role in mitotic checkpoint silencing was the conformational conversion experiment performed by Corbett lab researchers. This group incubated TRIP13 WT, TRIP13 ATP-binding deficient mutant (KA), and TRIP13 ATP-hydrolysis deficient mutant (EQ) in the presence of p31^{comet}, O-Mad2 or C-Mad2, and ATP and viewed the anion-exchange elution profiles of different Mad2 conformers, p31, and p31-Mad2 complexes. Only in the presence of active TRIP13 WT, p31^{comet}, and ATP were they able to see C-Mad2 to O-Mad2 conversion (38). Previous work has shown that essentially all free-Mad2 in HeLa cells is in its open-inactive conformation (44). The Corbett lab adds that at physiological temperatures, almost all O-Mad2 will spontaneously convert to C-Mad2 within only a few hours. They propose that the ability of TRIP13 to disengage the safety-belt motif of Mad2 and convert it back to its inactive conformer strongly suggests that TRIP13 ATPase activity counteracts Mad2's spontaneous conformational conversion in order to maintain it in its open-state and guard against improper-timing of mitotic checkpoint activation and MCC assembly (38). The Mad1-Mad2 complex disassembly experiment was designed to identify whether TRIP13 and p31^{comet} play a role in stopping generation of new MCC formation by disassembling the Mad1-Mad2 template, whose function is required for amplification

and formation of free-MCC (13-14, 40). Due to the fact that no disassembly of Mad2 was observed in the presence of TRIP13 lysate and purified p31^{comet}, it can be concluded that the function of this enzyme with its adaptor protein is not promoting the dissociation of the Mad1-Mad2 template. This is assuming that our TRIP13 enzyme and our recombinant p31^{comet} protein are both functional and this needs to be assessed to complete the findings presented here. In regards to the MCC disassembly experiment that was performed, although a good starting-point has been established to view how the MCC is disassembled by the addition of these components, some modifications need to be made to be able to conclude the results. Firstly, before this experiment can be repeated, it is crucial that we perform an ATPase activity assay to determine the relative activities of our purified TRIP13 WT and EQ mutant. One method we can employ is the enzyme-coupled assay done by the Corbett group in their TRIP13 studies (38). Once our protein functionalities have been validated, we can modify the experiment in several ways. One thing that can be done is to increase the harshness of Streptactin bead washing once MCC has been immobilized (Fig. 6; Step 1). This may help clear off any unstably-bound MCC prior to incubation with TRIP13 and p31. Another thing I plan to do is to purify recombinant free-MCC to completion. We know this is possible to do from previous work in the lab but have so far been unable to accomplish this in the allotted time. This is due to such problems like our insect cell virus not working efficiently to infect our insect cell culture with expression of MCC, as well as the instability of MCC protein complex during the process of purification. If MCC can be purified to obtain a good yield and purity, and also exhibit a 1:1 ratio of all four proteins (BubR1, Cdc20, Bub3, and Mad2) by coomassie gel staining, this would be ideal for our disassembly assay in being able to use all purified components. TRIP13 is shown to be up-regulated in many different cancer types

compared to normal tissues and over-expression of TRIP13 in non-malignant cells has been shown to result in malignant transformation (39). Thus, identifying where TRIP13 and p31^{comet} exert their activities and how they function as proposed mitotic checkpoint silencers is crucial to understanding their roles in human health and disease.

Appendix

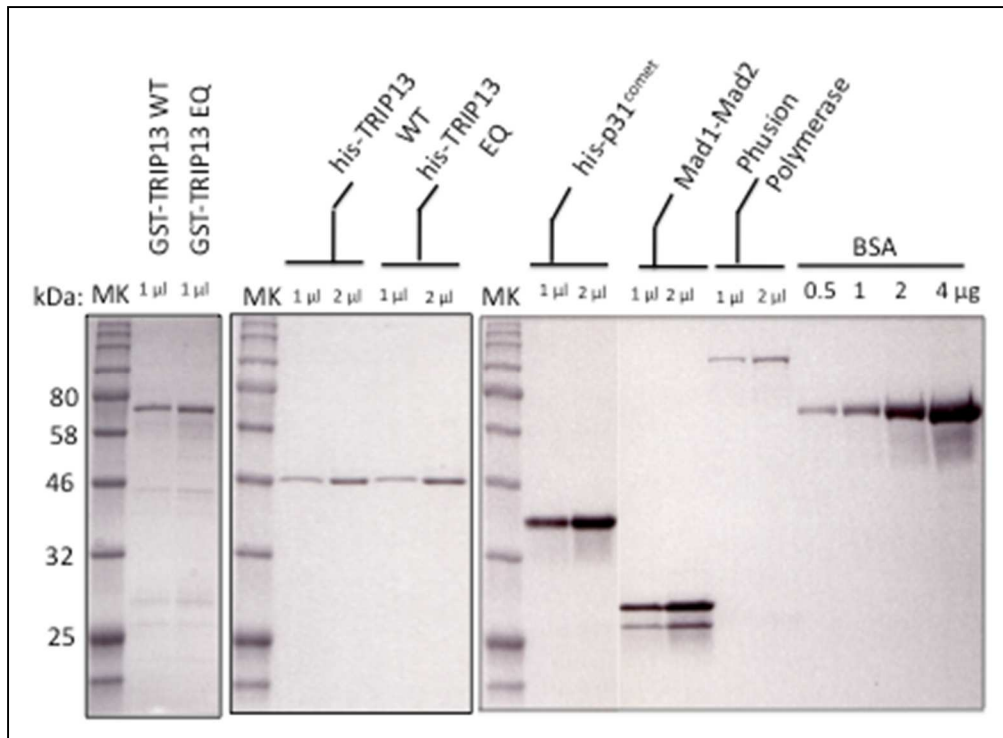


Figure 8. Supplemental: All protein purifications

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