Centromere-Associated Protein E: A Motor That Puts the Brakes on the Mitotic Checkpoint

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Abstract Cell cycle checkpoints have long been recognized as important nodes for regulating cell proliferation and maintaining genomic integrity. These checkpoints are often altered in cancer and represent promising points for therapeutic intervention. Until recently, direct targeting of the mitotic checkpoint has been an untapped area for cancer drug discovery. Regulation of the mitotic checkpoint is complex, but many of the critical players have been identified and functionally characterized. A substantial number of these proteins can be localized to the kinetochore, a structure located at the centromeric region of each mitotic chromosome. The kinetochore mediates chromosome attachment to spindle microtubules and subsequent chromosome movement. The mitotic checkpoint monitors microtubule attachment and chromosome position on the mitotic spindle, inhibiting progression into anaphase until proper attachment and metaphase positioning is achieved. Centromere-associated protein E is a kinesin microtubule motor protein that plays an essential role in integrating the mechanics of microtubule-chromosome interactions with mitotic checkpoint signaling, and has emerged as a novel target for cancer therapy.

Background

Ordered progression of the eukaryotic cell cycle from one phase to another is normally tightly regulated (1). Cancer is characterized by deregulated cell proliferation, often as a consequence of loss of specific brakes, or cell cycle checkpoints such as the mitotic checkpoint. A common characteristic of human cancers is abnormal numbers of chromosomes and centrosomes, thought to be caused, in part, by mitotic checkpoint dysfunction during tumor development (2). In addition to facilitating the genomic instability of the cancer, deregulated cell cycle checkpoints can also provide a foothold for selectivity of anticancer therapeutics.

During mitosis, a critical event is the transition from metaphase to anaphase when sister chromatids are separated and segregated to opposite poles of the cell. This transition is driven by the anaphase-promoting complex, or cyclosome (APC/C), an E3 ubiquitin ligase that mediates proteolysis of anaphase inhibitory proteins such as cyclin B and securin (Fig. 1). The APC/C is regulated by the mitotic checkpoint, which monitors the integrity of the mitotic spindle, and restrains the ubiquitin-mediated proteolytic activity of the APC/C, until all mitotic chromosomes have attached to spindle microtubules and chromosomes have achieved metaphase alignment. The hub of mitotic checkpoint signaling is the kinetochore, a large protein complex that assembles during mitosis on chromosomal regions known as centromeres and mediates the attachment of chromosomes to the mitotic spindle. There are several kinetochore proteins that bind the plus ends of spindle microtubules and additional proteins that regulate the stability of microtubule attachments (reviewed in ref. 3). It is at the kinetochore that an anaphase-inhibitory signal is generated, inhibiting the activity of the APC/C. Generation of this APC/C inhibitory signal is repressed upon interaction of the kinetochore with microtubules. Centromere-associated protein E (CENP-E) is the central player coordinating the mechanics of microtubule interaction with regulation of the APC/C.

There is now substantial evidence validating CENP-E as a cancer target. Efforts to identify inhibitors of CENP-E have led to the discovery of GSK923295, an allosteric inhibitor of the kinesin motor domain of CENP-E that recently entered phase I clinical trial. We review here the functions of CENP-E in cell cycle regulation and the relevance of CENP-E to cancer, and briefly summarize preclinical pharmacology of this first in class mitotic checkpoint modulator.

CENP-E Structure and Function

CENP-E is a large protein of ~312 kDa consisting of an NH_2-terminal kinesin motor domain tethered to a globular COOH-terminal domain via an extended "rod" domain predicted to form a coiled coil (4). Kinesin motors hydrolyze ATP to produce directed mechanical force along microtubules. In the case of CENP-E, the motor activity is involved in establishing and maintaining stable connections between mitotic chromosomes and the microtubules of the mitotic spindle. CENP-E exhibits a periodic accumulation and loss, with maximal levels found during late G_2 and M-phases of the cell cycle, and minimum levels in early G_1 (5). During interphase CENP-E is sequestered in the cytoplasm, gaining access to mitotic chromosomes only after nuclear envelope

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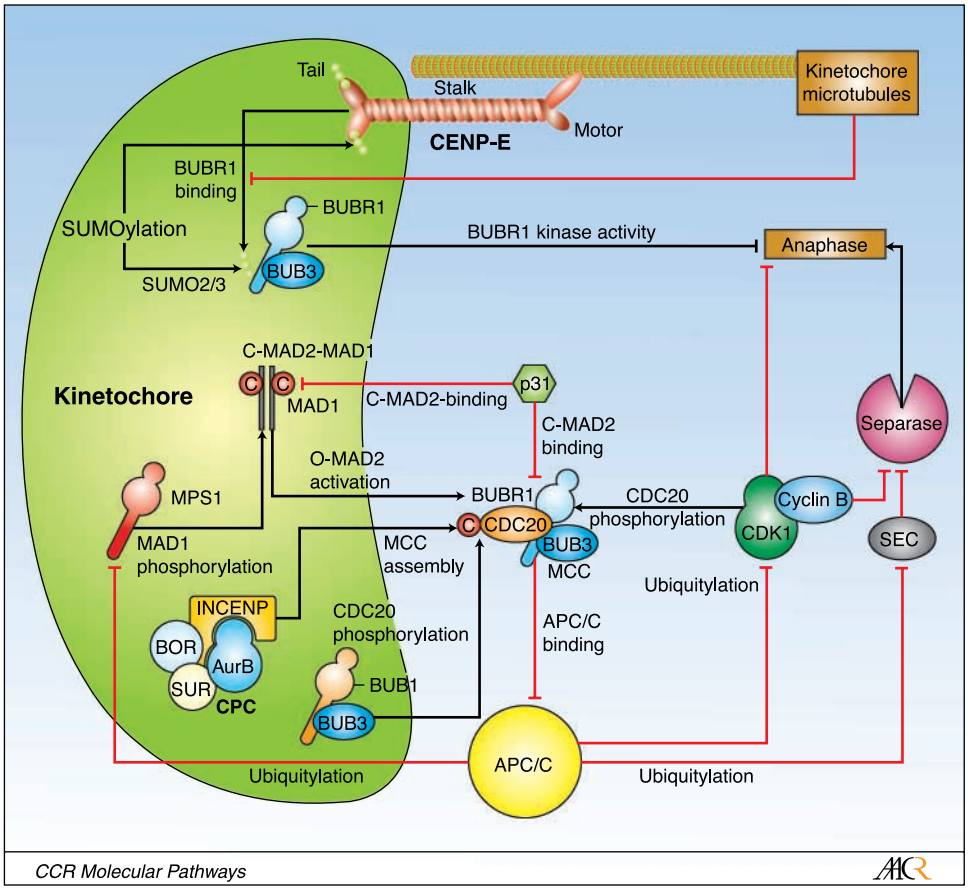

Fig. 1. CENP-E and kinetochore mitotic checkpoint signaling. Important regulatory pathways for mitotic checkpoint control at the kinetochore are shown. On each kinetochore, when microtubules are captured, CENP-E-mediated activation of BubR1 is suppressed, and formation of the APC/C inhibitory mitotic checkpoint complex is suspended, permitting APC/C-mediated protein ubiquitylation and degradation, allowing cell cycle progression to anaphase. Despite the detailed understanding of many aspects of checkpoint signaling, the pathway depicted is almost certainly oversimplified. Further detail on checkpoint regulation has been previously reviewed (3, 41). Adapted with permission from Musacchio et al. (3).

breakdown and entry into mitosis. During prometaphase and early anaphase CENP-E localizes to kinetochores of mitotic chromosomes (4–6). Late in mitosis, following sister chromatid separation, the abundance of most kinetochore proteins, including CENP-E, declines abruptly as the result of proteolysis. CENP-E is thus appropriately spatially and temporally positioned to integrate the regulation of the mitotic checkpoint with the mechanics of chromosome-microtubule interactions.

Observations in a wide variety of experimental systems support an essential role for CENP-E in achieving metaphase chromosome alignment during prometaphase. Depletion of CENP-E from cycling HeLa cells using antisense oligonucleotides or small interfering RNA targeting CENP-E mRNA has been shown to result in the accumulation of mitotic cells containing intact mitotic spindles with misaligned chromosomes (7–9). Similar results were obtained following microinjection of antibody directed against CENP-E or expression of a putative dominant negative mutant of CENP-E lacking the NH_2-terminal kinesin motor domain (10, 11). In Drosophila cells and in extracts prepared from *Xenopus* eggs, loss of the relevant CENP-E orthologue also results in a failure to achieve and/or maintain metaphase chromosome alignment (12, 13). Homozygous disruption of the CENP-E gene in developing flies and in mice produced early embryonic lethality as a result of mitotic abnormalities characterized by misaligned chromosomes (14, 15). The mitotic phenotype characteristic of CENP-E loss of function is a bipolar mitotic spindle with the majority zof chromosomes aligned at the metaphase plate. A minority of chromosomes remain clustered near either spindle pole. Whereas this lagging chromosome phenotype is consistent with a role for CENP-E as a kinesin motor required for the productive interaction of kinetochores with spindle microtubules (12, 16), the successful metaphase positioning of the majority of chromosomes points to either compensatory mechanisms of chromosome movement, or to a some distinct characteristic shared by those chromosomes unable to achieve proper alignment.

Although many molecular details of CENP-E motor function in chromosome alignment remain unknown, a requirement for CENP-E in satisfaction of the mitotic checkpoint in human cells is unambiguous. In all published studies in cultured human tumor cells, disruption of CENP-E function results in cell cycle arrest in prometaphase followed by cell death. This cell cycle arrest may be secondary to a failure of chromosome alignment, or alternately, may reflect a distinct role for CENP-E in the regulation of the E3 ligase activity of APC/C.

Restraint of cell cycle progression from metaphase to anaphase is enforced by the mitotic checkpoint complex, an inhibitor of the E3 ubiquitin ligase activity of the APC/C The mitotic checkpoint complex is a multiprotein complex consisting of BubR1, Bub3, Mad2, and Cdc20 (Fig. 1; refs. 17–20). BubR1 is a protein kinase, whereas the other three proteins are not enzymes. Together, as a complex, these proteins inhibit the ubiquitin ligase activity of the APC/C, but the individual contribution of each protein is not completely clear. All of these proteins can be found localized to the kinetochores of prometaphase chromosomes. A two-hybrid screen for proteins interacting with COOH-terminal portions of CENP-E identified BubR1 (21). Also identified in the two-hybrid screen were

CENP-E itself and another kinetochore protein, CENP-F/mitosin. Interaction of CENP-E with BubR1 has also been shown by coimmunoprecipitation from lysates of mitotic cells (7, 21). Subsequent studies have shown that BubR1 is required for cell cycle arrest induced by CENP-E dysfunction, as are the other well-characterized checkpoint proteins Mad2 and Mps1 (17, 20, 22). In these respects, cell cycle arrest following CENP-E loss of function is similar to that caused by antimicrotubule drugs, with one important distinction: CENP-E has a direct functional role in the regulation of BubR1 kinase activity (23–25). CENP-E stimulates the kinase activity of BubR1, and remarkably, this stimulation can be suppressed by the addition of microtubules (25). This suppression is mediated by the kinesin motor domain of CENP-E, as deletion of the motor domain abolishes the ability of microtubules to suppress the CENP-E stimulation of BubR1 activity. These findings have led to the proposal that CENP-E serves as the primary effector of the mitotic checkpoint, sensing kinetochore interactions with microtubules and transducing this signal through the suppression of BubR1 kinase activity and corresponding decreased inhibition of APC/C (25). Although this model is attractive, mitotic checkpoint complex–mediated inhibition of the APC/C cannot be dependent on the function of CENP-E; loss of CENP-E function results in cell cycle arrest in mitosis. Clearly, additional aspects of APC/C regulation, and the role of CENP-E in mitotic checkpoint complex function, remain to be elucidated.

Regulaton of CENP-E

The mechanisms through which the abundance, localization, and enzymatic function of CENP-E are regulated remain relatively unknown. Reported posttranslational modifications of human CENP-E include prenylation (26), sumoylation (27), and phosphorylation (28, 29). Prenylation is the covalent addition of lipid to a protein at the COOH terminus. It has been suggested to be important for the localization of CENP-E to kinetochores (26, 30), although the underlying mechanism has not been elucidated. Investigations into the cellular effects of the farnesyl transferase inhibitor lonofarnib/SCH 66336, which can inhibit protein prenylation, revealed a mitotic phenotype similar to that produced by dysfunction of CENP-E (26, 30). Further studies showed that levels of kinetochore-associated CENP-E were decreased in lonafarnib-treated cells, that a peptide containing the COOH-terminal CAAX motif from human CENP-E could be prenylated *in vitro*, and that CENP-E could be labeled in cells with [^{3}H]mevalonate (26). The significance of the prenylation of CENP-E remains unclear, but the observations reported in these two publications suggest a possible role in the regulation of CENP-E localization or abundance.

The modification of CENP-E by small ubiquitin-like modifiers (SUMO) 2/3 has recently been reported to regulate the association of CENP-E with kinetochores (27). SUMOylation is the covalent addition of SUMO peptides to a protein. Unlike ubiquitin, SUMO groups do not direct the degradation of target proteins, but can be involved in localization. Overexpression of the SUMO-specific isopeptidase SENP2 caused a prometaphase cell cycle arrest with misaligned chromosomes and dramatically reduced kinetochore-associated CENP-E. The authors showed SUMO-2/3 modification of a COOH-terminal 700 amino acid domain of CENP-E, localized a SUMO-interacting motif between residues 2307 and 2319 of this "tail" domain, and showed that mutation of this SUMO-interacting motif impaired the ability of the COOH-terminal tail domain to properly localize to kinetochores and to produce a mitotic arrest following overexpression. Two additional kinetochore proteins, BubR1 and Nuf2, were identified as substrates for SUMO-2/3 modification. The authors suggest that interaction of CENP-E COOH-terminal SUMO-interacting motif domain with polymeric SUMO-2/3 on these proteins may provide the basis for CENP-E localization to kinetochores.

Eight phosphorylation sites have been reported on CENP-E: T422, S454, S611, S1211, T1267, S2601, S2613, and S2616 (28). Phosphorylation of a COOH-terminal portion of CENP-E has been reported to regulate the association of that region with microtubules (29), although the specific site was not reported. Recently published findings from Espeut et al., working with *Xenopus* CENP-E, provide the first evidence for regulation of CENP-E kinesin motor domain activity. This group showed inhibition of the kinesin motor domain activity of CENP-E by a portion of the CENP-E COOH-terminus (residues 2717-2954). Phosphorylation of this COOH-terminal region by either cyclin B/Cdk1 or by MPS1 was sufficient to relieve this inhibition, whereas Plk1 and mitogen-activated protein kinase had no effect (31). The sites of CENP-E phosphorylation *in vitro* were not reported. Once these sites have been identified, it will be important to confirm their modification in human CENP-E and determine if any are misregulated in cancer.

The significance of the majority of these posttranslational modifications sites remains unknown. Particularly as CENP-E is exploited as a target for cancer therapy, it will be important to better understand the basis for regulation.

Clinical Translational Advances

CENP-E and cancer. As described above, CENP-E has an important role in mitotic progression and consequently is required for cellular proliferation. In previously unpublished studies, we have observed that the mRNA expression pattern of CENP-E shows a strong association with proliferation. In most normal tissues the expression of CENP-E is undetectable or very low. Those normal tissues with significant expression of CENP-E exhibit significant cellular turnover such as gastrointestinal epthelia (esophagus, small intestine, stomach, and colon), hematopoetic sites (spleen, thymus, and bone), or gametogenic tissue (testes). In cancer, CENP-E is frequently overexpressed in tumor tissue compared with normal tissue counterparts. In particular, lung adenocarcinomas and squamous cell carcinomas show 5-fold and 20-fold increases in expression over normal lung tissue, respectively. Several other tumors show similar increases in CENP-E mRNA expression compared with corresponding normal tissues (breast infiltrating ductal carcinoma, 3-fold; colon adenocarcinoma, 2-fold; ovary adenocarcinoma, 5-fold; pancreas adenocarcinoma, 4-fold). In contrast, prostate cancer has very low expression of CENP-E.

The pathways regulating CENP-E mRNA expression are not completely understood. Expression of CENP-E mRNA in normal tissues correlates well with other markers of proliferation such as KI67 or cyclin B expression (R^2 = 0.71 and 0.72, respectively). In tumors, however, the correlation of CENP-E expression with KI67 or cyclin B message expression is weaker (R^2 = 0.56 and 0.55, respectively), suggesting that CENP-E expression can

become dysregulated with respect to other cell cycle regulators. Interestingly, the correlation between CENP-E and another mitotic checkpoint gene, BubR1, is stronger ($R^2 = 0.67$) than the correlation with KI67, the widely used marker of proliferation. This may reflect a coordinated regulation of the checkpoint genes that is distinct from other cell cycle genes. There is little evidence of gene amplification at the CENP-E locus in cancer, but there are frequent DNA losses in that region.[3] There is also no evidence for somatic gain of function mutations in CENP-E that have been causally implicated in cancer. Nevertheless, CENP-E does interact directly and indirectly with other proteins known to have mutations that predispose to the development of cancer, including BubR1 and BRCA2 (32–35). Thus, CENP-E is not an oncogene in the classic sense. However, elevated expression of CENP-E in some cancers, an important role in genome stability, and reduced rates of cancer development in mice with reduced levels of CENP-E (see below) suggest the possibility that CENP-E overexpression may contribute to or enable tumor development.

Genetic studies of CENP-E in murine models of cancer have shown that it can be either tumor-promoting or tumor-suppressive depending on the context (36). In mice lacking one allele of CENP-E, reduced expression of CENP-E led to a higher frequency of aneuploidy in peripheral blood lymphocytes, splenocytes, and colonic epithelium. These mice exhibited a modest increase in the incidence of spontaneous lung adenomas and splenic lymphomas, suggesting a role for CENP-E in tumor suppression. Strikingly, these mice exhibited decreased rates of spontaneous occurrence of liver tumors, tumors induced by homozygous deletion of p19ARF or by treatment with the carcinogen 7,12-dimethylbenz(*a*)anthracene (36). These data suggest that in cancer cells with significant genetic damage, increasing aneuploidy via inhibition of CENP-E can have antitumor activity.

CENP-E inhibitors. To date, cancer drug discovery efforts focused on CENP-E have yielded one well-described drug candidate, GSK923295 (37–40). This is the only described inhibitor of CENP-E. GSK923295 is highly specific for CENP-E and inhibits the microtubule-stimulated ATPase of CENP-E kinesin motor domain with a K_i of 3.2 nmol/L (40). This inhibitor acts through an allosteric mechanism, stabilizing CENP-E kinesin motor domain in a complex with ADP, thus preventing nucleotide turnover and "locking" CENP-E in a state bound tightly to microtubules (40). GSK923925 entered phase I clinical studies in patients with solid tumors in August 2007. To our knowledge there are no other CENP-E inhibitors in development.

In vitro, GSK923295 inhibited the proliferation of a broad range of solid tumor and hematologic cell lines at concentrations of 10 to 150 nmol/L (38), causing cell cycle arrest in mitosis with apparently normal bipolar spindles with lagging chromosomes at the spindle poles (37). In many of those lines selected for more detailed study, mitotic arrest was followed by apoptotic cell death characterized by loss of mitochondrial membrane potential, accumulation of cleaved poly(ADP-ribose) polymerase and generation of sub-2n DNA content (37). Interestingly, primary cultures of bone marrow progenitor cells and nontransformed mammary epithelial cells were among the less sensitive cells. Although the basis for this reduced effect on normal cells is unclear, it suggests a potential for tumor selectivity of GSK923295.

The antitumor activity of GSK923295 has been investigated *in vivo* against a number of different human tumor xenografts (39). Histologic examination of established Colo205 xenografts in mice treated with GSK923295 revealed an abundance of abnormal mitotic figures and scattered apoptotic bodies 24 hours after dosing. The abnormal mitotic figures consisted of irregular metaphase plates with lagging chromosomes, a phenotype consistent with CENP-E inhibition. In addition, postmetaphase mitotic figures were extremely rare. Antitumor activity was observed against 10 of 11 tumor lines tested. Objective responses (tumor regression) were achieved with Colo205, H-460, A549, H1299, and SKOV3. Stable disease was observed with MCF-7 and MV-522. Evidence of tumor growth delay was observed with HT-29 and MX-1. Activity against MDA-MB-231 and HCT-116 was variable (growth delay/regression and delay/no activity, respectively).

Conclusion

CENP-E is the hub for mitotic checkpoint signaling, integrating the mechanics of mitotic spindle function with regulation of the mitotic checkpoint kinase BubR1. This checkpoint function, together with the overabundance of CENP-E in tumors relative to similar normal tissues, the low levels of expression in nonproliferating normal tissues, and the cancer-protective effect associated with reduced CENP-E activity in mouse models highlight the potential for targeting CENP-E for the treatment of human cancers. As with any new cancer therapy, the development of CENP-E inhibitors includes many challenges. Most prominent among these are the identification of patients with cancers that would be most responsive to treatment and the identification of effective drug combinations. The broad range of sensitivities across a diversity of human tumor cell lines, coupled with the growing knowledge of mitotic checkpoint and apoptotic pathways, lends some hope that it will be possible to derive markers that can predict response. Furthermore, this knowledge must be used to direct rational drug combinations. Based on current understanding, inhibitors of antiapoptotic pathway targets such Bcl-2, IAPs, and phosphatidylinositol 3-kinase have strong appeal as partner therapies for combination with GSK923295, as do approved therapies frequently used in combination with microtubule-directed therapies such as anthracyclines or trastuzumab.

As is the case for the majority of novel therapies in development today, the most important research challenge ahead for CENP-E inhibitors is the identification of markers predictive of patient benefit. Along the way, the use of small molecule inhibitors of CENP-E in the laboratory can also be expected to yield fundamental insights into mitotic checkpoint regulation that would be difficult to obtain using other approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[3] J. Greshock, personal communication.

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