Molecular Mechanisms that Maintain Genome Stability in Human Cells

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A hallmark of cancers is genome-instability; the failure to repair mutations in the genome and the failure to accurately segregate chromosomes during cell division. These defects provide the cancer cell with the ability to rapidly undergo genetic changes (evolution) so that they can continue to proliferate. Indeed, genome instability may explain why cancers frequently recur after chemo- or radiation therapy. Understanding the biochemical nature by which normal cells maintain the stability of their genome is of utmost importance to addressing how to more effectively treat cancers in patients.

Our laboratory's interest is to understand the mechanisms that specify accurate chromosome segregation during mitosis. Our research is particularly relevant to cancer research as drugs that inhibit mitosis are a major modality for anti-cancer therapy. Current drugs however, lack specificity as they target microtubules that provide functions that not only are critical for mitosis but also for other essential cellular processes such as vesicle transport, cell shape and locomotion. Our studies of how chromosomes segregate have revealed novel proteins that provide functions that are critical only during mitosis. As such, these proteins should be ideal candidates for the development of highly specific anti-mitotic drugs. We have focused our attention on characterizing the molecular composition and function of the kinetochore, as this is the structure on the chromosome that establishes connections with microtubules of the spindle in order to under proper separation. We have identified molecular motors and checkpoint proteins that reside at kinetochores and are interested in understanding how these proteins interact with each other to carry out complex kinetochore functions.

CENP-F is a novel microtubule binding that is essential for mitosis. Feng,[a] Yen

CENP-F is a 367 kDa kinetochore protein that was originally identified by a human autoimmune serum and independently isolated as Mitosin in a screen for proteins that interacted with the Rb tumor suppressor. CENP-F exhibits a very dynamic localization pattern whereby it undergoes transitions from the nuclear matrix, to the nuclear rim and then to kinetochores during the G2 phase of the cell cycle. Kinetochore localization persists until the onset of anaphase when it re-localizes to the spindle midzone. Immuno EM studies showed that CENP-F is concentrated at the outer kinetochore and may extend into the fibrous corona.

Despite its discovery over a decade ago, its functional importance has remained unanswered. With the advent of RNAi technology, we succeeded to deplete CENP-F from HeLa cells. Quantitative analysis of the magnitude of depletion by western blots and immunofluorescence microscopy showed that >95% of the normal level of CENP-F was depleted from the transfected cells. We characterized the effects of CENP-F depletion on mitosis by first monitoring the behavior of chromosomes during mitosis. Time-lapse videomicroscopy revealed that

chromosomes were able to congress towards the spindle equator with kinetics that were similar to control cells. Unlike normal cells that segregated the sister chromatids at anaphase, many chromosomes in cells depleted of CENP-F failed to segregate but instead were stranded in between the separating cells. The accumulation of lagging chromosomes indicate defects in the attachments between microtubules and the kinetochore.

We verified this possibility by comparing the integrity of the microtubule:kinetochore attachments between control and CENP-F depleted cells after cold treatment. Normal mitotic cells retained all of their microtubule attachments as their connections with the kinetochore stabilized them to cold treatment. In contrast, kinetochores depleted of CENP-F were unable to establish stable microtubule connections as they were not resistant to cold treatment. Furthermore, we found that cells depleted of CENP-F retained near normal levels of proteins that have previously been shown to be essential for microtubule:kinetochore attachments. Clues about how CENP-F might contribute towards kinetochore attachments came when we discovered that the N- and C-terminal portions of the protein co-localized with microtubules in transfected cells. We confirmed that the two regions of CENP-F were able to directly bind microtubules by testing bacterially expressed proteins for their ability to cosediment with microtubules that were purified from bovine brain. Using this *in-vitro* assay, we localized the microtubule binding activities to the N-terminal 300 and C-terminal 187 amino acids. We then determined that the C-terminal microtubule binding domain was capable of stimulating the rate of tubulin polymerization. The combined results strongly indicated that CENP-F's microtubule binding activity is important for kinetochores to establish microtubule attachments *in vivo*.

We also discovered that CENP-F contributes to the mitotic checkpoint as cells depleted of CENP-F cannot sustain a prolonged mitotic delay when their spindle is disrupted. Unlike classical mitotic checkpoint proteins whose loss results in no mitotic delay in response to unaligned chromosomes, cells depleted of CENP-F are still able to delay mitosis. The duration of the delay is short suggesting that CENP-F might affect the threshold level of checkpoint activity that is required to maintain a prolonged arrest. When cells depleted of CENP-F were stained for the presence of mitotic checkpoint proteins at kinetochores, we discovered that there was an across the board reduction (~30%) in their levels. The reduced amounts of mitotic checkpoint proteins at kinetochores depleted of CENP-F would likely limit the capacity to generate sufficient amounts of the "wait anaphase" signal to maintain a prolonged mitotic delay.

Identification and characterization of a new protein of the inner centromere. Huang, Feng,[a] Yen, in collaboration with Rattner[b]

Tripin is a mammalian gene of unknown function but was recently shown to share some motifs with the Shugoshin (Sgo), an evolutionarily conserved protein that localizes at kinetochores and is responsible for preventing premature separation of the paired centromeres. Additionally, Sgo1 in frogs was reported to bind to microtubules and functional studies reveal a dual role in specifying microtubule attachments at kinetochores in addition to its role in holding together sister centromeres. We generated antibodies specific for tripin and immunofluorescence staining revealed that it was localized to kinetochores. Its localization pattern was coincident with MCAK, a microtubule depolymerizing enzyme that is believed to be responsible for severing defective attachments at kinetochores. At kinetochores that have not established microtubule connections, tripin is localized in between the pair of sister kinetochores and appears as a single focus of staining. Upon microtubule attachment, tripin relocalizes towards each of the sister kinetochores and thus appearing as a band that spans the width of the centromere. Through the use of microtubule inhibitors, we demonstrated that the redistribution of Tripin from the inner centromere (single foci) towards the kinetochores (bar) depends on tension that is produced by opposing poleward forces that are generated by the kinetochore pair.

Functionally, we used siRNA to deplete ~95% of the Tripin from Hela cells. Time-lapse videomicroscopy showed that the chromosomes were delayed in aligning at the spindle equator. This delay reflects the failure of the kinetochore to establish connections with microtubules of the spindle. Although chromo-

somes eventually align, the attachments were defective as we found examples where one kinetochore was attached to microtubules from opposite poles, and both kinetochores were attached to the same pole. These attachments if not corrected to normal bipolar attachments prevent the chromosome from being segregated during anaphase. The progeny cells are thus aneuploid.

Given the similarity in the localization patterns between Tripin and MCAK, we examined whether their localization depended on each other. Cells depleted of Tripin failed to localize MCAK to kinetochores. The absence of MCAK at kinetochores can explain why loss of hSgo2 leads to an accumulation of defective kinetochore:microtubule attachments. Beyond this, we found at the EM level that the loss of hSgo2 resulted in a perturbation of the higher order structure of the kinetochore. The combined molecular and ultrastructural defect thus contributes to the inability to establish proper kinetochore attachments.

Mapping the pathways that specify the assembly of the kinetochore. Liu, Yen, in collaboration with Rattner[b]

The kinetochore is a macromolecular complex that localizes at the centromere of mitotic chromosomes and is a massive structure that is visible under the electron microscope (EM) as a trilaminar stack of plates that formed along the outer edge of the centromere. We previously reported that many of the kinetochore proteins in human cells exhibited a distinct temporal pattern of localization that begins in the G2 phase of the cell cycle and persists through mitosis. This staining pattern correlates with EM images that show kinetochore trilaminar plates are only visible during mitosis. The combined data suggested that kinetochores are assembled and disassembled during each mitosis.

We completed a study where we examined the interactions amongst twenty proteins with respect to the assembly to the centromere/kinetochore complex in human cells. These proteins include constitutive centromere proteins CENP-A, CENP-B, CENP-C, CENP-H, CENP-I; inner centromere protein Aurora B; microtubule interacting proteins: dynein-dynactin complex (represented by p150^{glued}), CENP-E, CENP-F and MCAK; mitotic checkpoint proteins: BUB1, BUBR1, MAD1, MAD2, hMPS1, hZW10, hROD as well as HEC1, NUF2, and hMis12. Amongst this group, Aurora B was classified as a "chromosomal passenger protein" or "inner centromere protein," but its role in kinetochore-microtubule attachment, mitotic checkpoint signaling and recruitment of kinetochore proteins led us to include it in our analysis.

We found that CENP-A, a histone H3 variant that is specifically associated with centromeric chromatin, occupies the top of a hierarchy that directs three major pathways specified by CENP-C, CENP-I and Aurora (**Figure 1**). Each pathway consists of sub-branches that intersect to form nodes that may coordinate the assembly process. Complementary EM studies found that the formation of kinetochore trilaminar plates depends on the CENP-I/NUF2 branch while CENP-C and Aurora B affect the size, shape and structural integrity of the plates.

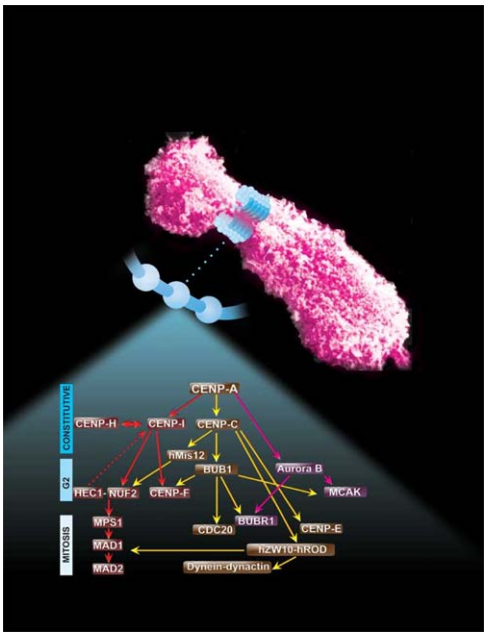

Figure 1. Schematic depiction of the network of interactions amongst 20 kinetochore proteins within a single kinetochore module. Multimerization of the modules generates the higher order organization of the kinetochore.

hMis12 complex exhibits a new cell cycle pattern of localization to the centromere. Liu, Yen

Among over 100 centromere/kinetochore proteins in human cells, only a small fraction constitutively localize at the centromere throughout the cell cycle. The majority are temporarily

recruited to the kinetochore as it initiates assembly from late G2 to mitosis. We report here the hMis12 complex, consisting of hMis12, hNnf1, hDsn1 and hNsl1 proteins, display a distinct localization pattern. It dissociates from kinetochores in telophase but rebinds to centromeres in early G1. More interestingly, analysis of its stability at centromeres by FRAP (Fluorescence Recovery After Photobleaching), showed that it exhibited a fast turnover during interphase but becomes virtually immobile during mitosis. This change in turnover probably reflects a change in affinity of the Mis12 complex with components of the centromere-kinetochore complex when cells enter mitosis. We analyzed and found that mutations at several amino acids of hMis12 altered its localization and dynamics pattern, suggesting they might be important for anchoring the hMis12 complex onto centromeres.

Biochemical characterization of the mechanism of the mitotic checkpoint. Hershko, Liu, Yen

The mitotic checkpoint is a failsafe mechanism that prevents cells with misaligned chromosomes from prematurely exiting mitosis and become aneuploid. Molecular and genetic studies from many labs have shown that the E3 ubiquitin ligase, APC/C, is inhibited by the mitotic checkpoint proteins. We previously biochemical purified an inhibitory factor from Hela cells that potently inhibited the APC/C *in vitro*. This inhibitor factor consists of a complex of the checkpoint proteins hBUBR1, hBUB3, cdc20 and Mad2.

To further dissect the biochemical mechanism by which the mitotic checkpoint inhibits the APC/C, we are refining a cell-system that partially recapitulates this activity. Crude extracts prepared from mitotically arrested Hela cells will degrade APC/C substrates such as cyclin B1 and securing. Kinetic studies showed there was a reproducible lag before the substrate was degraded. This lag reflects the residual checkpoint inhibitor activity that was present in cells at the time the extracts were prepared. If the extracts are pre-incubated for several hours, we observed that the substrate was rapidly degraded upon introduction. These data show there is a labile factor that normally inhibits the APC/C. Preliminary efforts to purify this inhibitory factor are consistent with the MCC. Ongoing efforts are to identify the components in the extracts that contribute towards the delay in the activation of the APC/C that was observed in the extracts.

Characterizing the molecular basis for aneuploidy in cancer cells. Beeharry, Yen

The overt differences between 'normal' cells and cancerous cells are minimal which makes the design and application of 'cancer cell-specific' therapies especially challenging. Many of the common drugs used to treat many cancers target mitosis by blocking the formation of the mitotic spindle. Targeting the mitotic phase of the cell cycle y is thought to be particularly advantageous due to the high replicative rate of tumor cells. The taxanes (paclitaxel), which act to stabilize microtubules, and the vinca alkaloids, (vinblastine or vincristine) which inhibit the polymerization of tubulin, are commonly used as 'first line' therapy for the treatment of ovarian and many other cancers. Furthermore, taxol is the drug of choice in patients resistant to platinum compound based therapies. Nonetheless, many patients relapse, indicating that tumor cell resistance is a major obstacle to effective treatment. Many questions remain about the precise mechanisms of anti-mitotic drugs and resistance. The importance of the integrity of the mitotic checkpoint, in the context of human cancers, is highlighted by the findings that many cancer cells exhibit an impaired mitotic checkpoint and by-pass a mitotic arrest in response to microtubule disrupting drug treatment. One of the aims of this project is to understand why agents that disrupt microtubule dynamics are effective in killing some tumor cells, but refractive in others. Our recent experiments in a number of ovarian or leukemic cell lines have shown that cells have different responses to anti-mitotic agents. We have measured the relative amounts of the seven essential mitotic checkpoint proteins as well as associated checkpoint and/or kinetochore proteins in asynchronous and drug treated cells. In this way we aim to identify whether or not the strength/length of the mitotic arrest is the summation of the biochemical activities of checkpoint proteins. Interestingly, our preliminary data suggests that the strength/duration of mitotic arrest reliably correlates with the levels of BubR1, a key mitotic checkpoint protein. Thus we are following up on the

role of BubR1 in regulating cellular fate following anti-mitotic drug treatment. We hope that enhanced understanding of Taxol and vincristine/vinblastine effects on the mitotic spindle, especially in light of more complete molecular dissection of the mitotic apparatus, may permit us to enhance drug activity, overcome resistance and/or develop novel agents targeted to the mitotic components.

CENP-F specifies the assembly of nucleoporins to kinetochores. Yen, in collaboration with Doye[c]

We previously showed that kinetochore proteins hMPS1, Mad1 and Mad2 are localized to the nucleoplasmic side of the nuclear pore complex in interphase cells. Others then discovered that some nucleoporins (Nup's) also localize to kinetochores in mitosis. A yeast two-hybrid screen for proteins that interact with Nup107 identified a C-terminal portion of CENP-F. Immunoprecipitation experiments conducted in HeLa lysates confirmed the yeast two-hybrid data. The significance of this interaction was tested by examining if the localization of Nup107 depended CENP-F. Quantitative immunofluorescence revealed cells depleted of CENP-F were unable to recruit Nup107 to kinetochores. However, localization of CENP-F to kinetochores did not depend on Nup107. Functionally, Nup107 was found to specify accurate chromosome segregation as cells depleted of Nup107 failed to align their chromosomes properly. The mechanism by which Nup107 specifies kinetochore microtubule attachments is being pursued.

Validating kinetochore proteins as targets for novel anti-cancer drug development. Lau, Yen, in collaboration with Einarson[§]

We are comparing the mitotic checkpoint potential of a large panel of human cancer cell lines in an effort to understand mechanisms of chromosome instability in cancer. Chromosome aneuploidy is a hallmark of the majority of cancers but mechanistic insights into this defect remains poorly understood. We are comparing how different cancer cells lines respond to the microtubule-destabilizing drug, nocodazole. FACS analysis combined with time-lapse videomicroscopy revealed that there was a wide variance in the duration of the mitotic delay after nocodazole treatment. Western blots comparing the steady-state levels of mitotic checkpoint proteins Bub1, BubR1, Bub3, Mad1, Mad2, and Mps1 revealed that the majority of cells expressed comparable levels of these proteins even though the duration of the mitotic delay varied. Thus, the duration of the mitotic checkpoint is specified not only by mitotic checkpoint proteins but other components whose identities remain to be identified.

To quantitate the efficiency of chromosome segregation more accurately, we have generated stable cell lines that express gfp:H2B. The gfp:H2B fluorescently labels the chromosomes in vivo and allows us to directly visualize their dynamic behavior during mitosis. Quantitative differences in the efficiency of chromosome segregation amongst different cancer cell lines may provide clues about mechanistic differences in the structural and regulator y proteins involved in chromosome segregation.

Publications

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